CHARACTERIZATION OF THE TETRASPANIN PROTEIN TSPAN18

by

DARIO COLOMBO

A thesis submitted to The University of Birmingham for the degree of DOCTOR OF PHILOSOPHY

Department of Cardiovascular Sciences
School of Clinical and Experimental Medicine
The University of Birmingham
September 2010

-

ABSTRACT

Tetraspanins are a superfamily of four-transmembrane proteins. They associate laterally with one another and with other transmembrane proteins to form membrane microdomains, in which associated proteins are regulated. Tetraspanins regulate diverse processes such as cell signalling and fusion, intracellular trafficking, viral infection and cancer.

The primary aim of this thesis was to study a previously uncharacterized tetraspanin, Tspan18. Unlike other tetraspanins, over-expression of Tspan18 in lymphocyte cell lines activated an NFAT/AP-1 reporter, which responds to calcium and MAPK signalling. In DT40 B cell lines, Tspan18 signalling was independent of Lyn, Syk, PLCγ2 and IP_3 receptors, which are essential for B cell receptor signalling. However, Tspan18 signalling did require extracellular calcium and the calcium-activated phosphatase calcineurin, which activates NFAT. Additional studies that included the Jurkat T cell line showed that Tspan18 could not activate the MAPK-responsive AP-1 promoter, but instead mimicked the effect of the calcium ionophore ionomycin. For the secondary thesis aim, over-expression of Tspan18 or other tetraspanins did not affect signalling by the platelet collagen receptor GPVI.

Finally, Tspan18 mRNA was found to be relatively highly expressed in endothelial cells and peripheral blood leukocytes. Together these data suggest a role for Tspan18 in calcium signalling on these cell types.

ACKNOWLEDGEMENTS

Thanks to Mike Tomlinson, for his immense patience, time, enthusiasm, optimism and day-by day supervision, which have been always great and much appreciated; thanks also for being an inspirational team leader.

Thanks to Steve Watson, for having given me the opportunity to be part of the Watson lab for three years. Thanks also for his guidance, supervision and encouragements.

Thanks to all the present and past members of the Watson lab, who made these years special. In sparse order: Majd, for being an amazing friend, technology buddy (one day, the systems will converge…), and a fantastic lab technician. Neil, for being a great tetraspaner, Ban, for always having looked properly after me, Jun, for the incredible friendship, the long hours spent talking about music, photos, and antibodies on the New Street-University-New Street train, Chris and Karis (CKK), for being my "adoptive family" and for pushing me well behind what I believed were my limits, Andy and Jocelyin, for their friendship, support, encouragements, feedbacks, for answering all my questions, and for offering their help during difficult times, Simon, for having been always positive, Gemma, for the Medici and bunny advices, Alex and Sonia, for their French touch, Craig, for his friendship and for his jokes, Stevie T, for all his microscope-related advices, Jen, for her several cups of tea and for all the molecular biology help, Stuart, for being a great office mate, Yotis, for being an endless source of encouragements, Galye, for keeping the lab together with fun and enthusiasm, Beata, for looking after all the lab equipments and being so nice.

Thanks also to Ilaria, for the huge support, and Piera, for having been a good friend. Thanks to Alison and Andrew at The Open University, for they encouragements, patience and support.

Finally, thanks to my family, Magda, Primo and Corinna, to whom this thesis is dedicated to, without the immense day-by-day support and encouragements this adventure would not have been possible.

Thanks to the British Heart Foundation, which financially supported this work, and to The University of Birmingam.

-

To my Family…

TABLE OF CONTENTS

LIST OF ILLUSTRATIONS

LIST OF TABLES

ABBREVIATIONS

ADAM10	A disintegrin and metallopeptidase 10
ADP	Adenosine diphosphate
AEBSF	4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride
AP-1	Activator protein 1
ATP	Adenosine-5'-triphosphate
BCR	B cell receptor
β-gal	β-galactosidase
BSA	Bovine serum albumin
BS3	Bis-sulfosuccinimidyl suberate
CHAPS	3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate
CRP	Collagen related peptide
CVX	Convulxin
DAG	Diacylglycerol
DMEM	Dulbecco/Vogt modified Eagle's minimal essential medium
DMSO	Dimethylsulphoxide
ECL	Enhanced chemiluminescence
EGTA	Ethylene glycol tetra-acetic acid
ER	Endoplasmic reticulum
ERK	Extracellular signal regulated-kinase
FACS	Fluorescent-activated cell sorting
FBS	Fetal bovine serum
FcR	Fc receptor
FITC	Fluorescein isothiocyanate
FLAG	Epitope tag with sequence MDYKDDDK
GEM	Glycosphingolipid-enriched microdomain, or lipid raft
GPVI	Glycoprotein VI
GTP	Guanosine-5'-triphosphate
HIV	Human immunodeficiency virus
HTLV-1	Human T-lymphotropic virus type 1
HVC	Hepatitis C virus
Icrac	Calcium release-activated current
IgG	Immunoglobulin G
IgSF	Immunoglobulin superfamily
IP_3	Inositol 1,4,5-trisphosphate
IP_3R	Inositol 1,4,5-trisphosphate receptor
ITAM	Immunoreceptor tyrosine based activation motif
lacZ	Lac operon which encodes for β-galactosidase
LAT	Linker for activated T cells
mAb	Monoclonal antibody
MAPK	Mitogen-activated protein kinase
NES	Nuclear export sequence
NFAT	Nuclear factor of activated T cells
PAR	Protease-activated receptor
PCR	Polymerase chain reaction
PH	Pleckstrin homology
PKC	Protein kinase C

PLC	Phospholipase C
PMA	Phrobol 12-myristate 13-acetate
PRP	Platelet rich plasma
PTK	Protein tyrosine kinase
PVDF	Polyvinylidene fluorite
qPCR	Quantitative real time polymerase chain reaction
RPMI	Roswell Park Memorial Institute medium
RT-PCR	Reverse-transcriptase polymerase chain reaction
SAGE	Serial analysis of gene expression
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SH2	Scr homology 2
SH3	Scr homology 3
SOC	Store-operated channel
SOCE	Store-operated calcium entry
STIM1	Stromal-interacting molecule 1
STIM2	Stromal-interacting molecule 2
TBS-T	Tris buffered saline-tween 20
TCR	T cell receptor
WT	Wild type

CHAPTER 1
GENERAL INTRODUCTION

1.1 TETRASPANINS

1.1.1 Overview

Tetraspanins are a large superfamily of evolutionarily conserved surface membrane proteins that have four transmembrane domains. A vast range of organisms, including plants, fungi and vertebrates, expresses tetraspanins. Humans have 33 tetraspanin members and studies have revealed that they are expressed in all cell types, each of which usually expresses multiple superfamily members. Some tetraspanin proteins are expressed in most cell types, such as CD9 and CD81, while others are expressed in only certain cell types, such as Tspan32 and CD37 in leucocytes.

The most distinctive feature of tetraspanin proteins is that they can form lateral associations with multiple molecular partners and with each other, organizing the surface membrane proteins in dynamic microdomains, referred to as “tetraspanin microdomains” or the “tetraspanin web.” This membrane organization, which is distinct from “lipid rafts,” can regulate important cell functions, such as the immune response, cell adhesion, mammalian fertilization, fungal invasion of rice leaves, cell fusion, trafficking and signal transduction (Wright, Moseley et al. 2004; Hemler 2005; Levy and Shoham 2005; Berditchevski and Odintsova 2007; Charrin, le Naour et al. 2009).

1.1.2 Structure of tetraspanin proteins

Tetraspanin proteins have four transmembrane domains, linked together by a small extracellular domain and a large second extracellular domain. Both the amino and the carboxy-termini are cytoplasmic and typically less that 10 amino acids in length (Figure 1.1). Tetraspanins are small transmembrane proteins, composed of 200-250

amino acids with a molecular weight of 25-50 kDa, and protrude only 4-5 nm above the membrane. Although several classes of molecules have four transmembrane domains they are not classified as members of the tetraspanin family unless they contain certain structural features. Tetraspanins have a number of conserved residues including one glycine and from four to eight cysteine residues in the large extracellular loop. Within this region, tetraspanin proteins contain a conserved CCG motif (where C is cysteine, G is glycine), that is characteristic of this superfamily, together with a PXSC motif (P is proline, X is any amino acid, S is serine and C is cysteine) and an EGC motif (E is glutamic acid, G is glycine, C is cysteine) (Levy and Shoham 2005).

Tetraspanin proteins can undergo post-translational modification such as glycosylation and palmitoylation. N-glycosylation can increase the size of a tetraspanin protein by up to 35 kDa (Tarrant, Robb et al. 2003). Tetraspanin proteins contain several cysteine residues at the interface between the plasma membrane and cytoplasm, which are palmitoylation sites (Kovalenko, Yang et al. 2004). Site-directed mutagenesis of all the juxtamembrane cysteine residues of CD9 and CD151 resulted in the disruption of tetraspanin-tetraspanin interactions, suggesting that palmitoylation plays a central role in tetraspanin microdomain assembly (Yang, Kovalenko et al. 2004).

In 2006, the Kong research group used cryo-electron microscopy to obtain structural information to 6 Angstroms of a complex composed of the uroplakin tetraspanins Ia and Ib and their molecular partners, the uroplakins II and IIIa. (Min, Wang et al. 2006). The structure is a very packed conformation, and, interestingly, tetraspanins

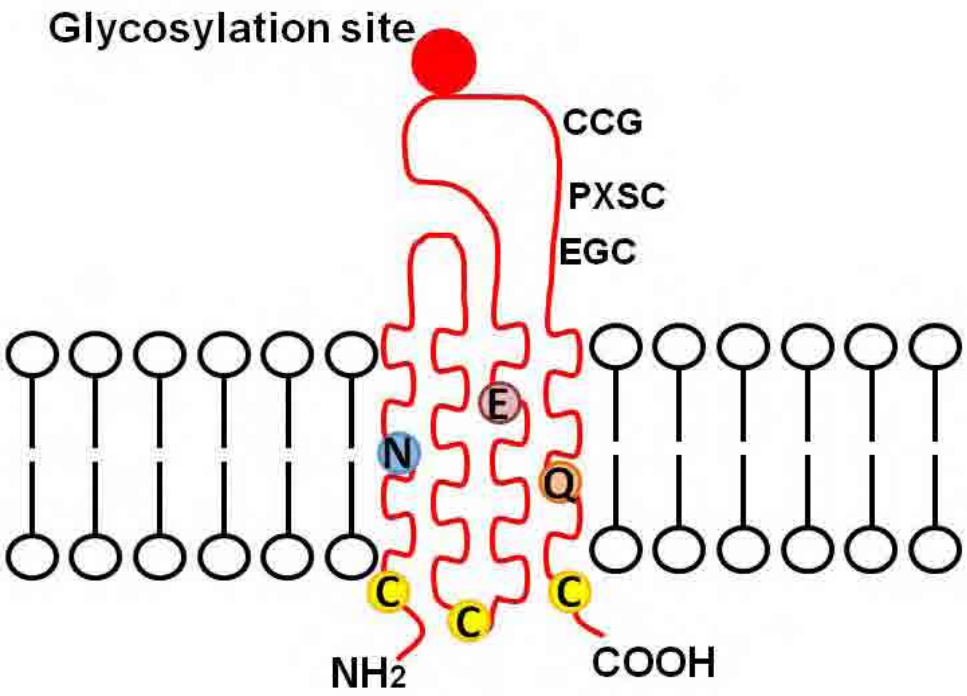


Figure 1.1 Schematic representation of a tetraspanin protein. Tetraspanin proteins contain four transmembrane domains, a small extracellular loop, a large extracellular loop and short amino and carboxy termini, both cytoplasmic. In the large extracellular loop, tetraspanin proteins contain the characteristic conserved CCG motif (where C is cysteine, G is glycine) a conserved PXSC motif (P is proline, X is any amino acid, S is serine and C is cysteine) and a conserved EGC motif (E is glutamic acid, G is glycine, C is cysteine). Tetraspanin proteins contain three other conserved residues in the transmembrane domains: an asparagine (N), a glutamic acid (E) and a glutamine (Q). Tetraspanin proteins are usually glycosylated on between one and three N-linked sites (represented by a red filled circle), and cytoplasmic cysteine residues can be palmitoylated (in yellow).

form interactions with the respective molecular partners both at the interface of the large extracellular loop and in the transmembrane region (Figure 1.2).

Kitadokoro *et al.* solved the crystal structure of the soluble portion of the CD81 large extracellular domain (Kitadokoro, Bordo et al. 2001). CD81 extracellular domain is composed of five α-helices arranged in "stalk" and "head" sub-domains. Two disulphide bridges stabilize the structure, involving the cysteine residues of the CCG motif and other conserved cysteine residues of the large extracellular loop (Figure 1.3).

Sequence alignment comparison performed by the Bolognesi group in 2001 suggest that the overall structural organization of the large extracellular loop might be largely conserved among tetraspanin proteins (Kitadokoro, Bordo et al. 2001). Moreover, ClustalW2 sequence alignment of all human tetraspanin proteins reveals that certain tetraspanins form subgroups that are particularly highly related, such as CD9, CD81 and Tspan2 (Figure 1.4).

The Seigneuret group, combining computational modelling and crystallographic data of the large extracellular loop of CD81, predicted the complete three-dimensional structure of CD81. The computational model shows an overall cylindrical mushroom-like shape with a packed structure (Seigneuret 2006). The transmembrane domains appear to be organized as a four-stranded left-handed coiled coil, organized as an approximately square bundle, giving a tight packing of the transmembrane domains.

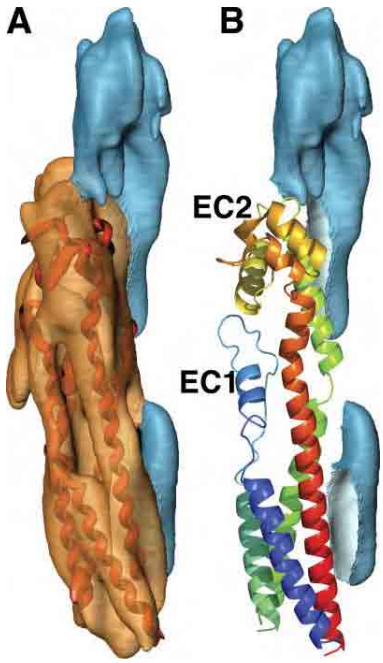


Figure 1.2 Structure of the tetraspanin uroplakin Ia and its molecular partner, the uroplakin II. (A) The molecular model of the tetraspanin uroplakin Ia (orange) is fitted into the electron density map of its molecular partner, the uroplakin II (blue). (B) The density of the tetraspanin uroplakin Ia tetraspanin is removed to show the model's relation to the density of the single-spanning transmembrane protein partner. The model is coloured so that the colour spectra start with blue at the NH_2 terminus and end with red at the COOH terminus of the tetraspanin protein. Taken from Min et al (Min, Wang et al. 2006).

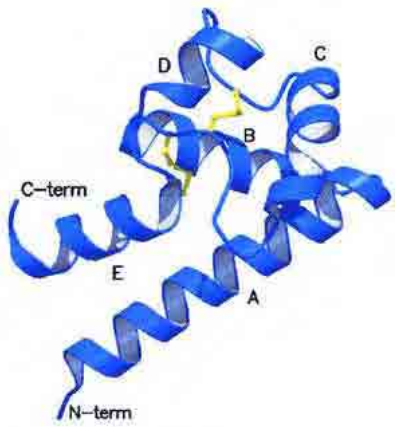


Figure 1.3 Crystal structure of the human CD81 extracellular loop. The view highlights the head subdomain localization relative to the NH_2- and COOH- terminal helices (α-helices A and E, respectively), and the labelling of secondary structure elements. The two disulfide bridges are shown in yellow. Taken from (Kitadokoro, Bordo et al. 2001).

The small extracellular loop appears to be composed of a small β-strand, while the amino and carboxy terminal regions of CD81, close to the intracellular membrane surface, appear to have a non-ordered structure. The large extracellular loop appears to accommodate the small extracellular loop in a hydrophobic conserved groove, giving the molecule an overall compact and cylindrical shape (Figure 1.5). The transmembrane region is mostly composed of hydrophobic amino acids, while the portion of the molecule exposed to the aqueous extracellular phase appears to be mostly hydrophilic, despite the presence of some hydrophobic regions. Combining these structural observations, it was hypothesized that the hydrophobic regions are involved in the interaction with other CD81 molecules, forming CD81 dimers, while the hydrophilic regions are involved in interactions with other partner proteins (Seigneuret 2006).

1.1.3 Tetraspanin web hypothesis and classification of tetraspanin interactions

Tetraspanin proteins have the capacity to interact with each other and with specific partner molecules. Most of the partner proteins play a receptor role, or couple receptors to signalling pathways. Specific partners include integrins, growth factor receptors, immunoglobulin superfamily proteins (IgSF) or signalling molecules. This unique capability leads to the ability to create supra-molecular complexes, referred to as “tetraspanin microdomains” or “tetraspanin webs” (Rubinstein, Le Naour et al. 1996). The tetraspanin microdomains provide a platform on which membrane and signalling proteins can perform their biological roles.

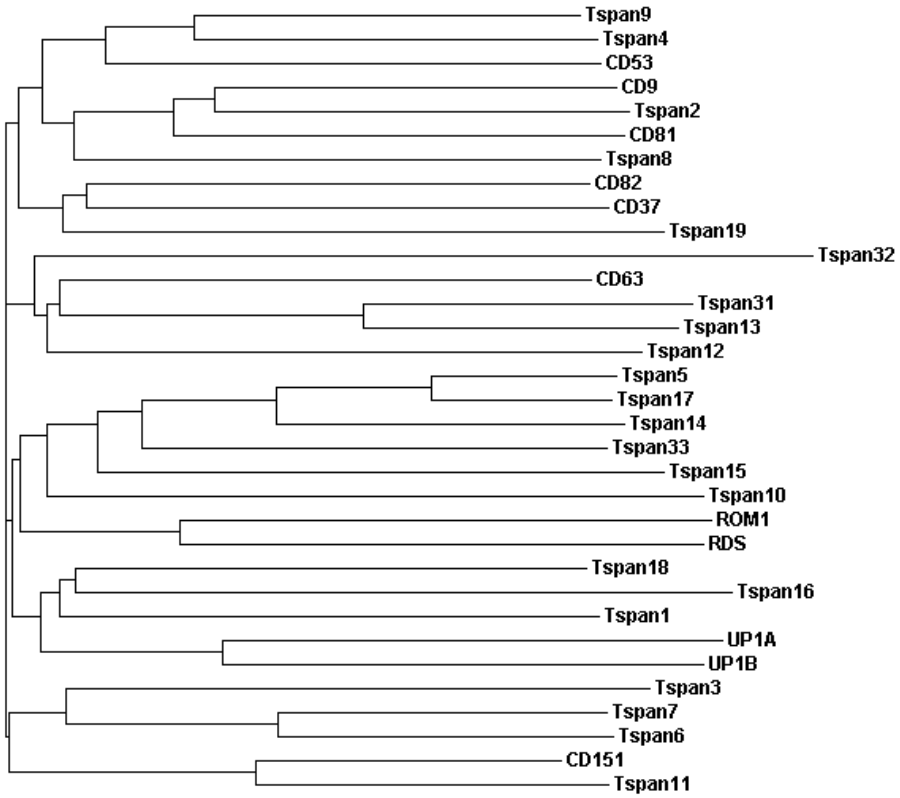


Figure 1.4 ClustalW2 phylogram tree of all human tetraspanin proteins.

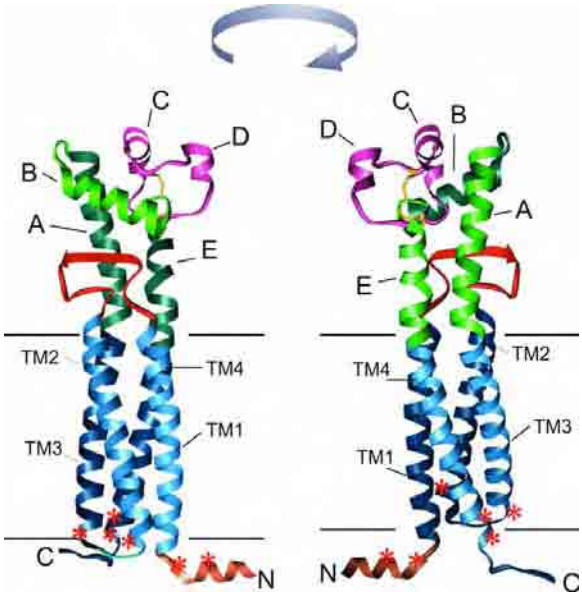


Figure 1.5 Ribbon-and-stick representation of the CD81 tertiary structure and topology. Transmembrane domains are represented in blue, the small extracellular loop represented in red, and the large extracellular loop is represented in green and pink. Different transmembrane regions are indicated (TM1-4) as well as different large extracellular loop sub-domains (A-E). The red asterisks represent probable palmitoylation sites. Taken from (Seigneuret 2006; Charrin, le Naour et al. 2009).

Combining the use of chemical cross-linkers and different detergents with immunoprecipitation techniques, the interactions that tetraspanin proteins establish with their molecular partners have been classified. Robust, direct interactions between tetraspanins and specific partner proteins are maintained in strong detergents, such as Triton X-100, in the absence of Ca^{2+} and Mg^{2+}. Less robust interactions, such as those between individual tetraspanins, are disrupted by Triton X-100, but are preserved in milder detergents, such as Brij 96 or Brij 97. Weaker interactions are only detected in very mild detergents, such as CHAPS (Hemler 2005; Levy and Shoham 2005). The direct interactions of several tetraspanin proteins with different molecular partners are summarised in Table 1.1.

In 2006, the Thali group demonstrated that CD63 and other tetraspanins, on the surface of HeLa cells, cluster at a large number of discrete sites, each of which extends over a few hundred nanometres. Interestingly, these clusters, or 'microdomains,' do not necessarily contain all the tetraspanins that were tested for, suggesting heterogeneity between microdomains (Nydegger, Khurana et al. 2006). This observed punctiform organization of the tetraspanin microdomains is distinct from the previous "web" or network hypothesis of tetraspanin surface organization. The idea that tetraspanins form discrete microdomains is further confirmed by single-molecule fluorescence microscopy, used to track a single CD9 molecule and to demonstrate that this molecule can dynamically enter and exit from a CD9 enriched microdomain, distinct from lipid rafts, at the cell surface (Espenel, Margeat et al. 2008). Furthermore, the Sanchez-Madrid group showed that the adhesion proteins VCAM-1 and ICAM-1, the receptors for leukocyte integrins, are recruited by inclusion within specialized preformed tetraspanin-enriched microdomains at the

surface of endothelial cells, thus acting as adhesive platforms for circulating leukocytes (Barreiro, Zamai et al. 2008).

A feature of tetraspanin proteins is that they can form distinct partnerships in different cell types. For example, in B cells CD81 associates with CD19, a member of the B cell receptor (BCR) co-receptor complex (Bradbury, Kansas et al. 1992), while in T cells, CD81 associates with CD4 and CD8 (Todd, Lipps et al. 1996). There is also variability in the stoichiometry of association. Some associations between tetraspanin proteins and relative partners are highly stoichiometric, such as the tetraspanins uroplakin Ia and Ib and their specific partners uroplakin II and uroplakin IIIa (Liang, Riedel et al. 2001), while immunoprecipitation studies conducted on other tetraspanins have demonstrated that not all tetraspanins have such a strong stoichiometry with their partner molecules (Hemler 2008). Tetraspanin proteins can also form associations with other tetraspanins, which are retained in mild detergents. At present, it is not known whether all tetraspanin proteins expressed in one cell type can associate with each other (Hemler 2008).

1.1.4 Specific tetraspanin functions

It has been proposed that tetraspanin proteins are involved in a variety of physiological and pathological processes in different cell types. Tetraspanins are reported to play a role in protein maturation and trafficking (Hu, Liang et al. 2005), in regulation of signalling (Lau, Wee et al. 2004; Goschnick, Lau et al. 2006), in sperm-egg fusion (Rubinstein, Ziyyat et al. 2006), in cancer (Le Naour, Andre et al. 2006) and in virus infection (Bartosch, Vitelli et al. 2003). The tetraspanin proteins that are

best studied are those for which an antibody or a knockout mouse is available. The results of these studies are described in the following sections.

Tetraspanin	Partner protein	Function	References
CD9	CD9	CD9-CD9 dimer may be a basic structural unit	(Kovalenko, Yang et al. 2004)
	EWI-2 (IGSF8, CD316)	Modulates integrin-dependent cell motility and/or spreading	(Charrin, Le Naour et al. 2003) (Stipp, Kolesnikova et al. 2003)
	EWI-F (C9P-1, FPRP)	Function unknown	(Stipp, Kolesnikova et al. 2003)
	Claudin 1	CD9 stabilizes expression of non junctional Claudin-1	(Kovalenko, Yang et al. 2007)
	HB-EGF	CD9 upregulates both diphtheria toxin binding and mitogenic functions of HB-EGF	(Iwamoto, Higashiyama et al. 1994) (Higashiyama, Iwamoto et al. 1995)
CD81	CD81	CD81-CD81 dimer might be a basic structural unit	(Stipp, Kolesnikova et al. 2001)
	EWI-2	Modulates integrin-dependent cell motility and/or spreading; also CD81 supports maturation and surface expression of EWI-2	(Charrin, Le Naour et al. 2003) (Stipp, Kolesnikova et al. 2001)
	CD19	CD81 supports maturation and surface expression of CD19	(Shoham, Rajapaksa et al. 2006) (Horvath, Serru et al. 1998)
	$\alpha_4\beta_1$ integrin	CD81 supports a4b1 integrin adhesion and strengthening	(Serru, Le Naour et al. 1999)
CD151	CD151	CD151-CD151 dimer may be a basic structural unit	(Kovalenko, Yang et al. 2004)
	$\alpha_3\beta_1$ integrin	CD151 affects integrin-dependent adhesion and motility	(Zijlstra, Lewis et al. 2008)
	$\alpha_6\beta_1$ integrin	CD151 affects integrin-dependent adhesion and strengthening	(Lammerding, Kazarov et al. 2003)
	$\alpha_6\beta_4$ integrin	CD151 affects integrin-dependent adhesion and motility	(Sterk, Geuijen et al. 2002)
	$\alpha_7\beta_1$ integrin	unknown function	(Sterk, Geuijen et al. 2002)
CD63	Syntenin-1	endocytosis regulation	(Latysheva, Muratov et al. 2006)
uroplakin Ia uroplakin Ib	uroplakin II uroplakin IIIa	trafficking and ER exit trafficking and ER exit	(Hu, Liang et al. 2005) (Hu, Liang et al. 2005)
ROM1	Peripherin/Rds	photoreceptor viability and morphogenesis	(Boesze-Battaglia et al. 2007)

Table 1.1 Directly associated tetraspanin partner proteins. Adapted from Hemler 2008.

Signalling: Several research groups have reported that tetraspanin proteins are involved in signalling processes. CD81 was shown to be an essential component of the CD9/CD21/CD81 co-receptor complex. Upon BCR stimulation, CD81 is found to be essential for the prolonged localization of the BCR/CD19/CD21 complex to lipid rafts and for sustained signalling. This appears to be due to an increase in CD81 palmitoylation within the lipid raft microdomains (Cherukuri, Shoham et al. 2004). However, a recent study contradicts this observation, convincingly showing that B cell receptor-induced calcium mobilization, tyrosine phosphorylation and proliferation are enhanced in the absence of CD81, suggesting that this tetraspanin is a negative regulator of B cell receptor signalling (Sanyal, Fernandez et al. 2009). In antigen-presenting cells, the tetraspanin CD37, a specific immune cell tetraspanin, is reported to interact with and negatively regulate the C-type lectin dectin-1, the major β-glucan receptor. CD37 appears to be important for dectin-1 stabilization at the plasma membrane and for the negative regulation of dectin-1 mediated interleukin 6 (IL-6) production (Meyer-Wentrup, Figdor et al. 2007). Moreover, in a previous study conducted by the same research group, CD37 deficient mice show T cell hyperproliferation in response to T cell receptor cross-linking (van Spriel, Puls et al. 2004). Mice deficient in CD151 and Tspan32 show a similar phenotype, suggesting that tetraspanin microdomains negatively regulate T cell receptor signaling (Tarrant, Groom et al. 2002; Tarrant, Robb et al. 2003; Wright, Moseley et al. 2004). Both the tetraspanins CD151 and Tspan32 have also been found to positively regulate the "outside-in" signaling of platelet integrin $\alpha_{IIb}\beta_3$. Indeed, CD151 and Tspan32-deficient platelets exhibit impaired kinetics of clot retraction, impaired platelet aggregation at low doses of various agonists, and impaired platelet spreading on fibrinogen (Lau, Wee et al. 2004; Goschnick, Lau et al. 2006). Moreover, CD151 and

Tspan32-deficient platelets show normal “inside-out” integrin $\alpha_{IIb}\beta_3$ activation and normal platelet adhesion on fibrinogen (Lau, Wee et al. 2004; Goschnick, Lau et al. 2006). These data suggest that CD151 and Tspan32 regulate $\alpha_{IIb}\beta_3$ "outside-in" integrin signalling events in platelets.

Other tetraspanins have been found to play a role in signalling. For example, CD82 is involved in the negative regulation of hepatocyte growth factor (HGF) receptor c-Met signalling (Takahashi, Sugiura et al. 2007). Moreover, the tetraspanin CD151 is able to modify the PI 3-K (phosphoinositide 3-kinase)/Atk pathway and the FAK (focal adhesion kinase)/Src kinase/p130 pathway (Takeda, Kazarov et al. 2007).

Regulation of integrins: Tetraspanin regulation of integrins has been the subject of several studies, which have focused on the relatively strong and specific functional interaction between CD151 and laminin-binding integrins. In 3T3 cells, CD151 can strongly associate with and regulate the integrin $\alpha_6\beta_1$, the major laminin-1 receptor at the cell surface, by selectively enhancing the adhesion strengthening of $\alpha_6\beta_1$ binding to laminin-1 (Lammerding, Kazarov et al. 2003). The Hemler group additionally reported, in lung endothelial cells lacking CD151, a reduced level of AKt phosphorylation, and its downstream target eNOS (endothelial nitric oxide synthase) in response to adhesion on laminin. This is accompanied by impaired localisation of laminin-binding integrins to tetraspanin microdomains and impaired endothelial cell angiogenesis (Takeda, Kazarov et al. 2007). Moreover, the Hemler group demonstrated that elevated expression of CD151 is associated with almost one third of breast cancers, and that CD151-α6 complexes promote tumour progression (Yang, Richardson et al. 2008). Finally, the Berditchevski group demonstrated that CD151 can regulate $\alpha_6\beta_1$ glycosylation, suggesting that CD151 plays an important role in

post-translation modification of this integrin (Baldwin, Novitskaya et al. 2008). Taken together, these data demonstrate that CD151 regulates adhesion strengthening, signalling and post-translational modification of laminin-binding integrins.

Protein maturation and trafficking: The apical surface of mammal urothelium contains four major integral membrane proteins, uroplakins Ia, Ib, II and IIIa, which are specialized in providing the barrier function of the bladder. Uroplakins Ia and Ib are tetraspanins which specifically associate with their molecular partners uroplakins II and IIIa, respectively. The Sun research group showed that the uroplakin tetraspanins can induce conformational changes in their partners, leading to ER-exit, protein stabilization and cell surface expression (Hu, Liang et al. 2005). In addition, the Levy group reported that in B cells the tetraspanin protein CD81, in addition to being part of the CD19/CD21/CD81 BCR co-receptor complex, associates with its molecular partner CD19 early in CD19 biosynthesis and is essential for normal CD19 glycosylation and endoplasmic reticulum exit (Shoham, Rajapaksa et al. 2003). Furthermore, CD81 plays multiple roles in regulating CD19 intracellular trafficking, processing and membrane functions in B cells (Shoham, Rajapaksa et al. 2006). Taken together, these data support the hypothesis that tetraspanin proteins play multiple roles in processing, intracellular trafficking and membrane function of their molecular partners.

Cancer: The Rubinstein group, using a CD9 immunoprecipiation strategy followed by proteomics, performed a comprehensive characterization of the tetraspanin web in human colon cancer cells (Le Naour, Andre et al. 2006). This work lead to the identification of 32 tetraspanin-associated proteins; these can be classified into adhesion molecules, membrane proteases, signaling proteins and tetraspanins. This work also established, using both tumor cell lines and patient samples, that several

components of the tetraspanin web are differentially expressed during tumor progression, suggesting a role in cancer. Indeed, a number of tetraspanin proteins are linked to tumor progression. For example, transfection of Tspan8 and CD151 promotes metastasis formation (Zoller 2006). CD151 overexpression has been reported in several studies, as discussed previously, and has been observed in several tumor types, including breast, pancreatic and colorectal cancer. As already described, CD151 appears to accelerate breast cancer by regulating α6 integrin function, suggesting that CD151 could be a potential therapeutic cancer target (Yang, Richardson et al. 2008; Zoller 2009). Despite these data, not all tetraspanin proteins have been found to promote metastasis and tumor formation. For example, the tetraspanin Tspan13 inhibits tumor proliferation in MDA-MB-231 cells (Huang, Sossey-Alaoui et al. 2007); expression of CD9 in a colon cell line delays the appearance of detectable tumors; and CD82 is downregulated in advance stages of cancer, and impairs cell mobility (Zoller 2009). Despite these findings, the precise molecular mechanisms by which tetraspanins are involved in cancer are still largely unknown.

Viral receptors: Tetraspanins play an important role in hepatitis C virus (HCV) entry and in human immunodeficiency virus (HIV) entry (Martin, Roth et al. 2005). In 1998 it was found that the HCV envelope glycoprotein E2 binds the tetraspanin protein CD81, and CD81 was addressed as a first putative HCV receptor (Pileri, Uematsu et al. 1998). Following this initial observation, other studies have confirmed that HCV entry is CD81 dependent and anti-CD81 monoclonal antibodies inhibit HCV entry in a hepatoma cell line (Bartosch, Vitelli et al. 2003; Cormier, Tsamis et al. 2004; Zhang, Randall et al. 2004). Moreover, HCV binding to CD81 is necessary but not sufficient for HCV infection (Cocquerel, Voisset et al. 2006). Several research group

have identified other molecules that are necessary for HCV entry, including the scavenger receptor class B type I and the tight junction protein claudin-1 (Dubuisson, Helle et al. 2008). More recently, occludin, another tight junction protein, was identified as a fourth crucial HCV entry factor (Ploss, Evans et al. 2009). Furthermore, it has been suggested that HCV might use all of these four proteins as an entry route to hepatocytes (Pietschmann 2009). Altogether, these data demonstrate that CD81 plays a critical role, as part of a multi-step mechanism, in HCV entry, although precise mechanisms regulating HCV entry are still under active investigation.

A physical link between tetraspanins and HIV-1 was first demonstrated through studies of viral trafficking in monocyte-derived dendritic cells (Garcia, Pion et al. 2005). In particular, internalised virus did not traffic to lysosomes, but instead to a tetraspanin-enriched compartment that was distinct from late endosomes and multivesicular bodies. Moreover, upon engagement with a T cell, the virus re-localised to the tetraspanin-containing infectious synapse (Garcia, Pion et al. 2005; Nydegger, Khurana et al. 2006; Jolly and Sattentau 2007). A functional link between tetraspanins and HIV-1 was then demonstrated when soluble forms of tetraspanin large extracellular loops were found to inhibit HIV-1 infection of macrophages (Ho, Martin et al. 2006), while tetraspanin knockdown increased infection of T cells (Gordon-Alonso, Yanez-Mo et al. 2006). In addition, CD63 over-expression was shown to inhibit viral infection of T cells by causing a loss of surface expression of the HIV-1 co-receptor CXCR4 (Yoshida, Kawano et al. 2008). Interestingly, the incorporation of tetraspanins into released viral particles reduced infectivity (Sato, Aoki et al. 2008) and infected T cells appeared to maximise infectivity by down-regulating tetraspanin expression (Krementsov, Weng et al. 2009). However, in a

recent study, the Thali group proposed that HIV-1 has evolved to utilise tetraspanins at the virological synapse to prevent cell-cell fusion and resulting syncytia formation, which is thought to be detrimental to viral spread *in vivo* (Weng, Krementsov et al. 2009).

Cell fusion: Tetraspanins are involved in two distinct fusion processes, the sperm-egg fusion process and syncytium formation.

A role for tetraspanins in sperm-egg fusion has been identified using a knockout mouse approach. The fertility of mice lacking CD9 appears to be severely reduced but not completely impaired. This is due to a defect in sperm-egg fusion and appears to be oocyte specific (Le Naour, Rubinstein et al. 2000). Mice lacking both CD9 and CD81 are completely infertile, suggesting that CD9 and CD81 play complementary roles in the sperm–egg fusion process (Rubinstein, Ziyyat et al. 2006). Later experiments demonstrated that CD9 might regulate the distribution of some membrane proteins into clusters, including the laminin-binding integrin $\alpha_6\beta_{1,}$ which may play a role in gamete fusion (Ziyyat, Rubinstein et al. 2006). Moreover, CD9 is expressed on mouse oocyte microvilli, which are involved in the sperm-egg fusion processes. In wild type mice, microvilli appear long and thin, while in CD9 null mice microvilli appear uniform, short and thick. This altered morphology might explain why the CD9 null mice show impaired sperm-egg fusion (Runge, Evans et al. 2007). Despite these findings, the precise role of tetraspanin proteins in the sperm-egg fusion process has not been elucidated yet.

The second cell fusion process that tetraspanins are involved in is the formation of a syncitium, which is defined as a cell-like structure with a large cytoplasmic mass containing multiple nuclei. Antibodies to CD82 block human T-cell lymphotropic

virus type 1 (HTLV-1) -induced syncytium formation in T lymphoblastoid cells (Fukudome, Furuse et al. 1992). Moreover, more recent data suggests that overexpression of CD82 inhibits syncytium formation, in addition to cell-to-cell virus transmission (Pique, Lagaudriere-Gesbert et al. 2000). CD9 and CD81 have been found to play a role in multinucleated giant cells (MGC). These cells form from the fusion of monocytes and macrophages as a response to external bodies. Antibodies against CD9 and CD81 enhance fusion of human monocytes and mouse macrophages in vitro (Takeda, Tachibana et al. 2003). Moreover, CD9 and CD81 knockout macrophages show enhanced MGC fusion, and the double CD9/CD81 knockout shows spontaneous MGC formation (Takeda, Tachibana et al. 2003). The Monk research group, in a more recent study aimed at identifying the precise role of tetraspanins in fusion processes, confirmed previous findings that CD9 and CD81 antibodies enhance MGC formation. Moreover, using recombinant proteins corresponding to the large extracellular loop of tetraspanin proteins, this group demonstrated that CD63, CD9 and CD151 all appear to play a role in MGC formation (Parthasarathy, Martin et al. 2009).

Tetraspanin proteins in lower organisms: Several studies have demonstrated the role of tetraspanin proteins in lower organisms, although the mechanisms behind the following data are not yet understood.

In the nematode *Caenorhabditis elegans*, the lack of the tetraspanin protein TSP-15 causes abnormalities of the hypodermis, including dissociation of the cuticles, indicating that TSP-15 functions in the maintenance of epithelial cell integrity (Moribe, Yochem et al. 2004).

In the plant *Arabidopsis thaliana*, deletion of the TORNADO2 gene, which encodes one of 17 tetraspanins in this species, leads to reduced complexity of the leaf venation network, asymmetric leaf growth and aberrant venation pattern (Cnops, Neyt et al. 2006). In addition, TORNADO2 appears to be involved in the maintenance of the radial pattern of tissue differentiation in the root (Cnops, Wang et al. 2000).

The genetic analysis of *Drosophila melanogaster* has revealed that this organism expresses 35 tetraspanin proteins (Todres, Nardi et al. 2000). Deletion of the neuronal tetraspanin late bloomer results in delayed motoneuron synapse formation with target muscles (Kopczynski, Davis et al. 1996). In contrast, a more recent study has demonstrated that the deletion of up to 25% of tetraspanin proteins does not perturb progression of the *Drosophila* life cycle, suggesting that molecular compensation exists for many tetraspanin proteins in this organism (Fradkin, Kamphorst et al. 2002).

In fungi, three tetraspanin families have been identified so far. These are Pls1 tetraspanins, of which Pls1 is required for pathogenicity of the plant pathogenic ascomycetes, *Magnaporthe grisea*, *Botrytis cinerea* and *Colletotrichum lindemuthianum*; Tsp2 tetraspanins, whose function is unknown; and Tsp3 tetraspanins, which are involved in the infection process of the plant pathogenic fungus *M. grisea* (Lambou, Tharreau et al. 2008).

These specific examples demonstrate that tetraspanin proteins are expressed in a vast range of organisms and are implicated in the regulation of a variety of cell functions.

1.2 CALCIUM SIGNALLING

Calcium is a very versatile second messenger, which modulates a variety of cellular processes (Berridge, Bootman et al. 2003) (Figure 1.6). Calcium signals are both responsible for short term cellular responses, such as secretion and contraction, and long term cellular responses, such as cell division, apoptosis and growth (Soboloff, Spassova et al. 2007). The plasma membrane and the membrane of many organelles separate compartments with different calcium concentrations: in the extracellular environment calcium concentration is around 2mM, in the ER (or in the sacroplasmatic reticulum in muscle cells) calcium concentration is around 100-700 μM, while in the cytoplasm it is in the order of 100 nM (Clapham 2007). Calcium signals are thus derived by fluxes of calcium across the membranes. Signals are a balance between "on" reactions, which are fluxes from internal stores or from the extracellular environment to the cytoplasm, and "off" reactions, which involve the activation of calcium pumps which transport calcium against the concentration gradient and restore basal conditions (Berridge, Bootman et al. 2003). Each cell type expresses a particular set (or "toolkit") of calcium-related proteins, such as receptors, transducers, channels, effectors, buffers and pumps. The expression of the specific toolkit creates a different system with unique spatial and temporal characteristics, which dictates the cellular calcium response (Berridge, Bootman et al. 2003).

Calcium entry mechanism: calcium can enter from the extracellular environment in the cytoplasm by various channels, which have different properties and different kinetics. Caclum entry involves voltage-dependent and voltage-independent calcium channels. Voltage-operated channels (VOCs) are found in excitable cells, and are responsible for fast calcium fluxes that regulate muscle contraction or exocytosis of neuronal cells. The mechanism that drives the aperture of the calcium channel is the

difference in electrical potential that exist between the intracellular and extracellular environments. The kinetic of the voltage-operated channels is extremely high, whth each channel transporting up to one million calcium ions per second (Clapham 2007). The channel is kept closed by a negative-charged helix-turn-helix paddle containing positive charged arginines, which opens upon change of membrane potential(Long, Campbell et al. 2005).

As the name suggest, in the voltage-independent calcium channels the aperture of the channel is not regulated by voltage. This class of channels include several members, including the receptor-operated channels, the second messenger-operated channels and the store-operated calcium channels. The receptor-operated channels (ROCs) are opened upon direct binding of a specific molecule to the calcium channel. Examples of ROCs include the N-methyl-D-aspartate receptor (NMDAR), which responds to glutamate, the ATP receptor $P2X_7$. Second-messenger-operated channels (SMOCs) respond to specific second messengers. Specific transduction pathways between receptors and calcium channels, which are physically separated, exist according to the type of the receptor and tissue. Store calcium depletion from the ER is responsible for the calcium-release activated current (CRAC), which allow a calcium flux from the extracellular environment into the cytoplasm. The CRAC current is activated only by a decline in ER calcium concentration and it is the major calcium entry mechanism in B and T cells

Plasma membrane receptors: calcium signaling can be triggered by the activation of specific plasma membrane receptors, such as G-protein-coupled receptors or the tyrosine kinase-coupled receptors (Clapham 2007). The activation of these receptors triggers calcium signals through the PLC protein family. Thirteen different PLC isoforms have been identified in mammals, and further complexity is provided by

alternative splicing variants for each (Suh, Park et al. 2008). PLC isoforms are tissue and cell specific, can mobilize calcium and are activated by different mechanisms. These includetyrosine kinase-coupled receptors which activate PLCγ, increase in calcium concentration which activates PLCδ, Ras which activates PLCε, and G-protein coupled receptors which activate PLCβ (Berridge, Bootman et al. 2003). Upon activation, PLC generates IP_3 and DAG (see sections 1.2.5 and 1.2.6).

Calcium release from internal stores: the IP_3 receptors and the ryanodine receptors are the two calcium channels responsible for the release of calcium from the ER or the sarcoplasmic reticulum into the cytoplasm. These channels are expressed on the ER or sarcoplasmic membrane surface and release calcium from intracellular stores in response to the activation of intracellular signaling pathways (Kushnir, Betzenhauser et al. ; Berridge, Bootman et al. 2003; Taylor, Rahman et al. 2009). While IP3 receptors are thought to be restricted to the ER and sarcoplamic reticulum, recent data suggests that ryanodine receptors can also be expressed on the plasma membrane, where they mediate calcium entry in certain cell types (Rosker, Meur et al. 2009; Taylor, Prole et al. 2009).

Calcium-binding proteins: Only a small proportion of the calcium inside the cytoplasm remains free, as intracellular calcium is bound by a variety of cytoplasmic proteins, called “effectors” or “buffers,” which have different properties and different expression patterns. Calcium can bind calcium-binding proteins such as calmodulin, but also calcium-regulated enzymes, such as PKC, or transcription factors, such as calcineurin (Berridge, Bootman et al. 2003). Upon calcium binding, effectors become active, and can trigger their biological responses.

Pumps and exchangers: following the transient increase in cytoplasmic calcium concentration, pumps and exchanges have the role to restore and maintain the basal calcium concentration. Pumps have also the role of ensuring that the internal stores are fully loaded. The two major pumps that perform this function are the plasma membrane calcium ATP-ase (PMCA), which actively pumps calcium from the cytoplasm to the extracellular environment, and the sarco(endo)plasmic reticulum calcium ATP-ase (SERCA), which pumps the calcium into the ER or sarcoplasmic reticulum (Berridge, Bootman et al. 2003).

Calcium in organelles: Calcium stores are not limited to the ER and the sarcoplasmic reticulum. Calcium is also present in other organelles, such as mitochondria and lysosomes. Mitochondria store mM of calcium, but calcium flux is regulated by a mechanism that is distinct from that which regulates calcium flux in the ER. (Guibert, Ducret et al. 2008). Calcium is pumped into the mitochondria by a calcium uniporter, named the mitochondrial calcium channel (MiCa), and is exported from the mitochondria by the sodium-calcium exchanger (NCX) (Berridge, Bootman et al. 2003). Lysosomes may also store calcium in certain cell types, since in sea urchin eggs, nicotinic acid adenine dinucleotide phosphate (NAADP) can mobilize calcium from lysosome-related organelles (Churchill, Okada et al. 2002) .

Spatial and temporal organization: Calcium is a widespread signaling mechanism and all cell types depend on calcium signals. These signals involve a combination of calcium release from intracellular stores and calcium influxes across the plasma membrane (Putney 2005).

Many calcium signals are organized in multicomplexes, that can function as an autonomous unit or module, or sequential switches (Berridge, Bootman et al. 2003).

Moreover, as the cell cytosol is enriched in calcium buffers, this results in relatively low calcium motility inside the cytoplasm. This allows the creation of local “calcium clouds” with high local calcium concentration (Taylor, Prole et al. 2009).

Calcium signals differ in the length of their responses, depending on the stimulus that generates the signal and the calcium “toolkit” employed by the cell. The response spans from milliseconds (synaptic transmission or cardiac contraction), seconds (metabolic reactions), or hours and days (transcription factor activation and egg fertilization) (Berridge, Bootman et al. 2003).

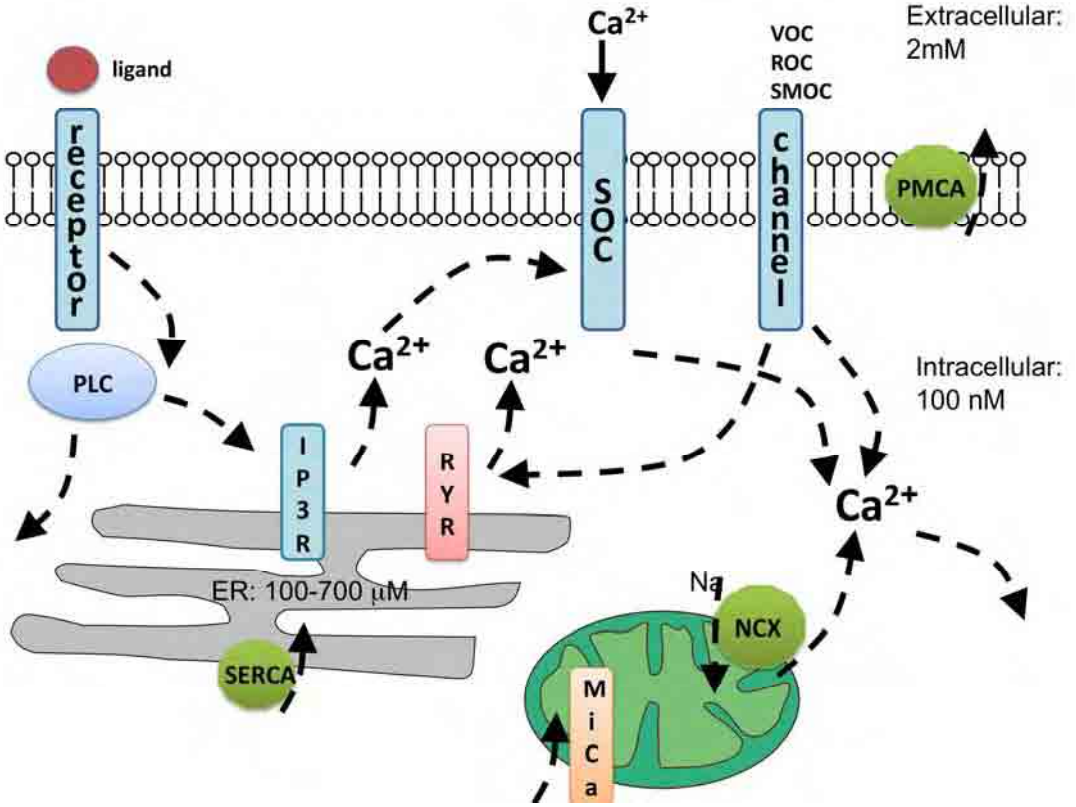


Figure 1.6: schematic representation of calcium fluxes. Under basal conditions, the extracellular calcium concentration is 2 mM, the intracellular calcium concentration is 100nM, while the endoplasmatic reticulum (ER) calcium concentration is 100-700μM. These differences create gradients that are responsible for different calcium fluxes. Voltage-operated channels (VOCs), receptor-operated channels (ROCs) and second messenger-operated channels (SMOCs), store operated channels (SOCs) and the transient receptor potential (TRP) channels, allow the calcium to enter from the extracellular environment into the cell. IP_3 receptors (IP_3Rs) and ryanodine receptors (RYRs) allow the calcium to flow from the ER (or the sacroplasmic reticulum) into the cytoplasm, and the mitochondrial calcium uniporter (MiCa) allows calcium movement from the cytoplasm to the mitochondria. Upon activation, calcium concentrations are restored by the plasma membrane calcium ATP-ase (PMCA), and the sarco(endo)plasmic reticulum calcium ATP-ase (SERCA) and the mitochondrial calcium potassium exchanger (NCX).

1.3 ITAM RECEPTOR SIGNALLING

1.3.1 Overview

A major function of cell surface receptors is to respond to appropriate stimuli and to generate intracellular signals. Such signals often lead to the nucleus where they are responsible for changes in gene expression and ultimately for generating different cellular behaviours. The B and T lymphocyte antigen receptors recognize different antigens and play different roles in immunity, but share a common signalling mechanism (Figure 1.7). Both of them contain a specific signalling motif in their cytoplasmic tails, known as the immunoreceptor tyrosine-based activation motif (ITAM), which is essential for signal transduction (Grande, Bannish et al. 2007). Other immune recognition receptors, such as some Fc receptors, contain and signal through ITAM motifs. These immune receptors share some of the signalling proteins and propagate the receptor signal in a similar way (Samelson 2002). On platelets, for example, the FcRγ chain, which contains a single ITAM motif, is coupled to glycoprotein VI (GPVI), forming the platelet collagen receptor GPVI/FcRγ complex.

1.3.2 ITAM motif

The ITAM motif was first described by Reth in 1989 and consists of the sequence YXX(L/I)$X_{6–12}$YXX(L/I) (in single-letter amino acid code, where Y is tyrosine, L is leucine, I is isoleucine and X represents any amino acid) (Reth 1989). The ITAM motif is expressed both by transmembrane proteins and cytoplasmic proteins, the latter of which are less common (Grande, Bannish et al. 2007). Transmembrane proteins containing ITAM motifs are expressed specifically in hematopoietic cells and are part of immunoreceptor complexes. These include Igα and Igβ chains of the B cell receptor (BCR) complex, CD3 and ζ chains of

the T cell receptor (TCR) complex and the β and γ chains of the FcεRI complex (Grande, Bannish et al. 2007). Cytoplasmic proteins that contain an ITAM motif are expressed in both hematopoietic and non-hematopoietic cells and include the proteins ezrin and radixin. These cytoplasmic proteins require recruitment to the membrane in order to be able to generate a signal (Grande, Bannish et al. 2007).

1.3.3 Proximal B and T cell receptor signalling

Upon engagement of the BCR or TCR, Src family protein tyrosine kinases are activated by a mechanism that is not entirely understood. Their phosphorylation of ITAM motifs provides docking sites for downstream effector molecules and adapter proteins via their tandem Src homology 2 (SH2) domains (Grande, Bannish et al. 2007). SH2 domains contain a positively charged pocket that binds the phosphorylated ITAM tyrosine and a hydrophobic pocket that binds the non polar leucine or isoleucine (Grande, Bannish et al. 2007). Two major effectors of the B and T cell receptors are the protein tyrosine kinases Syk in most hematopoietic cells

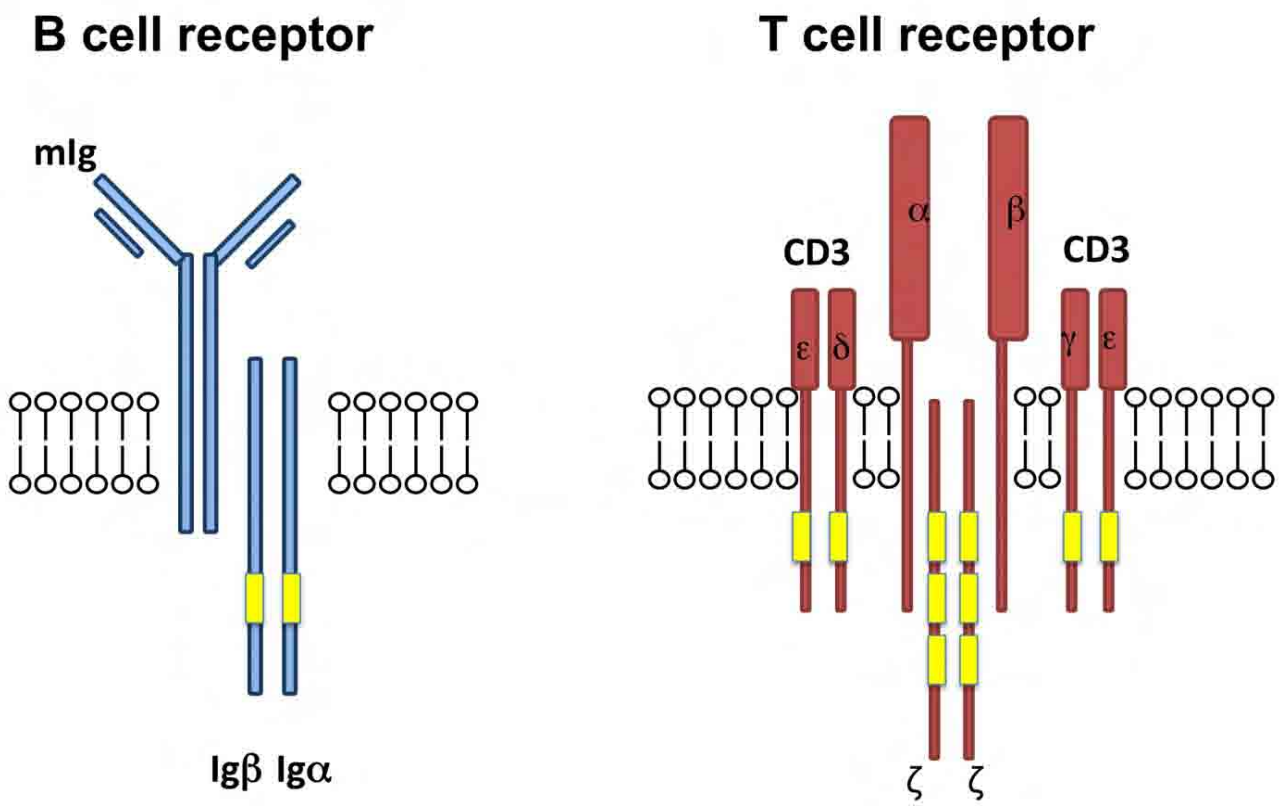


Figure 1.7: Schematic representation of the B and T cell receptors. The yellow boxes represent ITAM motifs.

and the related ZAP-70 in T cells, each of which possess tandem SH2 domains for ITAM binding. Such binding and activation of Syk/ZAP-70 is a fundamental step in ITAM-mediated signal transduction, as it amplifies signals, both by direct phosphorylation and activation of downstream signalling proteins and by recruiting other SH2-containing proteins to its own phosphorylated tyrosine-containing binding motifs.

1.3.4 Activation of PLCγ

Upon activation, Syk or ZAP-70 are responsible for propagating the signalling cascade. Both Syk and ZAP-70 engage a similar strategy, which involves the assembly of a "signalosome" which is responsible for the activation of PLCγ. Syk phosphorylates the adaptor protein BLNK in B cells, while ZAP-70 phosphorylates both LAT and SPL-76 in T cells (Figure 1.8) (Koretzky, Abtahian et al. 2006).

BLNK, LAT and SLP76 are adaptor proteins with no intrinsic enzymatic activity, but can assemble and activate multiple signalling molecules (Koretzky, Abtahian et al. 2006). They function as "scaffolds" for bridging signalling cascades, for activating signalling proteins and are key elements for signal amplification. BLNK and LAT/SLP76 contain multiple protein interaction domains and multiple phospho-tyrosines, that recruit SH2 domains, and proline motifs, that recruit SH3 domains (Myung, Boerthe et al. 2000). BLNK, LAT and SPL76 function as the docking site for several BCR and TCR signalling proteins, such as Vav, Nck, Btk/Itk, Grb2/Gads and PLCγ family members (Figure 1.8), which are responsible for activation of different downstream effectors. For example, the tyrosine kinases Btk, in B cells, and Itk, in T cells, are essential for the phosphorylation and activation of PLCγ enzymes (Koretzky, Abtahian et al. 2006).

PLCγ is one of the key proteins of the ITAM-induced signalling pathway, and is responsible for the cleavage of phosphatidylinositol 4-5-bisphosphate molecules, present in the plasma membrane, into the second messengers inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). Structurally, PLCγ contains a split pleckstrin homology (PH) domain, for plasma membrane localization via binding to inositol phospholipids, surrounding a central SH2-SH2-SH3 region (Wen, Yan et al. 2006). In the DT40 B cell line, the lack of PLCγ-2 resulted in the lack of downstream signalling and calcium mobilization, suggesting a key role for this protein in the BCR signalling pathway (Takata, Homma et al. 1995). Indeed, the two second messengers generated by PLCγ, IP_3 and DAG, trigger two distinct signalling cascades, the calcium and mitogen-activated protein kinase (MAPK) pathways, respectively (Berridge 1993). These two signalling cascades are the subjects of the following sections.

1.3.5 Calcium mobilization leads to NFAT activation

Calcium signals are essential in lymphocytes, as they regulate several cell functions, such as differentiation, effector function and gene transcription (Oh-hora and Rao 2008). IP_3 is responsible for the initial calcium release from the endoplasmic reticulum (ER) stores, which is followed by calcium influx across the plasma membrane. This is achieved by IP_3 engaging the IP_3 receptor on the ER. This causes the IP_3 receptor, which is a calcium channel, to open, resulting in a transient increase in intracellular calcium (Taylor, Rahman et al. 2009). This ER calcium store depletion is responsible for the opening of calcium release-activated calcium (I_{CRAC}) channels in the plasma membrane, leading to a sustained increase of intracellular calcium, by a mechanism which will be described in the following section (Oh-hora and Rao 2008).

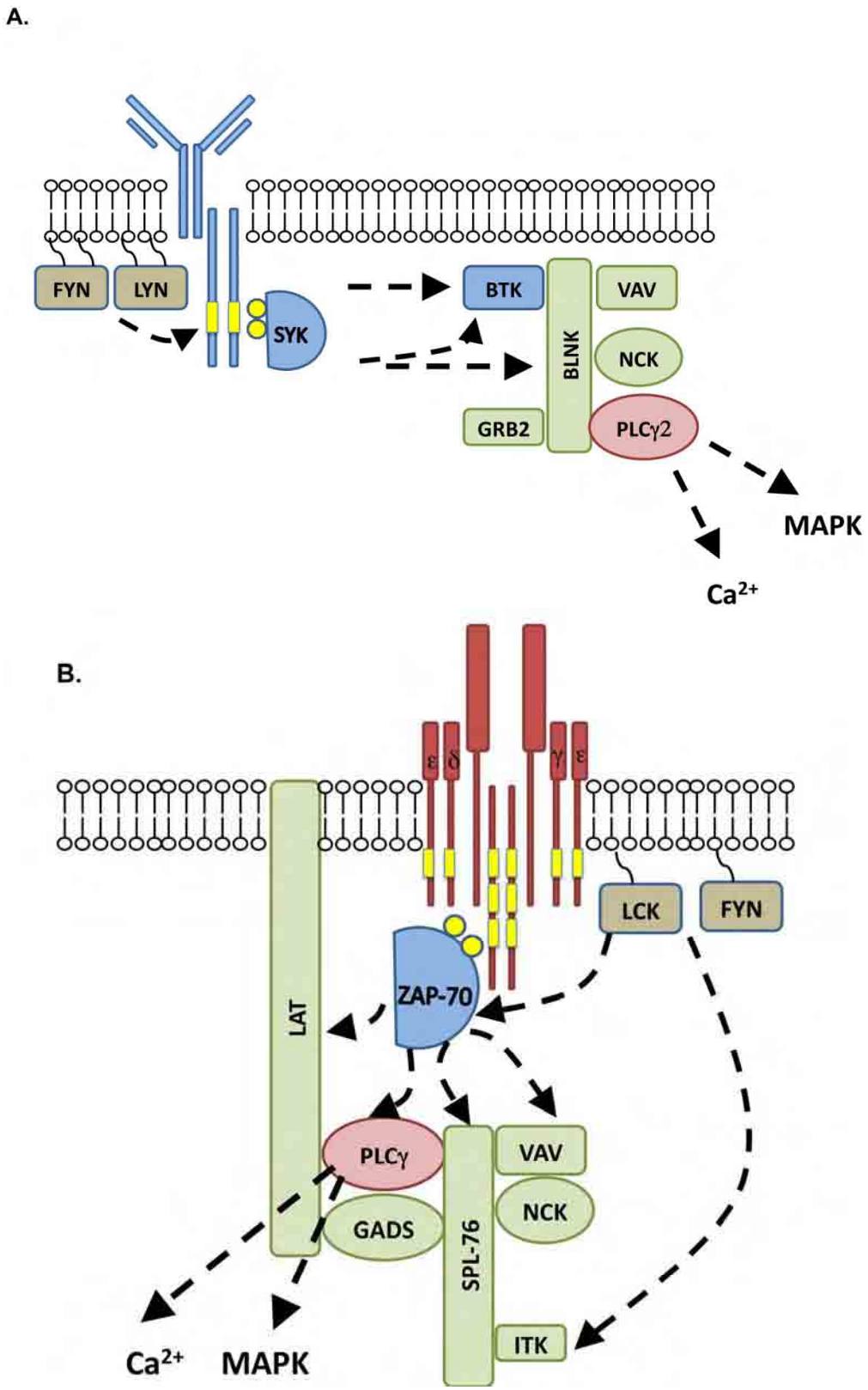


Figure 1.8: B cell receptor (panel A) and T cell receptor (panel B) signalling pathways. Square yellow boxes represent ITAMs motifs and yellow circles represent ITAM-binding SH2 domains.

The stromal-interacting molecule (STIM1) has recently been shown to promote calcium entry via I_{CRAC} channels following calcium release from ER stores (Liou, Kim et al. 2005; Roos, DiGregorio et al. 2005). STIM1 is a single-spanning transmembrane protein that is predominantly expressed on the ER membrane, and which functions as calcium "sensor" (Zhang, Yeromin et al. 2006). The EF-hand calcium-binding domain on the N-terminal luminal portion of STIM1 mediates this function (Liou, Kim et al. 2005). Upon depletion of calcium from the ER, bound calcium dissociates from the EF-hand motif of STIM1, and this elicits a conformational change in STIM1. STIM1 then moves laterally in the ER and forms punctuare accumulations, some in close proximity to the plasma membrane (Hogan and Rao 2007). STIM1 can subsequently induce opening of the I_{CRAC} channel (Hogan and Rao 2007).

The I_{CRAC} channel, responsible for outside-in calcium flux, is a multi-protein complex expressed on the plasma membrane (Figure 1.9). Although the precise protein complex has not been completely elucidated yet, a major subunit of the I_{CRAC} channel has been recently identified as Orai1 (Vig, Peinelt et al. 2006). Orai1 has four transmembrane domains and intracellular amino and carboxyl-terminus, and appears to form a functional I_{CRAC} channel following tetramerisation (Penna, Demuro et al. 2008). Moreover, a recent study by the Lewis research group demonstrated that STIM1 has a 107-residue I_{CRAC} activation domain that forms tetramers. This domain interacts with the amino and carboxy termini of Orai1, so inducing Orai1 tetramers. This study has therefore established that the direct binding of STIM1 to Orai1 is necessary for both the redistribution and activation of I_{CRAC} channels in response to calcium depletion (Park, Hoover et al. 2009).

The increase in cytoplasmic free calcium, generated by the calcium influx from the I_{CRAC} channel, regulates the activity of a large number of calcium-sensitive proteins. Calcium elevation leads to the activation of both calmodulin-dependent protein kinase II and the calmodulin-activated serine/threonine phosphatase calcineurin. One of the targets regulated by calcineurin is the transcription factor nuclear factor of activated T cells (NFAT), whose nuclear translocation is facilitated by dephosphorylation following interaction with a conserved PxIxIT motif in calcineurin (Liu, Masuda et al. 1999). De-phosphorylation results in the unmasking of a nuclear localisation sequence, leading to nuclear translocation and activation of transcription of target genes (Macian 2005). In the presence of sustained calcium elevation, sustained calcineurin activation maintains NFAT in a predominantly de-phosphorylated state in the nucleus. Once in the nucleus, NFAT can be re-phosphorylated by p38 MAPK, causing nuclear exit (Yang, Xiong et al. 2002). Five different members of the nuclear factor of activated T cells (NFAT) family have been identified: NFAT1, NFAT2, NFAT3, NFAT4 and NFAT5. NFAT1, NFAT2 and NFAT4 are expressed mainly in cells of the immune system where they regulate a large number of genes during the immune response (Macian, Lopez-Rodriguez et al. 2001).

NFAT1-4 contain a conserved regulatory domain in the amino-terminal region, which controls both NFAT cellular distribution and transcriptional activation, followed by a DNA-binding domain and a carboxy-terminal region (Macian, Lopez-Rodriguez et al. 2001; Macian 2005). NFAT5 possesses specific features that differentiate it from the classical NFAT1-4 members: NFAT-5 is the only non calcium-regulated NFAT protein. In lymphocytes, NFAT-5 regulates the expression of osmotic stress-induced cytokines, including tumour necrosis factor (TNF) and lymphotoxin-β (Macian 2005).

1.3.6 The MAP kinase pathway leads to AP-1 activation

The second product of PLCγ is DAG, which is a membrane glyceride composed of two fatty acid chains covalently bonded to a glycerol molecule. DAG is responsible for the activation of protein kinase C (PKC) and the Ras exchange factor RasGRP, leading to downstream Ras activation. Ras is a monomeric GTP-binding molecular switch that is activated when bound to GTP, and inactive when bound to GDP. Ras is localized to the plasma membrane via a farnesyl anchor, and upon activation can recruit and activate the serine/threonine kinase Raf. Raf phosphorylates and activates MEK, a dual specificity kinase which phosphorylates and activates Erk. Active Erk then translocates to the nucleus where its targets include the transcription factors c-Fos and c-Jun, which subsequently dimerize to form the activator protein 1 (AP-1) transcription factor complex, leading to gene transcription (Buday and Downward 2008).

1.3.7 NFAT/AP-1 promoter activation

Cooperation between NFAT and AP-1 proteins on composite DNA elements, which contain adjacent NFAT (T/AGGAAA/T) and AP-1 (TGAGTCA) binding sites, regulates the expression of a diverse group of genes, including interleukin-2, that are essential for a productive immune response (Figure 1.9) (Macian, Lopez-Rodriguez et al. 2001).

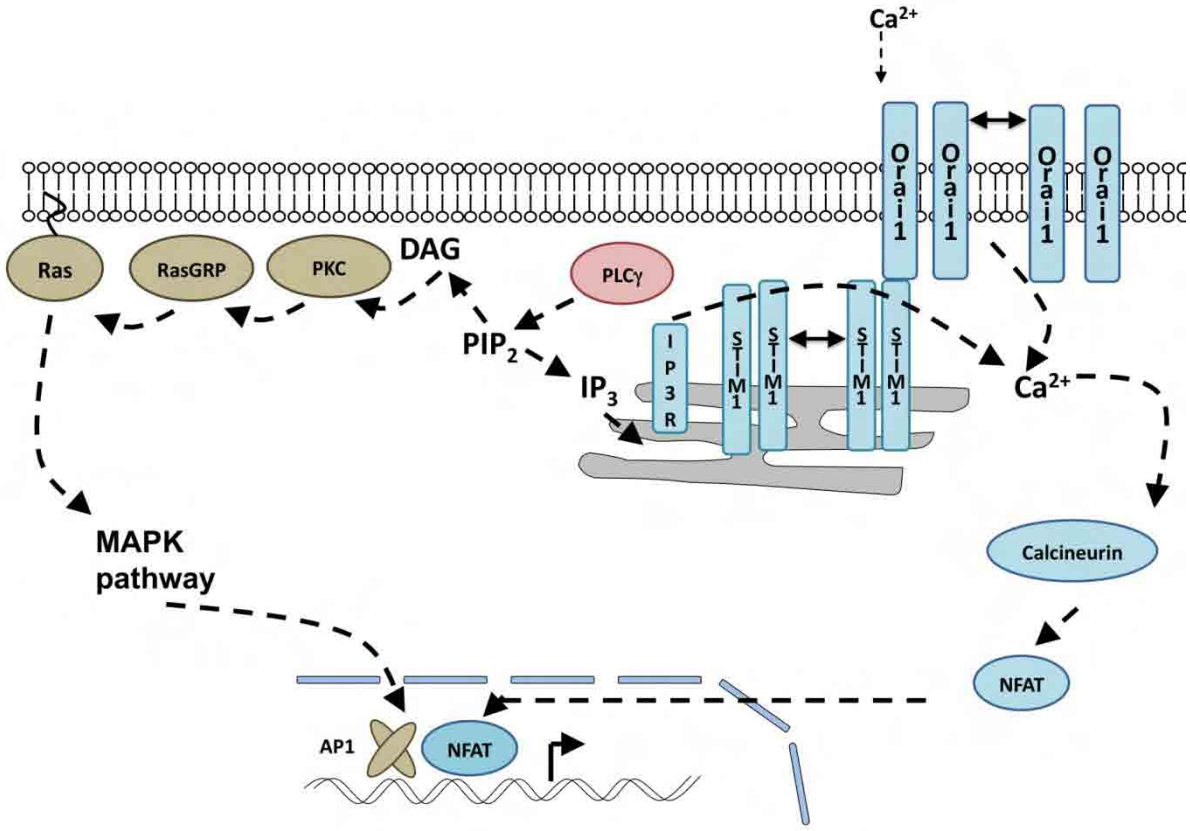


Figure 1.9 Schematic representation of lymphocyte signalling pathways downstream of PLCγ. The two PLCγ-induced second messengers, IP_3 and DAG, trigger two different signalling pathways. IP_3 binds to the IP_3-gated calcium channel on the ER membrane, inducing calcium release from the ER. The ER-resident calcium sensor, STIM1, undergoes a conformational change and re-localises to ER-plasma membrane junctions where it interacts with and induces Orai1 tetramers, so creating an I_{CRAC} channel which allows calcium entry across the plasma membrane. This increase in intracellular calcium is responsible for the activation of several calcium-sensitive proteins, including the phosphatase calcineurin which dephosphorylates NFAT. Upon de-phosphorylation, NFAT translocates into the nucleus. The second PLCγ product, DAG, activates protein kinase C (PKC) and RasGRP, leading to activation of Ras and the MAP kinase signalling pathway, the latter of which is responsible for the assembly of Fos and Jun heterodimers to form the AP-1 transcription factor. The co-operation of the NFAT and AP-1 transcription factors is required for the active transcription of interleukin-2 and many other important lymphocyte genes.

1.4 PLATELET COLLAGEN RECEPTOR GPVI AND PLATELET TETRASPANINS

1.4.1 Platelets

Platelets are of major interest to our group because of their central roles in haemostasis and thrombosis. Platelets are small and anuclear blood cells that circulate in a quiescent state but undergo rapid activation following damage to the blood vessel wall, leading to formation of a vascular plug and cessation of bleeding. This rapid response is mediated by a large repertoire of cell surface molecules, including immunoglobulin superfamily proteins, integrins, leucine-rich repeat and G-protein-coupled receptors. These receptors interact with a wide range of ligands such as extracellular matrix proteins, surface molecules on other cells and soluble agonists (Varga-Szabo, Pleines et al. 2008).

Platelets also play a role in the disease process termed atherosclerosis. This is the progressive thickening and hardening of the arterial wall leading to plaque formation, due to the deposition of fatty deposits under the endothelium and recruitment of inflammatory leukocytes. Plaque rupture and subsequent blockage of the artery by activated platelets can result in heart attack or stroke. However, it has recently emerged that platelet interactions with endothelial cells are an initiating event in atherosclerosis, and such interactions play a role throughout disease progression (Langer and Gawaz 2008). Indeed, at sites of turbulent blood flow or under inflammatory conditions, platelets can adhere to endothelium via selectins, integrins and immunoglobulin superfamily molecules. Activated platelets release chemokines and inflammatory cytokines, which stimulate endothelial cells to upregulate adhesion molecules and release further chemokines, which together recruit leukocytes and exacerbate inflammation (Langer and Gawaz 2008).

1.4.2 Platelet collagen receptor GPVI

Platelet glycoprotein VI (GPVI) is the major signalling receptor for collagen, the most thrombogenic component of the subendothelial matrix and atherosclerotic plaque. GPVI is composed of two Ig domains, an O-glycosylated stalk, a transmembrane region and a cytosolic sequence of 51 amino acids in human or 27 amino acids in mouse (Figrue 1.10) (Moroi and Jung 2004). GPVI is coupled to a disulphide-linked Fc receptor (FcR) γ-chain homodimer in the membrane via a salt-bridge between charged amino acids in the transmembrane regions (Feng, Garrity et al. 2005). FcRγ contains an ITAM motif, which couples GPVI activation to the activation of Syk in a similar manner to the antigen receptors for B and T cells (Figure 1.10) (Watson, Auger et al. 2005). The GPVI signalling pathway culminates in inside-out activation of integrins and platelet secretion (Nieswandt and Watson 2003). Critical to the understanding of platelet function are the way in which receptors such as GPVI are organised on the platelet surface and the manner whereby GPVI signals interact with those of other receptors to promote activation.

1.4.3 Regulation of platelet function by tetraspanins

Identification of platelet tetraspanins is difficult due to the lack of antibodies to most tetraspanins and the relatively low amount of mRNA in the anucleate platelet. However, using serial analysis of gene expression (SAGE) and DNA microarrays, 19 tetraspanins were identified in megakaryocytes, the platelet progenitors (Protty, Watkins et al. 2009). Using antibodies, five tetraspanin proteins have been reported to be expressed on platelets: CD9

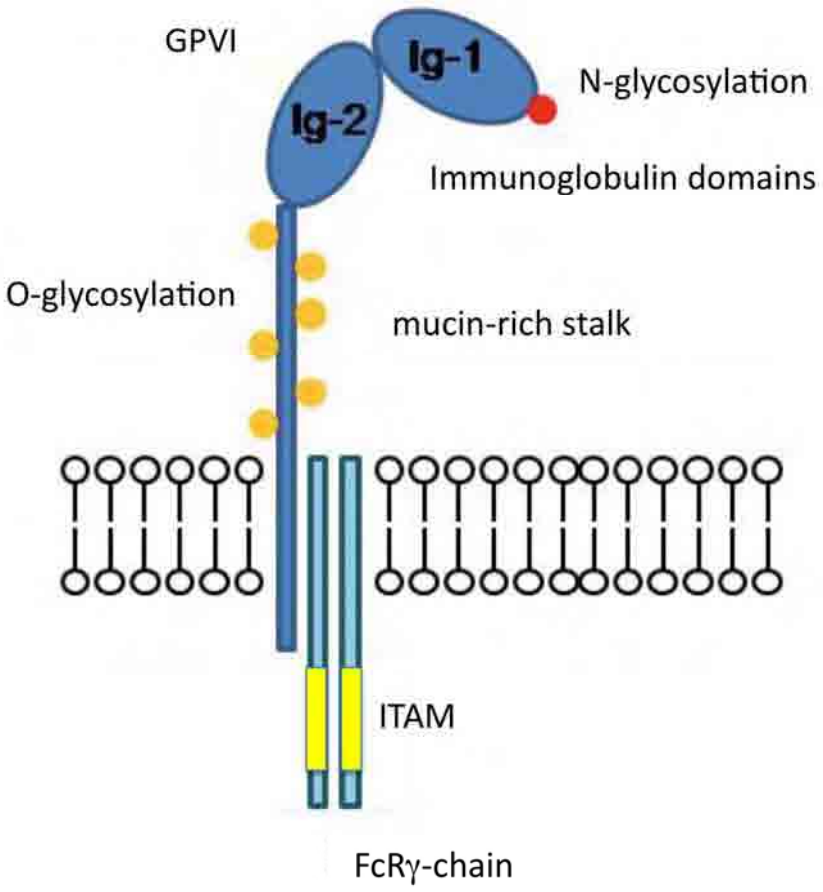


Figure 1.10 Schematic representation of the GPVI/FcRγ complex. The GPVI/FcRγ complex is the platelet collagen receptor. Structurally, GPVI it is composed of two Ig domains (one of which is N-glycosilated, in red), an O-glycosylated stalk (in yellow), a transmembrane region and a short cytosolic sequence. GPVI is coupled to a disulphide-linked Fc receptor (FcR) γ-chain which contain an ITAM motif (in yellow).

(Boucheix, Soria et al. 1983), CD63 (Nieuwenhuis, van Oosterhout et al. 1987), CD151 (Ashman, Aylett et al. 1991), Tspan32 (Robb, Tarrant et al. 2001) and Tspan9 (Protty, Watkins et al. 2008). Several other tetraspanins, including Tspan18, have been identified in platelets by proteomics (Lewandrowski, Wortelkamp et al. 2009), although confirmation awaits the generation of antibodies (Tomlinson 2009).

The roles of CD151 and Tspan32 were evaluated in platelets by the Jackson group using mice deficient in either of these tetraspanins. The two strains of mice had similar phenotypes, notably a mild bleeding defect detected by the tail bleeding assay, partially defective platelet aggregation to a variety of agonists, delayed clot retraction, impaired platelet spreading and partially defective *in vivo* thrombus formation. The authors also presented evidence that CD151 and Tspan32 each associate with αIIbβ3 and, as outlined in Section 1.1.4, speculated that these tetraspanins regulate "outside-in" $\alpha_{IIb}\beta_3$ signalling (Lau, Wee et al. 2004; Goschnick, Lau et al. 2006). However, our group have been unable to detect an interaction between $\alpha_{IIb}\beta_3$ and tetraspanins in human platelets (Protty, Watkins et al. 2008).

In contrast to CD151 and Tspan32, deletion of CD9 or CD63 has relatively minor effects on platelet function. CD9 is expressed at a level of approximately 49,000 copies per platelet (Protty, Watkins et al. 2008), which makes this tetraspanin the second most highly expressed protein at the platelet cell surface, behind $\alpha_{IIb}\beta_3$. Using CD9-deficient mice it has been demonstrated that CD9 negatively regulates the activation of the integrin $\alpha_{IIb}\beta_3$, although the phenotype is weak and leads to only marginally larger thrombi in an *in vivo* model (Mangin, Kleitz et al. 2009). The recent generation of the CD63-deficient mouse did not reveal a major role for this tetraspanin in platelets. Indeed, the only demonstrable phenotype was marginally enhanced platelet aggregation *in vitro*, in response to stimulation with collagen, ADP or a

thromboxane mimetic, but this was not accompanied by an effect on thrombus formation *in vivo* (Schroder, Lullmann-Rauch et al. 2009). Our group recently identified Tspan9 as the fifth platelet tetraspanin and showed it to be relatively specific to the megakaryocyte/platelet lineage, although its function is unknown (Protty, Watkins et al. 2008). In the same study, GPVI was found to be a component of tetraspanin microdomains on platelets (Protty, Watkins et al. 2008).

1.5 THESIS AIM

The initial aim of this thesis was to determine whether any platelet tetraspanins play a role in signal transduction. This was done by over-expressing tetraspanins in the DT40 model B cell line with an NFAT/AP-1 transcriptional reporter. NFAT/AP-1 reporter assays are a sensitive method to measure calcium and MAPK signal transduction. Our group had previously developed this model system to study signalling of the platelet collagen receptor GPVI (Tomlinson, Calaminus et al. 2007). The major aim of the thesis was developed from these initial experiments, and became the characterization of a novel tetraspanin protein, Tspan18, which was found to have the unique capability to activate NFAT/AP-1. A final aim of the thesis was to test if Tspan18, or any other platelet tetraspanin, could affect GPVI signalling in response to collagen.

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Antibodies and reagents

The antibodies used are described in Table 2.1. Firefly luciferin was from LuxBiotech (Edinburgh, UK). All general laboratory materials were purchased from Sigma (Poole, UK), and all tissue culture reagents and materials were purchased from PAA Laboratories (Somerset, UK).

Antibody	Source	Experimental concentrations
Mouse anti-FLAG epitope (clone M2)	Sigma	IP: 5 μg/ml WB: 10 μg/ml
Mouse anti-human GPVI (clone 204-11)	Dr. Masaaki Moroi	FC: 10μg/ml
Mouse anti-Myc epitope (clone 9B11)	Cell signalling technology	WB: 1 μg/ml
Donkey anti--rabbit IgG, HRP-conjugated	Amersham Pharmacia Biotech	WB: 1 μg/ml
Sheep anti-mouse IgG, FITC-conjugated	Sigma	FC: 15 μg/ml
Sheep anti-mouse IgG, HRP-conjugated	Amersham Pharmacia Biotech	WB 1 μg/ml
Rabbit anti-flag	Sigma	WB: 0.5 μg/ml

Table 2.1 Details of antibodies. Legend: IP: immunoprecipitation, WB: western blotting, FC: flow cytometry

2.1.2 Plasmids and constructs

The vector used for tetraspanin cloning was pEF6/*Myc*-His (Invitrogen) (Figure 2.1).

Human GPVI and FcRγ expression constructs were as described (Tomlinson, Calaminus et al. 2007).

The NFAT luciferase reporter construct contains three copies of the distal NFAT site from the IL-2 promoter (Shapiro et al., 1996) and was a gift from Prof. A. Weiss. The pEF6-*lacZ* construct was obtained from Invitrogen.

2.1.3 Inhibitors and agonists used in luciferase assays

EGTA: EGTA (Ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid) is a high affinity calcium chelating agent. EGTA was used at a concentration of 4 mM.

Cyclosporin A: Cyclosporin A is a drug currently used after transplantation, and it is a selective calcineurin inhibitor. The cyclosporin A mechanism of action involves the formation of a complex with its binding protein, cyclophilin (Iano Ikuko, 2003). Subsequently, the cyclosporin A-cyclophilin complex binds to and inhibits the activity of the protein phosphatase calcineurin, which is responsible for the dephosphorylation and subsequent nuclear translocation of NFAT proteins. Cyclosporin A was used at a concentration of 2mM.

PMA: PMA (Phorbol 12-myristate 13-acetate) is a powerful tumour promoter and a potent activator of protein kinase C (PKC) and RasGRP, which activate the MAP kinase pathway. PMA was used at a concentration of 50 ng/ml.

Ionomycin: Ionomycin is an ionophoric antibiotic that binds calcium and serves as an effective mobile carrier of this cation (Chao-min Liu and Theron E. Hermann, 1978). Ionomycin has become a useful tool over other ionophores for studies of calcium transport

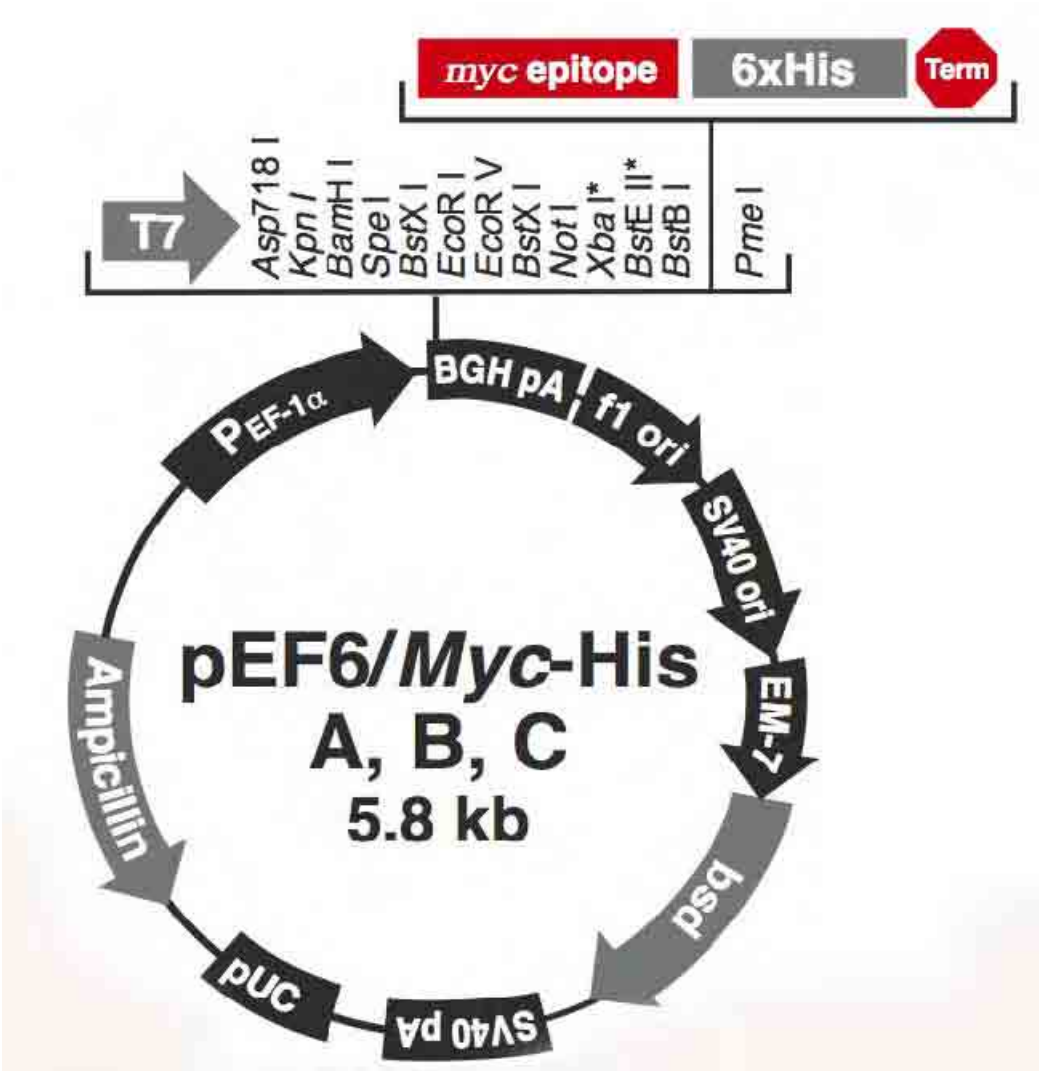


Figure 2.1 Map of the pEF6/*Myc*-His vector (Invitrogen).

across membranes. Ionomycin induces a rise in intracellular free calcium, although the precise molecular mechanism of action is still unknown. Ionomycin was used at 1 μM.

2.1.4 Cells and tissues

Wild type DT40 chicken B cells and DT40 deficient in Lyn/Syk (Takata et al., 1996), PLCγ2 (Takata et al., 1995) and IP_3R (Sugawara, Kurosaki et al. 1997) were kindly provided by Dr. T. Kurosaki (Kansai Medical University, Moriguchi, Japan).

Jurkat human T cells (clone E6.1) were a gift from Dr. A. Weiss (University of California, San Francisco, USA) and were previously characterized (Strauss et al, 1992).

Human aortic smooth muscle cells were a gift from Prof. R. Bicknell (Institute of Biomedical Research, University of Birmingham).

Human peripheral blood leucocytes were collected at the interface of red cells and plasma after centrifugation of citrated whole blood at 200 x*g* for 20 minutes.

DAMI cells are a human megakaryocyte-like cell line (Greenberg, Rosenthal et al. 1988) and were obtained from Prof. J. Frampton (Institute of Biomedical Research, University of Birmingham).

DG75 cells are an Epstein-Barr virus-negative human B-cell line that were obtained from Dr M. Rowe (Institute for Cancer Studies, University of Birmingham).

Primary human fibroblasts were a gift from Prof. R. Bicknell (Institute of Biomedical Research, University of Birmingham).

Human K562 are a myelogenous leukaemia cell line (Lozzio and Lozzio 1975) that was a gift from Dr Padma-Sheela Jayaraman (Institute of Biomedical Research, University of Birmingham).

HPB-ALL are a human T-cell line that was provided by Prof. J. Gordon (Institute of Biomedical Research, University of Birmingham).

The HEL human erythroleukaemia cell line was obtained from Prof. J. Frampton (Institute of Biomedical Research, University of Birmingham).

The HEK-293T human embryonic kidney cell line (Graham, Smiley et al. 1977) was obtained from Prof. J. Frampton (Institute of Biomedical Research, University of Birmingham).

Primary human hepatocyte total mRNA was a gift from Dr Peter Balfe (Institute of Biomedical Research, University of Birmingham).

Primary human umbilical vein endothelial cells (HUVECs) were obtained from Dr V. Heath (Institute of Biomedical Research, University of Birmingham).

MDA-MB-231 are a human epithelial cell line (Young, Cailleau et al. 1974) that was a gift from Dr F. Berditchevski (Institute for Cancer Studies, University of Birmingham).

HMEC-1 is a human microvascular endothelial cell line (Ades, Candal et al. 1992) that was a gift from Dr. V. Heath and Prof. R. Bicknell (Institute of Biomedical Research, University of Birmingham).

The RAJI human B cell line was obtained from Prof. J. Gordon (Institute of Biomedical Research, University of Birmingham).

The U937 monocyte cell line was obtained from Prof. J. Frampton (Institute of Biomedical Research, University of Birmingham).

The following mouse tissues were obtained by dissection of 6-8 week-old C57 BL/6 mice: brain, heart, kidney, liver, lung, muscle, spleen and thymus.

For peripheral blood leucocytes (PBL), human blood was taken from healthy and drug-free volunteers using sterile 1:10 (v/v) sodium citrate and 1:9 (v/v) acidic citrate dextrose (ACD) (120 mM sodium citrate, 110 mM glucose, 80 mM citric acid) as anti-coagulants. Whole blood was centrifuged at 200x*g* for 20 minutes, platelet-rich plasma (PRP) was removed and PBL were collected from the erythrocyte-plasma interface. The PBL were then washed twice with Tyrode's-Hepes buffer (134 mM NaCl, 2.9 mM KCl, 0.34 mM Na_2HPO_4, 12 mM $NaHCO_3$, 20 mM HEPES, 5mM glucose, 1 mM $MgCl_2$, pH 7.3).

2.2 CELL CULTURE

HEK-293T were grown in DMEM medium, supplemented with 10% heat inactivated fetal bovine serum (FBS), 100 units/ml penicillin, 100 μg/ml streptomycin, and 20 mM glutamine.DT40 cells were cultured in RPMI 1640 supplemented with 10% heat inactivated FBS, 1% chicken serum, 100 units/ml penicillin, 100 μg/ml streptomycin 50 μM β-mercaptoethanol and 20 mM glutamine. Jurkat cells were cultured in RPMI 1640 supplemented with 10% heated inactivated FBS, 100 U/ml penicillin, 100 μg/ml streptomycin and 20 mM glutamine.

All cells were kept at an exponential phase of growth and kept in 5% CO_2/95% air in a humidified incubator at 37°C.

2.3 FUNCTIONAL STUDIES

2.3.1 Transient cell transfection

DT40 and Jurkat cells were transfected by electroporation. 1 x 10^7 DT40 or Jurkat were used for each sample.

DT40 cells were counted and washed once with cytomix buffer (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 $MgCl_2$, pH 7.6) supplemented on the day of the experiment with 5 mM glutathione and 40 μM ATP.DT40 cells were resuspended in 400 μl of cytomix buffer and placed into an electroporation cuvette, together with the appropriate DNA constructs. Cells were electroporated using a GenePulser II (Biorad) set at 350 V, 500 μF, were left 5 minutes at room temperature, incubated 10 minutes on ice and resuspended in 8 ml of complete medium. Sixteen hours after transfection, cells were used for the experiment.

Jurkat cells were counted and washed once with serum free RPMI 1640 medium and resuspended in 400 μl of RPMI 1640 medium and placed into an electroporation cuvette, together with the appropriate DNA constructs. Cells were electroporated using a GenePulser II (Biorad) set 250 V and 950 μF, left 5 minutes at room temperature, incubated 10 minutes on ice and resuspended in 8 ml of complete medium. Sixteen hours after transfection, cells were used for the experiment.

HEK-293T cells were transfected by the calcium phosphate method. On the day before the transfection, 3 x 10^6 cells were seeded into 10 cm plates in 10 ml complete medium. For the transfection, 5 μg of DNA was added to 450 μl of sterile distilled water, followed by 63 μl of 2 M $CaCl_2$. Then 500 μl of 2X HEPES-buffered saline (280 mM NaCl, 10 mM KCl, 1.5 mM

Na_2HPO_4, 50 mM HEPES, 12 mM dextrose, pH 7.5) was added drop-wise to the solution containing the DNA, mixing between drops. The solution was left for 15 minutes at room temperature to allow the calcium phosphate-DNA precipitate to form, then added drop-wise to the cells. After 16 hours incubation, the supernatant was removed and fresh medium was added to the cells. Cells were incubated for an additional 24 hours, to allow the transfected proteins to be expressed, before harvesting by scraping.

2.3.2 Luciferase assay

DT40 or Jurkat cells were transfected with 20 μg of NFAT/AP-1- or AP-1-luciferase reporter constructs, and 2 μg of a β-galactosidase construct under the control of the EF-1α promoter, to act as a control for transfection efficiency. Sixteen hours after transfection, live cells were counted using Trypan blue exclusion, and the concentration was adjusted to 2×10^6 cells/ml for each sample. An aliquot of 250 μl was used for β-galactosidase assay and a second aliquot of 250 μl was used for flow cytometry (to determine GPVI surface expression, in GPVI experiments). The luciferase assay was performed in triplicate wells of a 96-well plate, using 1×10^5 cells per well in a volume of 100 μl. Cells were unstimulated or stimulated for a period of 6 hours with the following different stimuli and/or inhibitors: 50 ng/ml PMA, 1 μM ionomycin, 4 mM EGTA and 2 mM cyclosporin A. The assay was harvested by first lysing the cells for 5 minutes at room temperature using 11 μl of harvest buffer (200 mM potassium phosphate buffer, pH 7.8, 12.5% Triton X-100 and 4 mM DTT). Following lysis, 100 μl of cell lysate was added to 100 μl of assay buffer (200 mM phosphate buffer pH 7.8, 20 mM $MgCl_2$ and 10 mM ATP) in a white, opaque 96-well plate. The luciferase activity was measured using a Centro LB 960 microplate luminometer (Berthold Technologies, Germany)

after injection of 50 μl of 1 mM luciferin and measured over a 10 second period. Data were expressed as relative luciferase activation, following correction to control β-galactosidase activity, using the geometric mean and standard error of at least two independent experiments.

2.3.3 β-galactosidase assay

The β-galactosidase assay was performed using an aliquot of 5 x 10^5 cells, using the Galacto-Light^{TM} β-Galactosidase Reporter Gene Assay System (Applied Biosystems, Bedford, Mass, USA), following the manufacturer's protocol. β-galactosidase activity was measured in triplicate using a microplate luminometer (Centro LB 960, Berthold Technologies, Hertfortshire). All luciferase assay data were normalized to the relative β-galactosidase value.

2.3.4 Flow cytometry

Flow cytometry was used to assess the surface expression of GPVI following transient transfection. Samples of 5 x 10^5 cells were collected and stained in a 50 μl volume of FACS buffer (PBS, 0.2% (w/v) BSA, 0.02% (w/v) sodium azide) with 10 μg/ml mouse anti-human GPVI mAb for 30 minutes at 4°C. Cells were then washed once in 1 ml FACS buffer, and incubated for 30 minutes with 15 μg/ml FITC-conjugated anti-mouse IgG secondary antibody, in a 50 μl volume at 4^0C. Cells were then resuspended in 250 μl of FACS buffer containing propidium iodide at 2 μg/ml to label and allow gating out of dead cells, and analyzed by flow cytometry using a FACScalibur (BD Biosciences). Data were collected and analyzed using CellQuest software.

2.4 PROTEIN STUDIES

2.4.1 SDS polyacrylamide gel electrophoresis, western blotting and immunoblotting

Cells were lysed in 1% Triton X-100 lysis buffer (1% (v/v) Triton X-100, 10 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.01% (w/v) sodium azide). The following inhibitors were added to the lysis buffer just before use: 200 μg/ml AEBSF (4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride), 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 μg/ml pepstatin A, 10 mM NaF and 2 mM sodium orthovanadate. Following lysis for 30 minutes on ice, insoluble debris was removed by centrifugation for 10 minutes at 18,000 x*g* in a micro-centrifuge at 4^{o}C. Cell lysates were mixed with an equal volume of 2x Laemmli sample buffer.

Proteins were separated using 12% sodium dodecyl-sulphate (SDS)-polyacrylamide gels. Prestained molecular weight markers (Bio-Rad, Hemel Hempstead, UK) were used in all gels. Separation was performed at room temperature using a constant voltage of 125 mV. Upon protein separation, polyvinylidene fluorite (PVDF) membranes were quickly soaked in blotting buffer (25 mM Tris, 250 mM glycine, 20% methanol) and proteins were transferred to membranes by a Trans-Blot SD semi-dry transfer apparatus (Biorad), using a current of 110 mA for 25 minutes. Membranes were blocked for 1 hour at room temperature in TBS-T (20 mM Tris, 137 mM NaCl, 0.1% (v/v) Tween-20, pH 7.4) containing 5% (w/v) milk powder. The blocked membrane was stained with primary antibody in TBS-T containing 10% (w/v) bovine serum albumin (BSA) and 0.1% (w/v) sodium azide for one hour at room temperature under constant agitation, and washed three times in high salt TBS-T (TBS-T containing 500 mM NaCl) for 10 minutes each wash. The membrane was then incubated for one hour at room

temperature (or overnight at 4 ^{o}C) with HRP-conjugated secondary antibody, diluted in TBS-T containing 3% (w/v) milk powder. Membranes were washed five times, for five minutes each wash, in high salt TBS-T and developed using an enhanced chemiluminescence (ECL) kit (Amersham Bioscience, Buckinghamshire, UK), following the manufacturer's instructions, using Amersham Hyperfilm ECL (GE Healthcare) films and a Compact X4 film processor (Xograph Imaging Systems).

2.4.2 Lipid raft separations

A transfected 10 cm plate of HEK-293T cells was washed twice with 10 ml PBS and lysed for 30 minutes in 1 ml of ice-cold GEM lysis buffer (1% (v/v) Triton X-100, 25 mM Tris pH 7.5, 150 mM NaCl, 5 mM EDTA, 200 μg/ml AEBSF, 10 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, 10 mM NaF and 2 mM sodium orthovanadate). The supernatant was mixed with 1 ml ice-cold 80% (w/v) sucrose in GEM lysis buffer and laid at the bottom of an ultracentrifuge tube. In order to create a sucrose step gradient in the tube, 2 ml of 30% (w/v) ice-cold sucrose in GEM buffer was laid on top of the sample. On top of that was laid 1 ml of ice-cold 5% (w/v) sucrose in GEM buffer.

Samples were ultracentrifuged at 200,000 x*g* for 16 hours at 4 ^{o}C in a Beckman Coulter ultracentrifuge, using a Sw Ti 55 rotor (Beckman Coulter, High Wycombe). Following ultracentrifugation, 12 aliquots of 400 μl were collected from the top of the tube for each sample. In order to dissolve the lipid rafts, 1% (w/v) of N-Octyl-β-D-gluco-maltoside was added to each aliquot. Each sample was analyzed both by dot-blot and by electrophoresis.

2.4.3 Dot-blot analysis

100 μl of each aliquot from the lipid raft preparation was analyzed by dot-blot, using a Bio-dot microfiltration apparatus (Bio-Rad, Hertfortshire). Samples were immobilized on a nitrocellulose membrane and washed twice with TBS. The membrane was blocked with 5% (w/v) milk powder in TBS-T and probed with 0.33 μg/ml cholera toxin B subunit conjugated to peroxidase. The membrane was incubated for one hour at room temperature, washed five times, for five minutes each wash, with TBS-T and developed using ECL and film.

2.5 MOLECULAR BIOLOGY

2.5.1 Generation of tetraspanin constructs

Tetraspanin expression constructs were generated by Kate Fitzpatrick-Ellis and Dr Victoria Heath. In brief, tetraspanin cDNAs were generated by PCR and cloned into the pEF6/*Myc*-His A vector (Invitrogen), which had been modified to include an in-frame, upstream sequence for the FLAG epitope tag (amino acid sequence: MDYKDDDDK, M is methionine, D aspartic acid, Y tyrosine and K lysine) (Figure 2.1), between the Kpnl and BamH1 restriction enzyme sequences. The endogenous stop codons were retained for each tetraspanin construct to prevent translation of the Myc and His epitope tags.

2.5.2 CD9/Tspan18 chimera and Tspan18 isoform 1 constructs

The CD9/Tspan18 chimera construct was generated by a two-step PCR method (Higuchi et al., 1988). This construct contains cytoplasmic and transmembrane domains of human CD9 and small and large extracellular regions of human Tspan18. Tspan18 isoform 1 was generated by PCR from the IMAGE clone 3357734.

The oligonucleotides used to generate PCR products are detailed in Table 2.2.

Construct	Vector	Restriction sites	Primers
CD9/Tspan18 chimera	pEF6/*Myc*-His-A	*Bam*HI and *Eco*RV (vector), *Bgl*II and EcoRV (insert)	Forward 5'-TAGTAGGGATCCCCGGTCAAAGGAGGCACCAAG-3' Reverse 5'-TAGTAGGATGATTTAGACCATCTCGCGGTTCCT-3'
Tspan18 isoform 1	pEF6/*Myc*-His-A	*Bam*HI and *Xba*I (vector), *Bgl*II and *Xba*I (insert)	Forward 5'-TAGTAGGGATCCGAAGGCGACTGTCTGAGCTGC-3' Reverse 5'-TAGTAGTCTAGAGGCTGTGGCTCCGCGTGT-3'

Table 2.2 Oligonucleotides used to generate the CD9/Tspan18 chimera and Tspan18 isoform 1 constructs.

2.5.3 DNA amplification by PCR

Tetraspanin PCR products for cloning were generated in 100 µl reaction volumes using *Pfu*Turbo DNA polymerase (Stratagene) according to the manufacturer's protocol. Forward and reverse primers were used at a concentration of 0.4 pmol/µl, and dNTPs were used at a concentration of 0.2 mM each nucleotide. Standard PCR conditions were the following: denaturation at 93 ^{o}C for 5 minutes, annealing at 55 ^{o}C for 60 seconds, and elongation at 72 ^{o}C for 60 seconds (30 cycles). PCR products were separated on 1.5% agarose gels (prepared in TBE buffer: 89 mM Tris, 89 mM boric acid, 20 mM EDTA, 0.005% ethidium bromide, pH

8) and purified using a QiaQuick column (Qiagen) and the column was eluted with 50 μl of appropriate buffer according to the manufacturer's protocol.

2.5.4 Restriction enzyme digest

Digestion of DNA with specific restriction enzymes was used for both analytical purposes and for the isolation of specific fragments. Digestions were performed at the temperature and in the appropriate salt buffer suggested by the manufacturer for at least 3 hours. Digestion was followed by gel purification, using a Qiaex Kit (Qiagen).

2.5.5 Ligation of DNA into vectors

DNA fragments were ligated into the pEF6/Myc-His A plasmid in a reaction volume of 10 μl, containing 20 ng of linear vector, 3-6 times more insert in molar terms, and 1 unit of T4 DNA ligase (Roche) per reaction. Ligations were performed at room temperature for 5 minutes, employing a Quick Ligation Kit (Roche), and following the manufacturer's instructions.

2.5.6 Transformation of plasmid DNA into bacteria

Following ligation, 1 μl of the ligation mix was added to 50 μl of competent DH5α bacteria (Invitrogen) and placed on ice for 30 minutes. The mixture was then heat shocked at 37 $^{\circ}$C for 20 seconds, and placed again on ice for 2 minutes. After the addition of 200 μl of SOC medium (Invitrogen), bacteria were incubated for one hour at 37 ^{o}C, to allow the ampicillin antibiotic-resistance gene to be expressed. Bacteria were then spread on Luria-Bertani (LB)

agar ampicillin plates (1% NaCl, 1% Tryptone, 0.5% yeast extract, 1.5% agar, 100μg/ml ampicillin, pH 7 and incubated overnight at 37 °C.

Colonies were individually screened by PCR using one insert primer and one vector primer. Positive colonies were confirmed by electrophoresis gels. A positive colony was then inoculated in 250 ml of LB broth (1% NaCl, 1% Tryptone, 0.5% yeast extract, 100μg/ml ampicillin, pH 7), and grown for 16 hours at 37 °C under constant agitation. Plasmid DNA was purified according to the Plasmid Maxiprep Kit (QIAGEN, West Sussex, UK). The sequence of all the generated constructs was confirmed using a sABI Prism 3,700 DNA analyser (Functional Genomics Laboratory, The University of Birmingham, UK).

2.5.7 RNA extraction and conversion to cDNA

For cells and lines, $1x10^6$ cells were resuspended in 1 ml of TRIzol® Reagent (Invitrogen, Paisley, UK). For tissues, 50-100 mg of mouse tissue were added to 1 ml of TRIzol® Reagent and rotor-homogenized. Total RNA was extracted using the TRIzol® Plus RNA Purification Kit (Invitrogen) according to the manufacturer's instructions.Total RNA was quantified using a Nanodrop 1000 spectrophotometer (Thermo Scientific, Whaltman, USA). A 2 μg quantity of total RNA was collected after quantification and converted into cDNA using the Transcriptor High Fidelity cDNA Synthesis Kit (Roche), following the manufacturer's protocol.

2.5.8 Quantitative real time PCR (qRT-PCR)

A volume of 15 μl of the reaction mix containing the two specific gene primers (0.4 μl each), 12.5 μl qRT-PCR master mix and 0.4 μl of specific probe, were added to 10 μl of cDNA diluted 1:20. The probe contains two labels in close proximity to each other: a fluorescent reporter dye at the 5'- end and a fluorescent quencher at the 3'- end. When the probe is intact, the quenching label suppresses the fluorescent signal. When the probe is hybridized to its target sequence, it can be cleaved by the 5´-3´ exonuclease activity of the Taq DNA polymerase, which "unquenches" the fluorescent reporter dye and release it into solution. During each PCR cycle, the released fluorescent dye accumulates, boosting the fluorescent signal. The fluorescent dye is detected using a SYBR green I filter. A representative qRT-PCR experiment is presented in Figure 2.2.

The conditions for qRT-PCR amplification were the following: initial denaturation at 95^{o}C for 10 minutes, followed by 30 cycles of denaturation at 95^{o}C for 10 seconds, and elongation at 60^{o}C for 45 seconds. qRT-PCR was performed in a Rotor Gene 3000 qRT-PCR machine (Corbett Research), which performs the PCR amplification in a spinning rotor to generate a uniform distribution of the temperature across the samples. The primers and probes are shown in Table 2.3. Mouse and human data were normalized for the HPRT and actin housekeeping genes, respectively.

Gene	Primers	Probe
Human Tspan18 isoform 2	Forward 5'- GAAGGCGACTGTCTGAGCTGC-3' Reverse 5'-CTACTGGATGCCCCGGAAGCG-3'	Probe 53
Human Tspan18 isoform 1	Forward 5'-GAAGGCGACTGTCTGAGCTGC-3' Reverse5'- GGCTGTGGCTCCGAGTG-3'	Probe 11
Mouse Tspan18	Forward5'-GAGGGCGACTGTCTGAGCTG-3' Reverse5'-CTACTGGATGCCCCGGAAGAG-3'	Probe 66
Human actin	Forward5'- GCACCCAGCACAATGAAGA-3' Reverse5'- CGATCCACACGGAGTACTTG-3'	Probe 63
Mouse HPRT	Forward5'- GAAGCGGTAGCACTT-3' Reverse 5'-GTTCATCATCGCTAATCACGAC-3'	Probe 69

Table 2.3 qRT-PCR primers and probes. Probe numbers refer to the Human Roche Universal Probe Library.

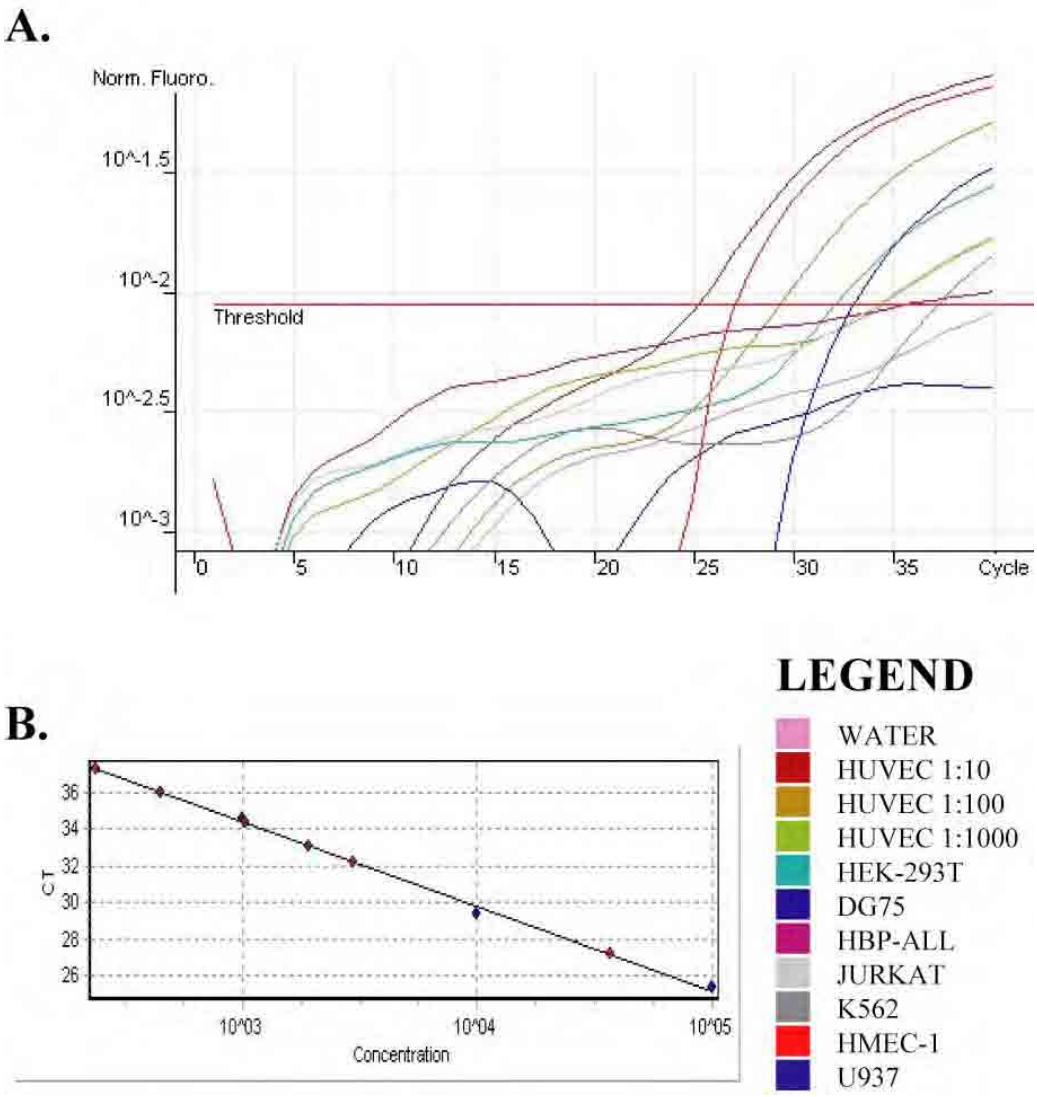


Figure 2.2 Representative example of a qRT-PCR experiment. Following PCR amplification of 11 samples, each was displayed as a curve on a graph of fluorescence intensity, which is proportional to the amount of PCR product, versus number of PCR cycles The threshold value was automatically calculated at a position above the background noise, and on the linear range of the positive samples (A.). A standard curve was drawn using the three dilutions of the positive HUVEC sample (shown as blue diamonds in panel B), using the number of PCR cycles at the threshold fluorescence versus an arbitrary measure of the sample dilution, labelled 'concentration' of the original cDNA. The other samples (shown as red diamonds in panel B) were quantitated relative to HUVEC using the standard curve B.).

2.6 ANALYSIS OF THE DATA

Western blotting and FACS data were representative of 2-5 experiments. qRT-PCR experiments were performed three times.

Luciferase assays were preformed between two and eight times, as indicated. Each luciferase reading was normalised for transfection efficiency by dividing by the relative β-gal value. These data failed to pass the D'Agostino-Pearson normality test, suggesting that they are not normally distributed. In order to employ parametric statistical tests, which are more robust than the equivalent non-parametric tests, data were and multiplied by the arbitrary value of 100, in order to obtain positive logarithmic data. Data were transformed into a logarithmic scale. Logarithmic data passed the D'Agostino-Pearson normality test, indicating that this transformation resulted in a normally distribuited data set. The normality of the logarithmic data was verified for each experiment (data not shown) and the standard deviations among replicate samples were calculated. Logarithmic data was analyzed by the analysis of variance (ANOVA) test. Statistical significance was determined by employing Tukey-Kramer's test, which compares all pairs of columns of normally distributed and independent data, and $P<0.05$ was taken as significant. Following statistical analysis, data were re-transformed into linear values for graphical representation.

CHAPTER 3

TSPAN18 OVER-EXPRESSION INDUCES NFAT ACTIVATION

3.1 INTRODUCTION

3.1.1 DT40 as a cell line model to study PTK-based signalling pathways

The DT40 chicken B cell line is an effective model to study receptors that signal via PTK-based signalling pathways, such as the ITAM-containing B cell receptor. This cell line expresses surface B cell receptor, of the IgM isotype, which is activated upon cross-linking, leading to calcium mobilization and apoptosis (Kurosaki, Maeda et al. 2000). Furthermore, DT40 is unique amongst cell lines in exhibiting a high propensity for homologous recombination, which has allowed the deletion of signalling components, either individually or in combination (Kurosaki 1997). Such work has played a pioneering role in dissection of the role of signalling molecules along the BCR signalling pathway.

In 1994, the Kurosaki group generated DT40 cells deficient in the Src family PTK Lyn and studied B cell receptor signalling in this mutant, reporting a weak, delayed but sustained signalling (Takata, Sabe et al. 1994). Similar BCR cross-linking on Syk-deficient DT40 revealed a lack of tyrosine phosphorylation of PLCγ2, resulting in the loss of both IP_3 generation and calcium mobilization. Moreover, Lyn/Syk doubly-deficient DT40 cells exhibited a complete defect in induction of tyrosine phosphorylation of any protein upon BCR ligation (Takata and Kurosaki 1996). These data indicate that Lyn and Syk play an essential initiating role in BCR signalling.

In 1996, the Kurosaki group characterized DT40 cells deficient in the PTK Btk (Takata and Kurosaki 1996). Following BCR cross-linking, Btk-deficient DT40 exhibited reduced PLCγ2 phosphorylation and a loss of IP_3 production and calcium mobilization, indicating that Btk is required for PLCγ2 phosphorylation and activation. Moreover, the same group generated and

characterized DT40 cells lacking PLCγ2 (Takata, Homma et al. 1995), reporting that, upon BCR cross-linking, PLCγ2 mutant cells completely failed to mobilize calcium. PLCγ2 hydrolyses the membrane inositol phospholipid PIP_2, resulting in generation of the second messengers DAG and IP_3. DAG activates protein kinase C and RasGRP which activate the MAPK pathway leading to AP-1 transcriptional activation (Chang and Karin 2001). IP_3 binds to the IP_3 receptors (IP_3Rs), which form the calcium channels located on the endoplasmic reticulum (ER), leading to calcium release from this internal store. Together these studies delineated a BCR-Lyn/Syk/Btk-PLCγ2-IP_3R pathway for induction of calcium mobilization. More recent studies using DT40 cells deficient in the ER-resident STIM1 protein (Prakriya, Feske et al. 2006) and cells and mice deficient in STIM1 and the calcium channel Orai1 (Park, Hoover et al. 2009), have demonstrated that STIM1 detects calcium depletion of the ER stores and binds to a tetramer of Orai1 in the plasma membrane, resulting in channel opening, calcium entry and activation of downstream transcriptional responses such as the calcineurin/NFAT pathway (Macian 2005; Oh-hora and Rao 2008).

3.1.2 NFAT/AP-1 luciferase assay

Our group has previously developed a cell line assay to measure GPVI/FcRγ signalling responses to collagen (Tomlinson, Calaminus et al. 2007) (Figure 3.1). The method involves the transfection of the DT40 chicken B cell line with the NFAT/AP-1-luciferase transcriptional reporter construct and expression constructs different receptors. DT40 chicken B cell line is transfected with the NFAT/AP-1-luciferase transcriptional reporter construct and expression constructs for GPVI and FcRγ. Sixteen hours post-transfection, cells are stimulated for six hours with the GPVI agonist collagen, or the positive control agonists PMA and

ionomycin, which bypass receptor proximal signalling to activate downstream MAPK and calcium pathways, respectively. GPVI/FcRγ stimulation with collagen induces the activation of PLCγ2 leading to downstream activation of MAPK and calcium pathways, both of which are required for optimal activation of the composite NFAT/AP-1/ promoter. In this cell line model, this leads to the generation of luciferase protein, which is measured using a luminometer that detects light production following oxidation of the luciferase substrate luciferin in the presence of ATP. A β-galactosidase construct is always included in each transfection, and β-galactosidase activity is used to correct for transfection efficiency. This method has been successfully used to study the signalling pathways of other platelet receptors, such as the C-type lectin receptors CLEC-2 and Dectin-1 (Fuller, Williams et al. 2007), and has been be employed in this chapter to evaluate if a tetraspanin protein can induce NFAT/AP-1 signalling.

3.2 AIM

The aim of this chapter was twofold. The first aim was to evaluate if any of the platelet-expressed tetraspanin proteins (CD9, CD63, CD151, Tspan32, Tspan9 and Tspan18) could induce signalling leading to activation of the NFAT/AP-1 promoter. This was done using the DT40 B cell line NFAT/AP-1-luciferase assay previously developed by our group (Tomlinson, Calaminus et al. 2007). The second aim was to study the signalling pathway of the tetraspanin Tspan18, which has been found to be able to induce NFAT/AP1 signalling during the first aim.

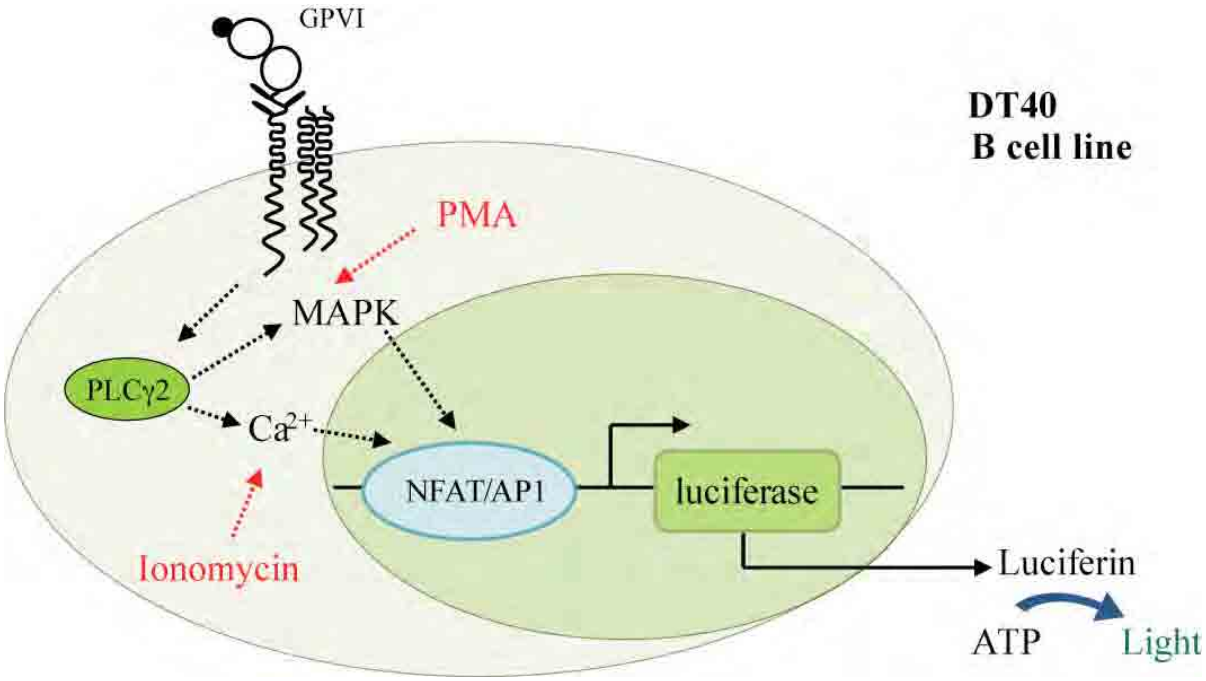


Figure 3.1 Model of the NFAT/AP-1 transcriptional reporter assay in DT40 cells. DT40 B cell line is transfected with the NFAT/AP-1-luciferase transcriptional reporter construct and expression constructs for GPVI and FcRγ. Cells are stimulated for six hours with the GPVI agonist collagen, or the positive control agonists PMA and ionomycin, which bypass receptor proximal signalling to activate downstream MAPK and calcium pathways, respectively. The activation of MAPK and calcium pathways are both required for the optimal activation of the NFAT/AP-1/ promoter.

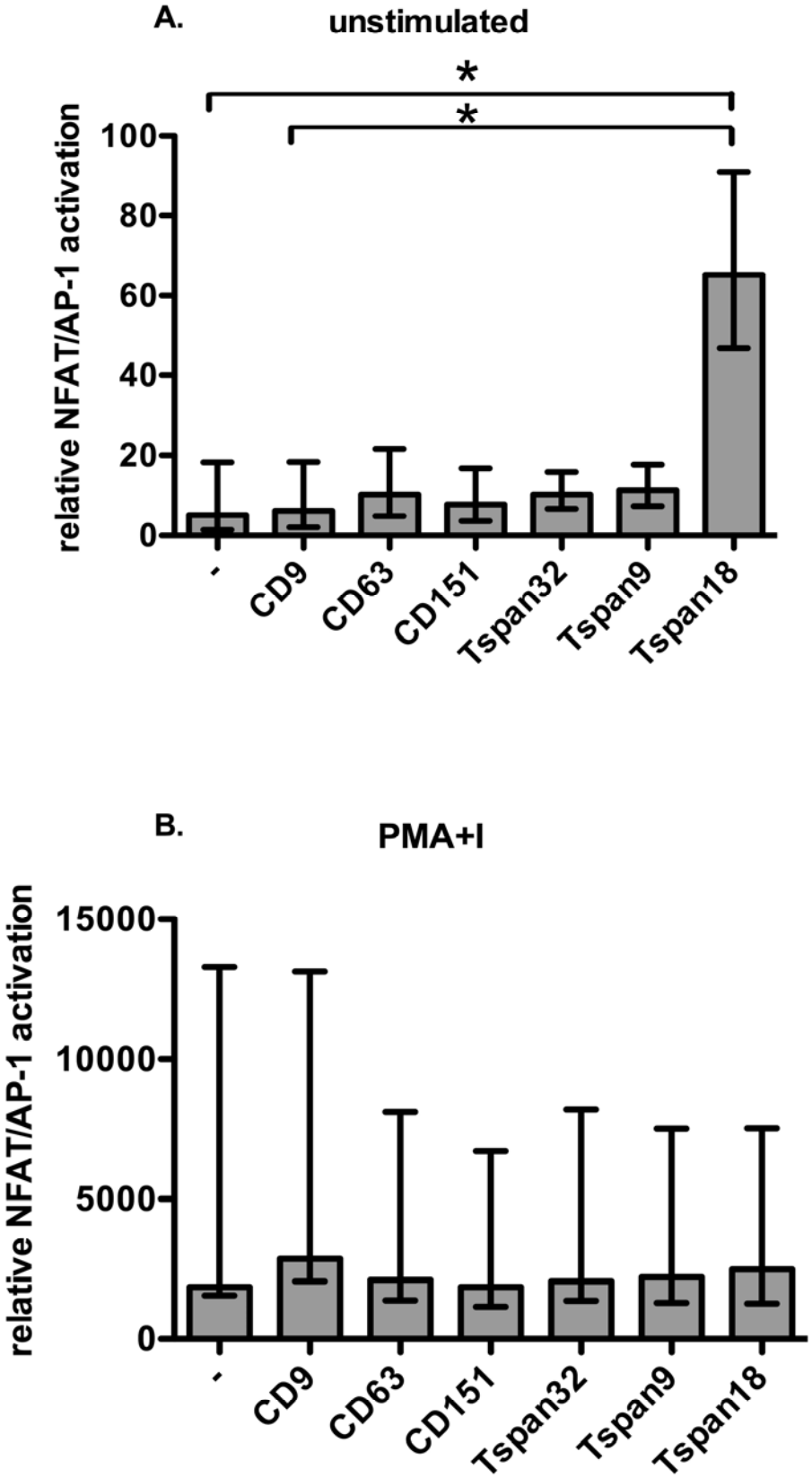
3.3 RESULTS

3.3.1 Tspan18, but not other platelets tetraspanin proteins, can induce NFAT/AP-1 signalling in the DT40 B cell line

In order to study if any of the tetraspanin proteins expressed on human platelets has an effect on NFAT/AP-1 signalling, a panel of FLAG-tagged tetraspanin proteins was individually co-transfected with the NFAT/AP-1 reporter in DT40 cells. The tetraspanins CD9, CD63, CD151, Tspan9 and Tspan32 were selected because they were known to be platelet-expressed, and Tspan18 was selected as a likely sixth platelet tetraspanin due to positive RT-PCR data from human platelets (V.L. Heath and M.G. Tomlinson, 2004, unpublished). The response of each tetraspanin protein was evaluated under basal conditions. Despite the Tukey's statistical analysis revealed CD9 and Tspan18 both significally induce NFAT/AP-1, only Tspan18, among the panel of tested tetraspanins, was able to substantially induce NFAT/AP-1 activation (Figure 3.2A). As a positive control, all transfection conditions responded robustly to PMA and ionomycin, albeit with a relatively high degree of variability between these three experiments, as shown by the large error bars, as one of the trhee experiment reported a stronger PMA+I response than the other two (Figure 3.2B). An aliquot of each transfected cell sample was lysed in 1% Triton X-100, analyzed by SDS-PAGE and blotted using an anti-FLAG antibody, in order to check the expression level of the transfected tetraspanin proteins (Figure 3.2C). Tetraspanins CD9, CD151 and Tspan9 were highly expressed, while CD63 and Tspan32 showed an intermediate expression level. The Tspan18 expression level was the lowest of the tested tetraspanin proteins, being barely detectable at this exposure (Figure 3.2C), which suggests that this tetraspanin is a powerful activator of NFAT/AP-1 signalling.

3.3.2 Tspan18 activates NFAT/AP-1 in a dose-dependent manner

Following the observation that Tspan18 can independently induce NFAT/AP-1 activation, the relationship between Tspan18 expression level and its ability to activate NFAT/AP-1 was investigated. DT40 cells were transfected with the NFAT/AP-1 reporter and different amounts of Tspan18 expression construct (2.5, 5, 10 and 20 μg for sample). Tspan18 induced NFAT/AP-1 activation in a dose-dependent manner (Figure 3.3A). Tukey's test demonstrated a significant increase in signalling over basal when samples are transfected with high doses of Tspan18 (10 and 20 μg), but despite the statistical analysis, previous experiments (Figure 3.2) showed 5μg of Tspan18 could induce a significant response over basal. This suggests a high variability among different experiments. The positive control stimulations with PMA and ionomycin were similar for each transfection (Figure 3.3B). Samples were analyzed Tukey's test, which did not show any significant difference. Taken together, these data strengthen the finding in this chapter that Tspan18 over-expression is sufficient to induce NFAT/AP-1 activation.


A.
unstimulated
*
*
relative NFAT/AP-1 activation
100
80
60
40
20
0
-
CD9
CD63
CD151
Tspan32
Tspan9
Tspan18
B.
PMA+I
relative NFAT/AP-1 activation
15000
10000
5000
0
-
CD9
CD63
CD151
Tspan32
Tspan9
Tspan18

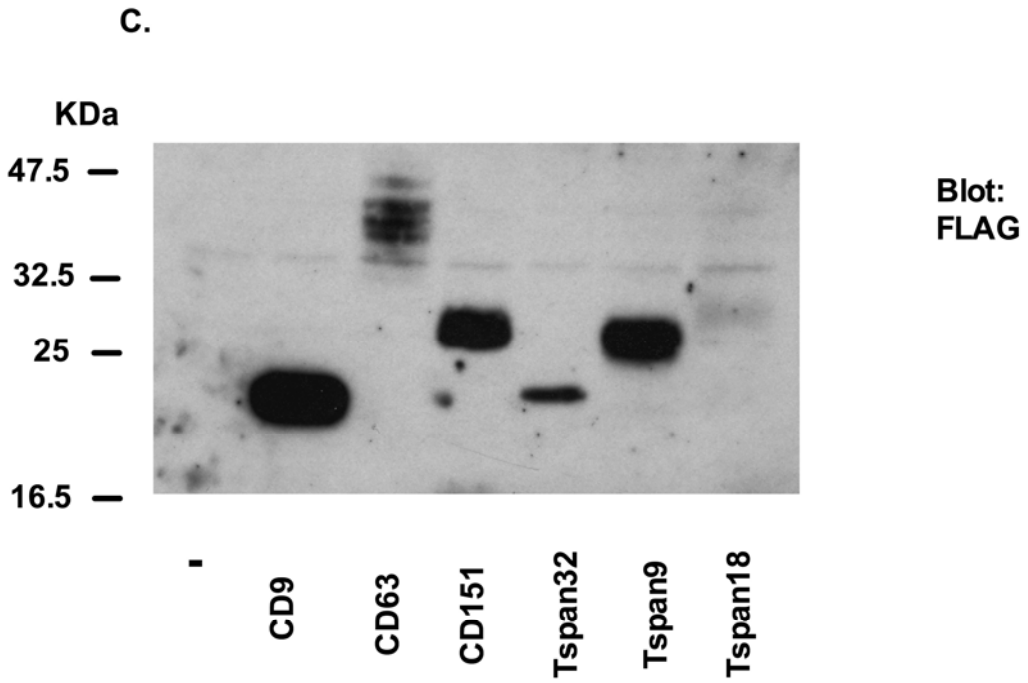


Figure 3.2 Tspan18 induces NFAT/AP-1 activation. DT40 cells were transfected with 20 μg of the NFAT/AP-1-luciferase reporter construct, 2 μg of β-galactosidase expression construct and 5 μg of the FLAG-tagged tetraspanin. Cells were unstimulated (A.) or were stimulated with PMA and ionomycin (B.). Data were normalized for β-gal values and analyzed by ANOVA test. Statistical significance is indicated by Tukey's test (**P*<0.05). Error bars represent the standard deviation of three independent experiments. Whole cell lysates were separated by SDS-PAGE, western blotted with an anti-FLAG antibody, and a representative blot is shown (C).

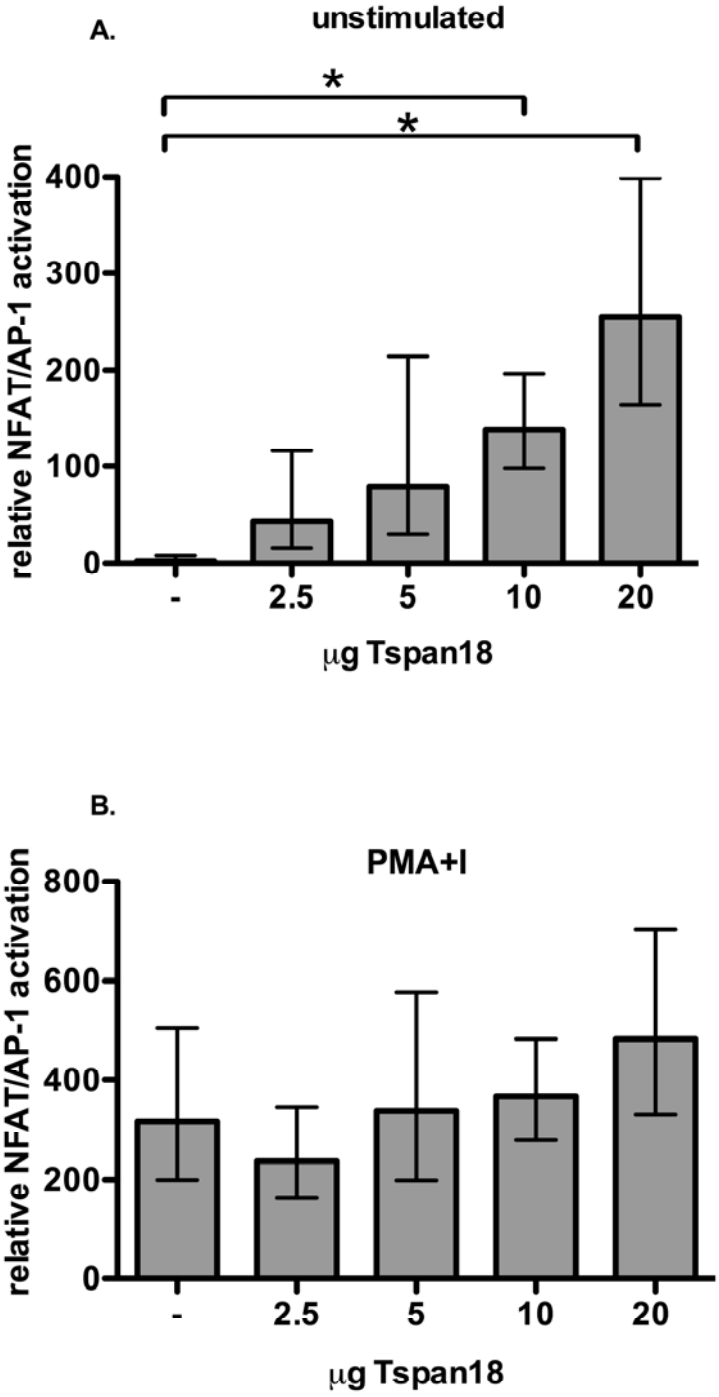
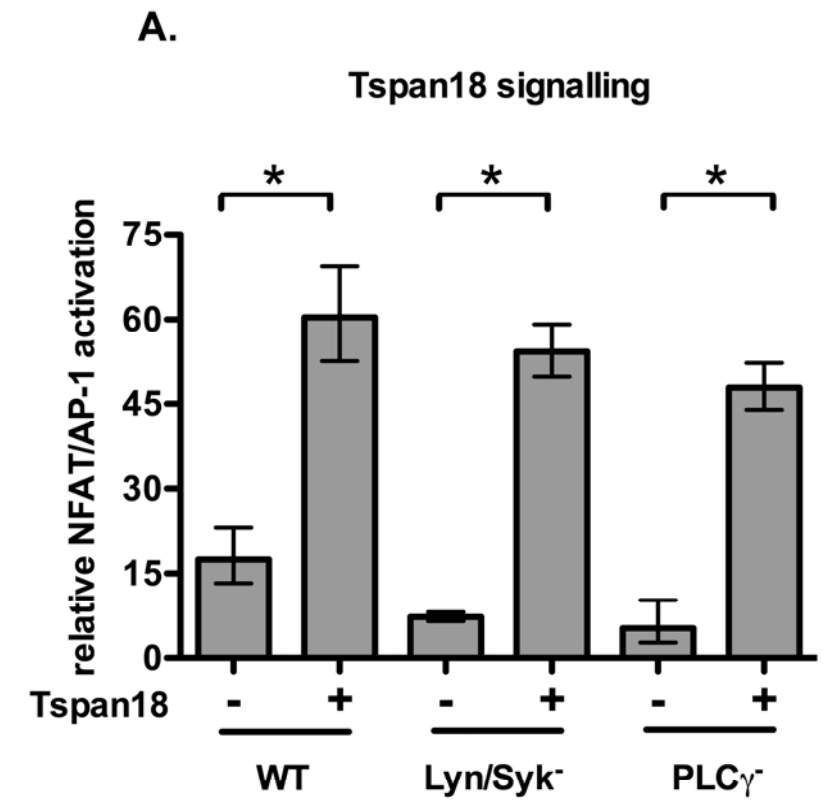


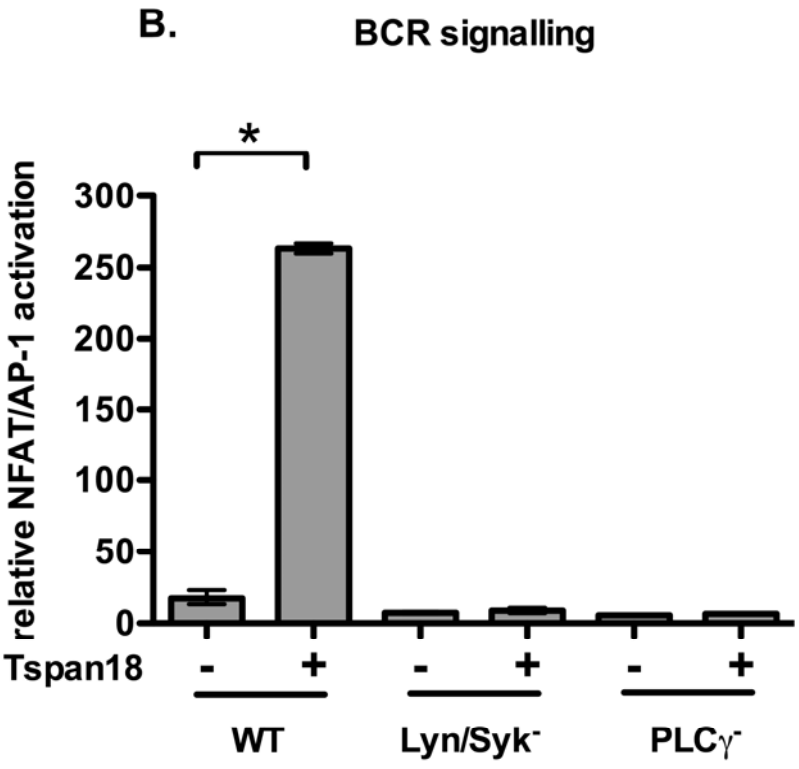
Figure 3.3 Tspan18 induces NFAT/AP-1 signalling in a dose-dependent manner. DT40 cells were transfected with the NFAT/AP-1-luciferase reporter, a β-galactosidase construct and 2.5, 5, 10 or 20 μg of Tspan18. Cells were left unstimulated (A.) or were stimulated with PMA and ionomycin (B.). Data were normalized for β-gal values and analyzed by ANOVA test. Statistical significance is indicated by Tukey's test (**P*<0.05). Error bars represent the standard deviation of two independent experiments.

3.3.3 Tspan18 signalling is Lyn/Syk- and PLCγ-independent

As tetraspanin proteins do not possess any enzymatic activity, it is likely that the observed NFAT/AP-1 activation is not directly due to Tspan18, but is instead a result of a Tspan18-associated signalling protein that is activated as a result of Tspan18 over-expression. If this theory is correct, and if the putative Tspan18 partner signals via a tyrosine kinase-activated PLCγ2 pathway, similar to the BCR, one would predict that signalling would be impaired in the absence of PLCγ2 or the major DT40 cell PTKs, Lyn and Syk. To address this, DT40 cells lacking in both the PTKs Lyn and Syk (Lyn/Syk$^-$) and PLCγ2 (PLCγ2$^-$), which were generated by homologous recombination (Ishiai, Sugawara et al. 1999), were transfected with Tspan18 and with the NFAT/AP-1 reporter construct. Tspan18 induced substantial NFAT/AP-1 activation in mutant cells lacking Lyn/Syk or PLCγ2 (Figure 3.4A). However, in control stimulations these mutant lines failed to respond to B cell receptor stimulation (Figure 3.4B), as has been observed previously (Tomlinson, Kane et al. 2004). In order to determine whether Tspan18 was expressed at similar levels in each cell type, whole cell lysates were analyzed by SDS-PAGE and blotted with a FLAG antibody. The result showed that Tspan18 expression was comparable in each sample (Figure 3.4C). Tspan18 was more readily detectable in this experiment than observed previously (Figure 3.2C), because four times more DNA was used in the transfection.

Taken together, these data indicate that Tspan18 does not activate NFAT/AP-1 in a manner similar to that of the BCR, which uses ITAMs, PTKs and PLCγ2.


A.
Tspan18 signalling
relative NFAT/AP-1 activation
75
60
45
30
15
0
*
*
*
Tspan18
-
+
-
+
-
+
WT
Lyn/Syk-
PLCγ-


B.
BCR signalling
relative NFAT/AP-1 activation
300
250
200
150
100
50
0
*
Tspan18
-
+
-
+
-
+
WT
Lyn/Syk-
PLCγ-

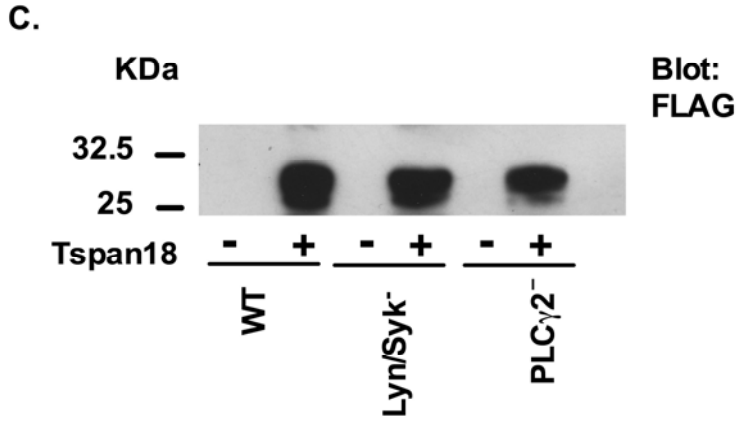


Figure 3.4 Tspan18 signalling is independent of Lyn, Syk and PLCγ2. Wild-type (WT) DT40 cells or cells deficient for Lyn and Syk or PLCγ2 were transfected with 20μg of the vector control or FLAG-tagged Tspan18 and with the NFAT/AP-1 reporter and β-gal control construct. Cells were left unstimulated (A.) or were stimulated with 4 μg/ml of anti-BCR antibody (B.) Data were normalized for β-gal values and analyzed by ANOVA test. Statistical significance is indicated by Tukey's test (**P*<0.05). Error bars represent the standard deviation of three independent experiments. An aliquot of whole cell lysate was analyzed by SDS-PAGE and blotted with the FLAG antibody (C.).

3.3.4 Tspan18 signalling mimics the calcium ionophore ionomycin

The two second messengers generated by PLCγ, IP_3 and DAG, trigger two distinct signalling cascades, the calcium and mitogen-activated protein kinase (MAPK) pathways, respectively. The optimal NFAT/AP-1 activation requires both calcium and MAPK pathways. To address whether Tspan18 is inducing calcium or MAPK signalling, or both, DT40 cells were transfected with either the NFAT/AP-1 or AP-1 reporter construct in the presence or absence of Tspan18, and cells were stimulated with PMA alone, ionomycin alone, or PMA in combination with ionomycin. For the NFAT/AP-1 reporter, in the absence of Tspan18, the agonists PMA, ionomycin and PMA plus ionomycin induced a robust activation (Figure 3.5A). This is consistent with the idea that calcium and MAPK signalling are required for optimal NFAT/AP-1 activation, but further shows that calcium alone can induce substantial NFAT/AP-1 activation, whereas MAPK alone has a minimal effect. Tspan18 over-expression induced significant NFAT/AP-1 activation (Figure 3.5A), similar to the level observed previously. However, the combination of Tspan18 and PMA yielded a synergistic response, while in contrast the combination of Tspan18 and ionomycin was not significantly different to ionomycin alone (Figure 3.5A). These results demonstrate that Tspan18 over-expression mimics the effect of ionomycin stimulation, albeit at a weaker level.

To determine whether Tspan18 over-expression can induce activation of the MAPK pathway, the AP-1-luciferase construct was used. Consistent with the idea that this is a MAPK-responsive reporter, maximal activation was observed with PMA regardless of the presence of ionomycin (Figure 3.5B). Tspan18 induced significant but relatively weak AP-1 activation (approximately 2.5-fold), similar to that induced by ionomycin (Figure 3.5B). The latter

suggests that calcium signalling can induce weak AP-1 activation in this DT40 cell line, perhaps due to some cross-talk with the MAPK pathway. The similar effect of Tspan18 and ionomycin suggest the idea that Tspan18 primarily activates calcium signalling leading to NFAT activation.

To determine whether Tspan18 activates NFAT/AP-1 in other cell types, the DT40 experiment shown in Figure 3.5A was repeated for the Jurkat T cell line, a widely used model system to study T cell signalling. In contrast to DT40 cells, Tspan18 over-expression did not induce significant NFAT/AP-1 activation in Jurkat cells. However, ionomycin also failed to activate substantial NFAT/AP-1 (Figure 3.6) in contrast to the activation observed in DT40 cells (Figure 3.5A). This suggests that in Jurkat cells, unlike DT40 cells, calcium signalling alone cannot induce substantial NFAT/AP-1 activation. However, a striking synergy was observed between PMA and Tspan18 and between PMA and ionomycin (Figure 3.6). These data support the conclusion from the DT40 data that Tspan18 over-expression activates NFAT by activating calcium signalling.

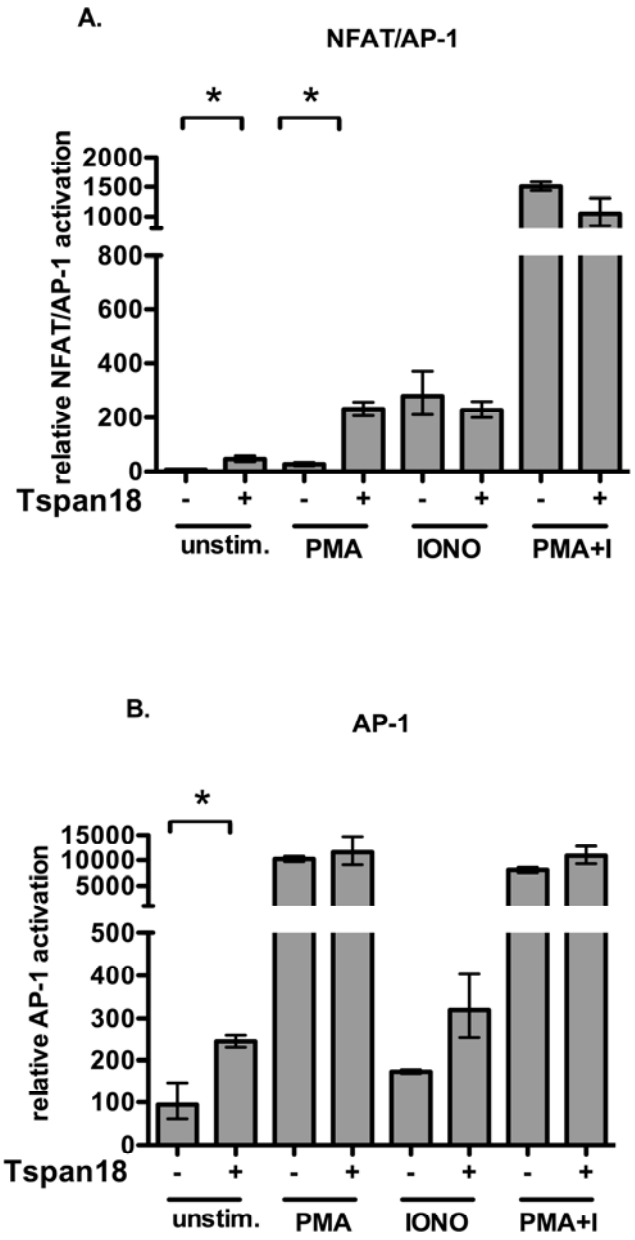


Figure 3.5 Tspan18 mimics ionomycin. DT40 cells were transfected with NFAT/AP-1 (A) or AP-1 (B) reporter constructs, and the β-gal control construct, in the presence or absence of 20 μg of the FLAG-tagged Tspan18 construct. Transfected cells were left unstimulated or were stimulated with PMA or ionomycin or with a combination of PMA and ionomycin. Data were normalized for β-gal values and analyzed by ANOVA test. Data were analyzed in pairs (with and without Tspan18 of unstim, PMA, iono and PMA+I) employing the Tukey's test (**P*<0.05). Error bars represent the standard deviation of three independent experiments.

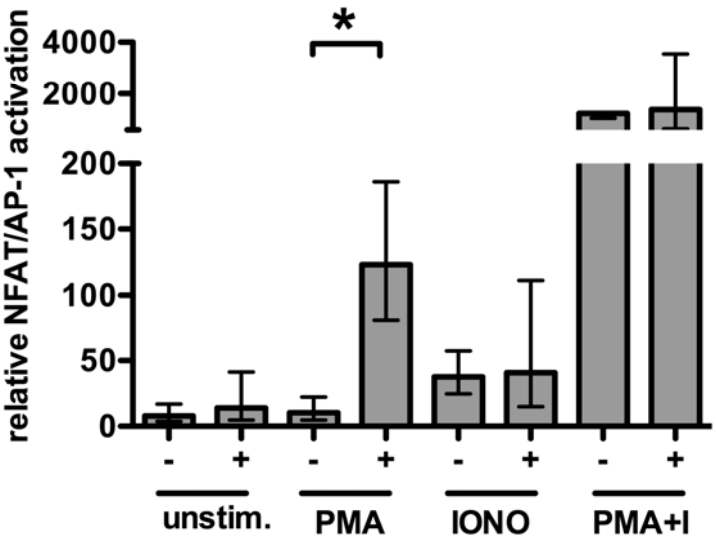
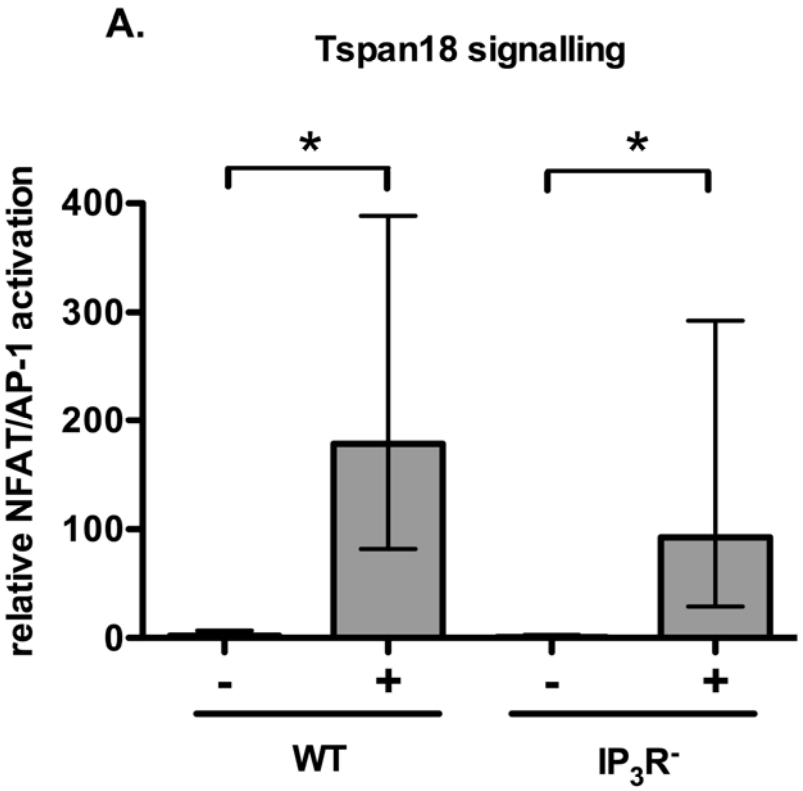


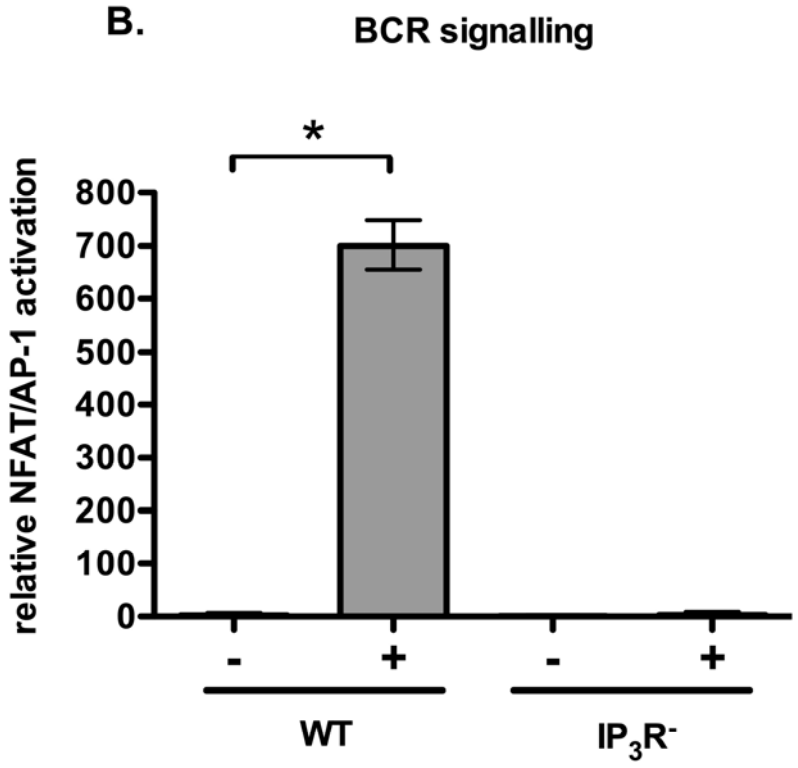
Figure 3.6 Tspan18 synergises with PMA to activate NFAT/AP-1 in Jurkat cells. Jurkat cells were transfected with the NFAT/AP-1-luciferase reporter and β-gal control constructs in the presence or absence of 5 μg of the FLAG-tagged Tspan18 construct. Cells were left unstimulated or were stimulated with PMA, ionomycin or a combination of PMA and ionomycin. Data were normalized for β-gal values and analyzed by ANOVA test. Data were analyzed in pairs (with and without Tspan18 of unstim, PMA, iono and PMA+I) employing the Tukey's test (**P*<0.05), but none of the analyzed samples pairs presented a significant difference. Error bars represent the standard deviation of three independent experiments.

3.3.5 Tspan18 signalling is IP_3R independent

The Tspan18-induced NFAT/AP-1 activation observed in Lyn/Syk- and PLCγ2-deficient DT40 cells suggests that Tspan18 exerts its effect downstream or independently of PLCγ2. In ITAM-based signalling pathways such as that used by the BCR, the activity of PLCγ2 generates the second messengers diacylglycerol and IP_3, the latter of which activates IP_3 receptors, which are IP_3-gated calcium channels in the endoplasmic reticulum. To test whether Tspan18 can activate NFAT/AP-1 independently of IP_3Rs, DT40 cells lacking in all three IP_3Rs, generated previously by homologous recombination (Sugawara, Kurosaki et al. 1997), were used. Tspan18-induced NFAT/AP-1 activation was reduced in the absence of the IP_3 receptors, but only by approximately 50% (Figure 3.7A), demonstrating that the IP_3 receptors are not essential for Tspan18 signalling. Control stimulations with anti-BCR antibody did not activate NFAT/AP-1 in the IP_3 receptor-deficient cells (Figure 3.7B), consistent with the idea that IP_3 receptors are essential components of the BCR signalling pathway leading to calcium release (Kurosaki, Maeda et al. 2000) .

In order to check Tspan18 expression, whole cell lysates of each sample were analyzed by SDS-PAGE and blotted with the FLAG antibody. The result showed a similar Tspan18 expression in wild-type and IP_3 receptor-deficient cells (Figure 3.7C). These results show that Tspan18-induced NFAT/AP-1 activation is partially dependent on the IP_3 receptors, but that they are not essential. This suggests a signalling role for Tspan18 downstream of IP_3 receptors.


A.
Tspan18 signalling
relative NFAT/AP-1 activation
400
300
200
100
0
*
*
-
+
-
+
WT
IP3R-


B.
BCR signalling
relative NFAT/AP-1 activation
800
700
600
500
400
300
200
100
0
*
-
+
-
+
WT
IP3R-

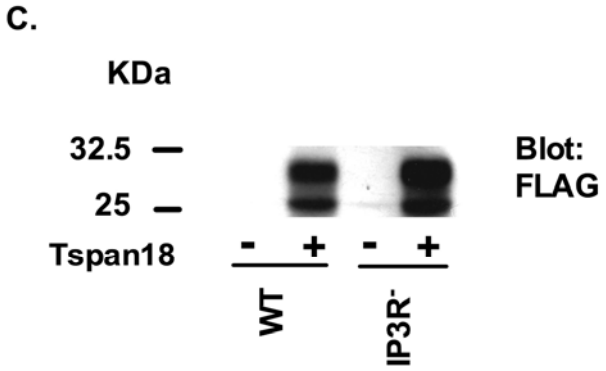
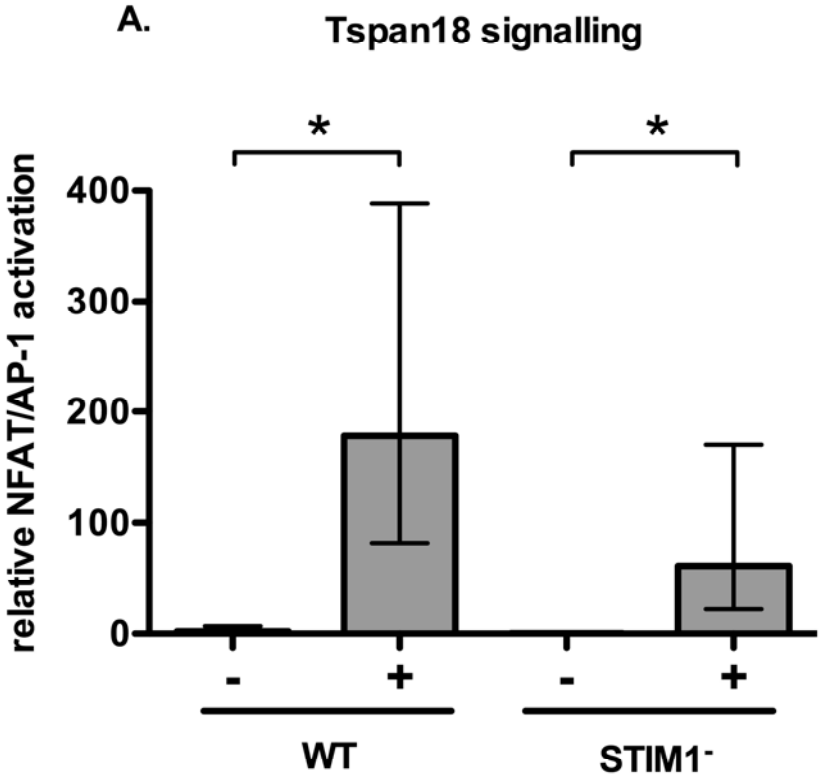
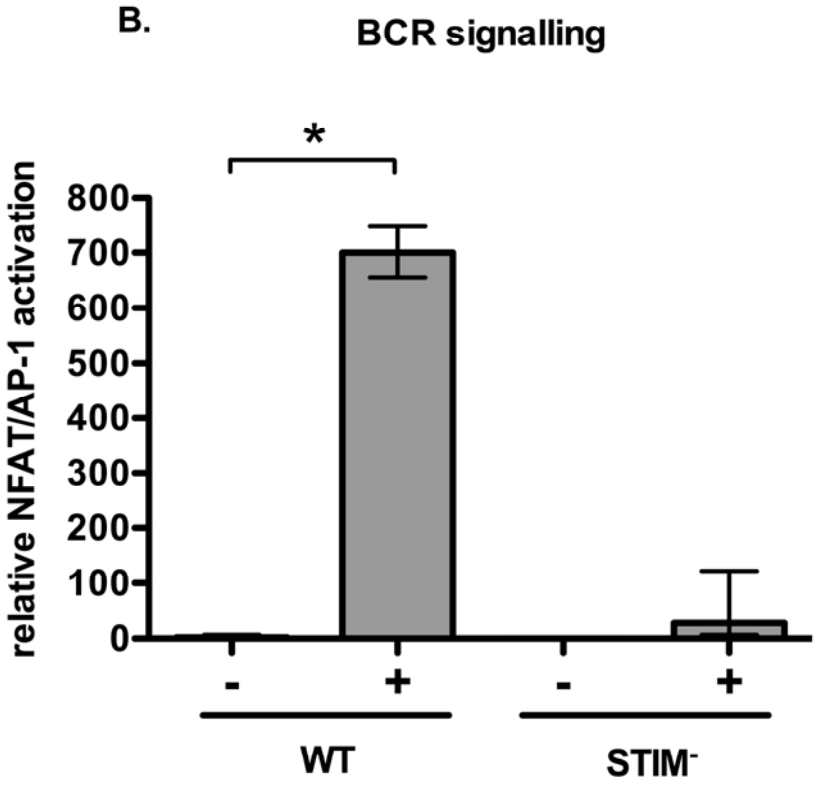


Figure 3.7 Tspan18 signalling is maintained in the absence of IP_3 receptors. Wild-type (WT) DT40 cells or IP_3 receptor-deficient DT40 cells were transfected with control vector or FLAG-tagged Tspan18, and with the NFAT/AP-1 reporter and the β-gal construct. Cells were left unstimulated (A.) or were stimulated with 4 μg/ml of BCR receptor antibody (B.). Data were normalized for β-gal values and analyzed by ANOVA test. Statistical significance is indicated by Tukey's test (**P*<0.05). Error bars represent the standard deviation of three independent experiments. . An aliquot of whole cell lysate was analyzed by SDS-PAGE and blotted with the FLAG antibody (C).

3.3.6 Tspan18 signalling is independent of STIM1

When IP_3 receptors release calcium, so emptying ER stores, the calcium sensor STIM1 induces clustering and opening of Orai1-containing plasma membrane I_{CRAC} channels, to cause calcium entry and sustained calcium elevation, leading to NFAT activation (Hogan and Rao 2007). To determine whether Tspan18 requires STIM1 for its activation of NFAT signalling, DT40 cells rendered deficient in STIM1 by homologous recombination (Baba, Hayashi et al. 2006) were utilised. Tspan18-induced NFAT/AP-1 activation was reduced by 60% in the absence of STIM1, but this reduction in signalling was not significant (Figure 3.8A). Moreover, in the absence of STIM1, BCR-induced NFAT activation was similarly impaired, but not completely abrogated (Figure 3.8B). As observed for the other mutants, expression levels of FLAG-tagged Tspan18 were similar between wild-type and STIM1-deficient cells (Figure 3.8C). Together these data suggest that either Tspan18 is acting downstream of STIM1, or that Tspan18 is upstream but there is some compensation for the lack of STIM1, such as by the other family member STIM2. The latter possibility is supported by the residual BCR signal in the absence of STIM1. Future experiments could be performed using STIM1/STIM2 doubly-deficient DT40 cells or using an RNAi knockdown approach.


A.
Tspan18 signalling
relative NFAT/AP-1 activation
400
300
200
100
0
*
*
-
+
-
+
WT
STIM1-


B.
BCR signalling
relative NFAT/AP-1 activation
800
700
600
500
400
300
200
100
0
*
-
+
-
+
WT
STIM-

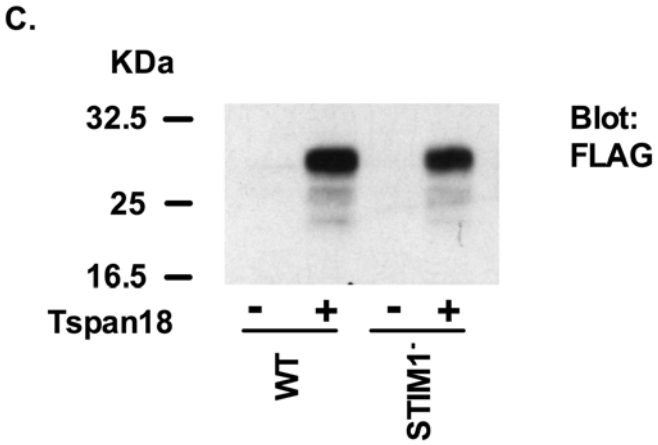
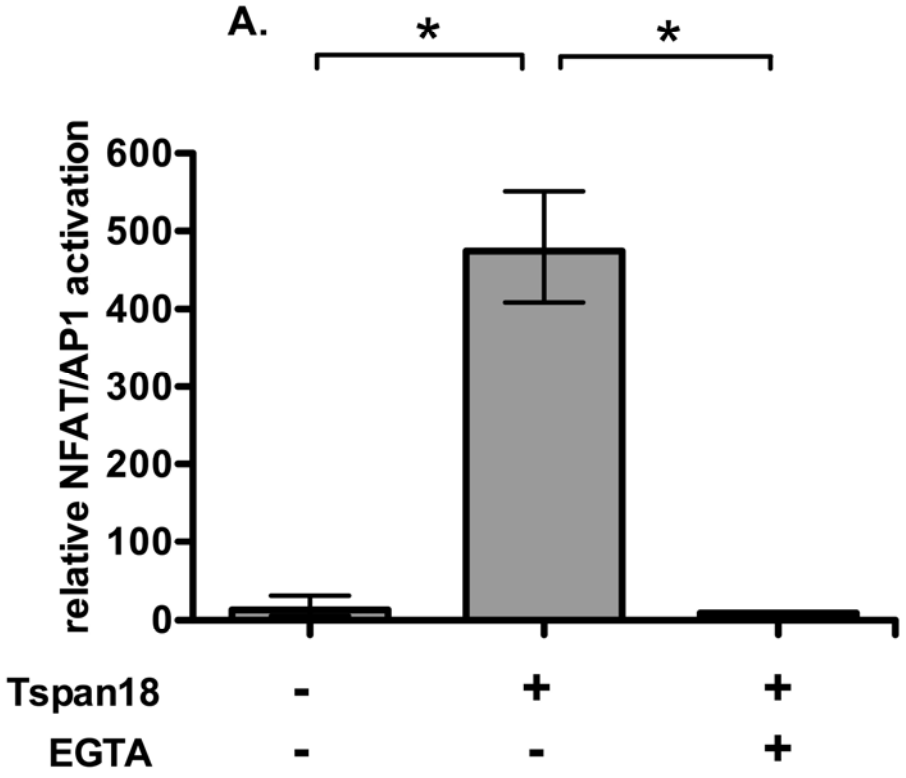


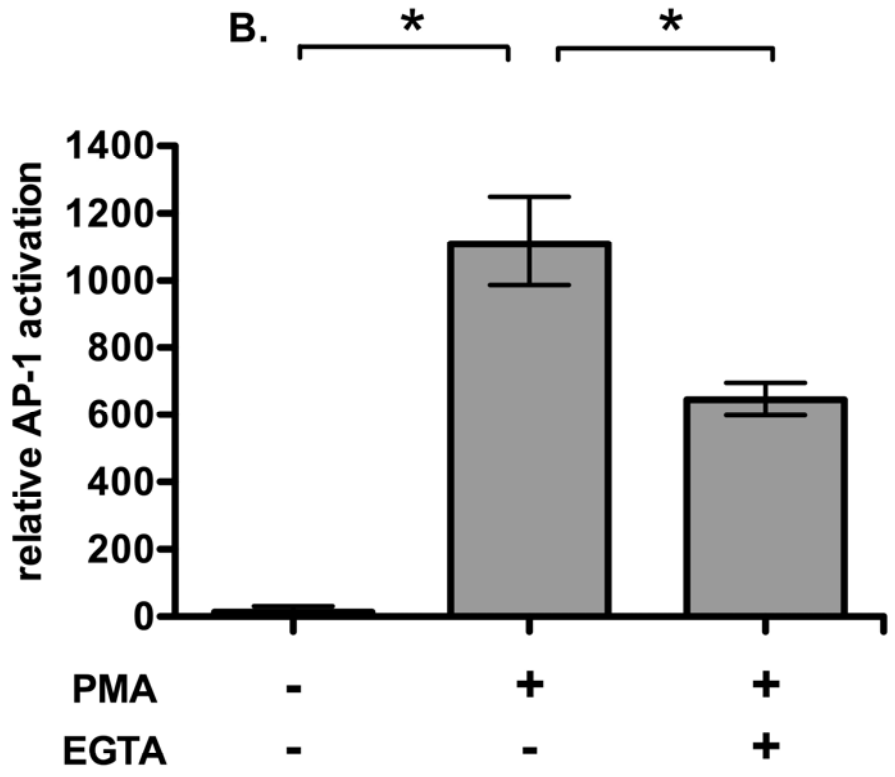
Figure 3.8 Tspan18 signalling is independent of STIM1. Wild-type (WT) or STIM1-deficient DT40 cells were transfected with the FLAG-tagged Tspan18 and with NFAT/AP-1 reporter and β-gal control construct. Cells were left unstimulated (A) or were stimulated with 4 μg/ml of BCR receptor antibody (B.) An aliquot of whole cell lysate was analyzed by SDS-PAGE and blotted with the FLAG antibody (C). Data were normalized for β-gal values and analyzed by ANOVA test. Statistical significance is indicated by Tukey's test (*P<0.05). Error bars represent the standard deviation of three independent experiments.

3.3.7 Tspan18 signalling is dependent on extracellular calcium

To distinguish the relative importance of calcium release from internal stores and extracellular calcium entry across the plasma membrane, Tspan18 signalling was investigated using the extracellular calcium chelator EGTA. DT40 cells were transfected with Tspan18 and with the NFAT/AP-1 reporter. Transfected cells were left untreated or were treated with 4 mM EGTA. The data demonstrated that Tspan18 signalling was completely blocked by the presence of EGTA (Figure 3.9A). Control stimulations with PMA and ionomycin also failed to induce NFAT/AP-1 activation in the presence of EGTA (Figure 3.9B).

As EGTA completely blocked Tspan18 signalling, this could be due to a global inhibition of signalling. In order to address this, the previously used AP-1 reporter was employed. The results demonstrated that EGTA was not toxic to the cells, as they retained partial AP-1 signalling capability (Figure 3.9C), and enforced the previous findings of Tspan18 involvement in calcium signalling. Moreover, the partial reduction in AP-1 signalling suggests that AP-1 signalling pathways might be partially calcium dependent, indicating a possible pathway cross-talk.


A.
*
*
relative NFAT/AP1 activation
600
500
400
300
200
100
0
Tspan18
-
+
+
EGTA
-
-
+


B.
*
*
relative AP-1 activation
1400
1200
1000
800
600
400
200
0
PMA
-
+
+
EGTA
-
-
+

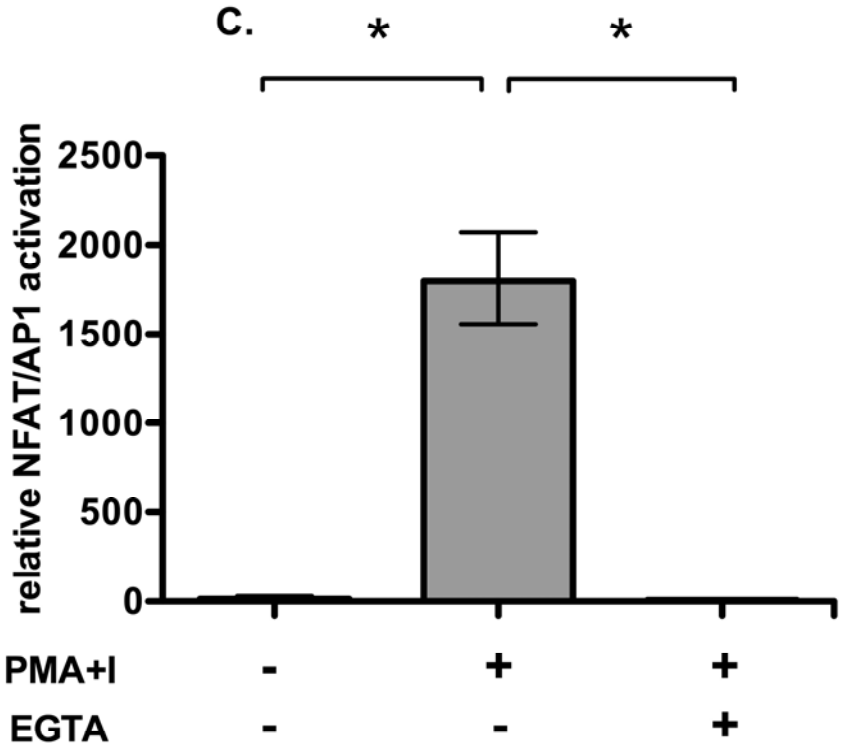


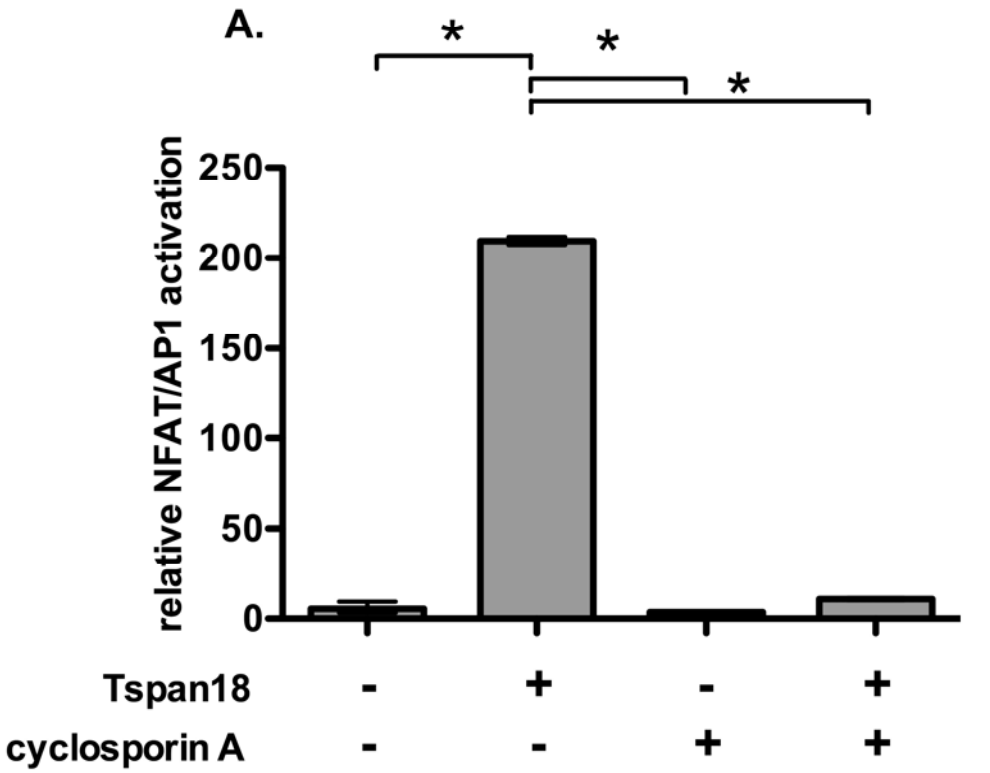
Figure 3.9 EGTA can block Tspan18 signalling. DT40 cells were transfected with the NFAT/AP-1 reporter and the -gal construct, and with 20 μg of the control vector or FLAG-tagged Tspan18. Cells were left untreated or treated with 4 mM EGTA (A). DT40 cells were transfected with the NFAT/AP-1 or AP-1 reporter, in the absence of Tspan18 (B and C, respectively). Cells were left unstimulated or stimulated with PMA and ionomycin (B) or PMA alone (C). EGTA was added immediately following the transfection. Data were normalized for β-gal values and analyzed by ANOVA test. Statistical significance is indicated by Tukey's test (**P*<0.05). Error bars represent the standard deviation of two independent experiments.

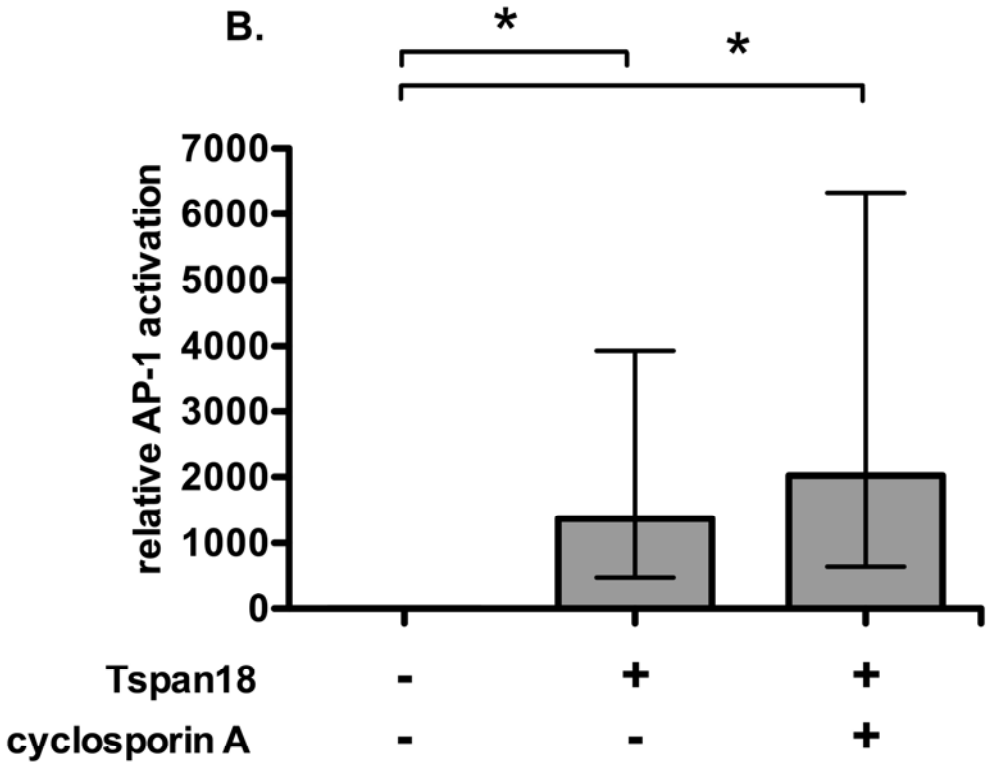
3.3.8 Tspan18 signalling is dependent on calcineurin activity

Calcineurin is a calcium-activated cytoplasmic phosphatase that can activate NFAT by dephosphorylation, resulting in its translocation into the nucleus. Calcineurin activity can be selectively blocked by the immunosuppressive drug cyclosporin A (Liu 2009). Therefore this drug was used to test whether Tspan18-induced NFAT/AP-1 activation was dependent on calcineurin activity.

DT40 cells were transfected with the NFAT/AP-1-luciferase reporter construct and either vector control or the Tspan18 construct, and cells were left untreated or treated with cyclosporin A. The data demonstrated that cyclosporin A was able to completely block NFAT/AP-1 activation induced upon Tspan18 over-expression (Figure 3.10A). These data suggest Tspan18 signalling is calcineurin dependent.

As a control for cyclosporin A treatment, the drug completely inhibited NFAT/AP-1 activation induced by the combination of PMA and ionomycin (Figure 3.10B). However, it is possible to speculate from these data that cyclosporin A was not specifically inhibiting calcineurin, but was instead globally inhibiting signalling by killing the cells, for example. However, AP-1 activation was unaffected by cyclosporin A (Figure 3.10C), suggesting that the drug is not toxic to the cells and strengthening the finding that Tspan18 signalling is calcineurin dependent.


A.
*
*
*
relative NFAT/AP1 activation
250
200
150
100
50
0
Tspan18
cyclosporin A
- + - +
- - + +


B.
*
*
relative AP-1 activation
7000
6000
5000
4000
3000
2000
1000
0
Tspan18
cyclosporin A
- + +
- - +

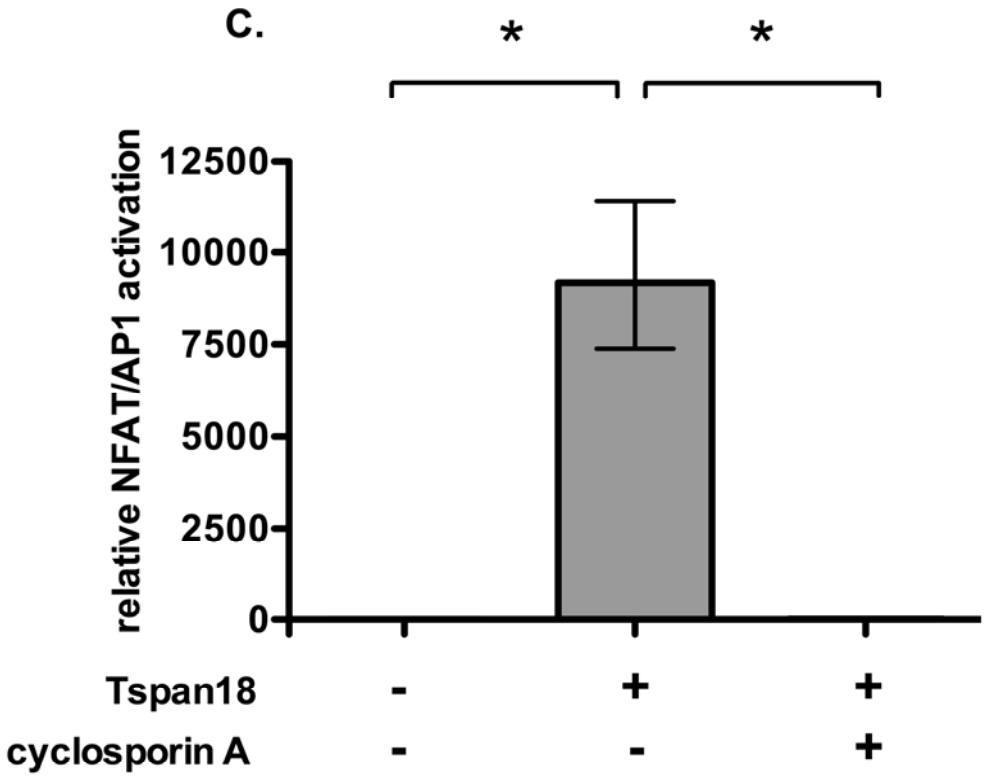


Figure 3.10 Tspan18 signalling is blocked by cyclosporin A. DT40 cells were transfected with β-gal control construct, with 20 μg of control vector or FLAG-tagged Tspan18, and with the NFAT/AP-1 (A and B) or the AP-1 reporter (C). Cells were left unstimulated or stimulated with PMA or PMA and ionomycin, in the presence or absence of 2 mM cyclosporin A, as indicated. Cyclosporin A was added to the medium immediately after transfection, while agonists were added 16 hours after the transfection. Data were normalized for β-gal values and analyzed by ANOVA test. Statistical significance is indicated by Tukey's test (**P*<0.05). Error bars represent the standard deviation of two independent experiments. .

3.4 DISCUSSION

In this chapter, the possibility that platelet-expressed tetraspanin proteins could induce NFAT/AP-1 signalling has been tested, and the mechanism by which Tspan18 over-expression induces NFAT/AP-1 activation was investigated using DT40 cells lacking in proteins involved in the BCR signalling pathway (Syk, Lyn, PLCγ2, IP_3Rs and STIM1), inhibitors (EGTA and cyclosporin A) and agonists (anti-BCR antibody, PMA and ionomycin). The results demonstrated that, among the tested platelet tetraspanin proteins, only Tspan18 is able to induce NFAT/AP-1 signalling. Tspan18-induced activation is independent of Syk, Lyn, PLCγ2, IP_3Rs and STIM1 but dependent on extracellular calcium and calcineurin. Moreover, Tspan18 over-expression mimics the effect of ionomycin, both in DT40 and in Jurkat cells, suggesting that Tspan18 activates NFAT/AP-1 by inducing calcium signalling.

A prediction of the original hypothesis was that Tspan18-induced NFAT/AP-1 activation would be abrogated in the absence of Lyn and Syk or PLCγ2, since BCR signalling is abrogated in cells lacking these signalling proteins. However, Tspan18 signalling was completely unaffected in these mutant DT40 cells. Moreover, the Tspan18 signal was not significantly affected in the absence of the three IP_3 receptors, which are key players in store-operated calcium entry leading to NFAT activation. These findings suggested that Tspan18 is exerting its effect downstream or independent of the IP_3 receptors, perhaps at the level of calcium entry or through activation of NFAT itself. The latter possibility was ruled out by the finding that cyclosporin A, an immunosuppressive drug that inhibits the NFAT-activating, calcium-activated phosphatase calcineurin, completely inhibited Tspan18-induced NFAT/AP-1 activation. This data further suggested that Tspan18 might be activating NFAT by inducing calcineurin activation or calcium signalling. The latter was supported by complete inhibition

of Tspan18 signalling by EGTA, which chelated extracellular calcium, so preventing store-operated calcium entry. Taken together, these data support the possibility that Tspan18 induces calcium signalling by activating a calcium channel or calcium channel regulator.

In recent years extensive research has been performed in the field of calcium signalling following the discovery of two players in the store-operated calcium entry (SOCE) mechanism, STIM1 and ORAI1. STIM1 detects calcium release from the ER stores and induces clustering of the calcium channel Orai1 and channel opening (Barr, Bernot et al. 2008). In this chapter it has been demonstrated that Tspan18-induced NFAT/AP-1 activation is only partially dependent on STIM1. It is possible to speculate that STIM2, whose role has not been fully elucidated yet, might play a role in calcium signalling, and might be able to partially compensate for the lack of STIM1. A possible strategy to test this hypothesis might be to evaluate Tspan18 signalling in DT40 cells lacking in both STIM1 and STIM2, that have been recently developed (Wang, Deng et al. 2009). A second interpretation of the result might be that Tspan18 function is located downstream of STIM1. However, the partial BCR-induced signal in STIM1-deficient cells suggests that they can compensate for the lack of this protein, thus making the Tspan18 data difficult to interpret. Studies using the agonists PMA and ionomycin, in both DT40 B cells and Jurkat T cells, demonstrated that Tspan18 over-expression mimics the effect of ionomycin. This was observed in a number of ways. Firstly, both ionomycin and Tspan18 induced substantial NFAT/AP-1 activation in DT40 cells. Secondly, both induced relatively weak NFAT/AP-1 activation in Jurkat, and similarly weak AP-1 activation in DT40 cells. Thirdly, in both cell types, Tspan18 and ionomycin each synergised with PMA to induce NFAT/AP-1 activation. In combination with the inhibition of Tspan18 signalling by calcineurin and EGTA, this strongly suggests that Tspan18 over-expression induces calcium signalling.

Two findings in the previous paragraph are worthy of further discussion. The first is the fact that DT40 and Jurkat were different in their capacity to activate NFAT/AP-1 in response to ionomycin or Tspan18: relatively strong activation was observed in DT40 and very weak activation occurred in Jurkat. The reason for this is not clear, but it is possible that calcium elevation in DT40 can induce a low level of AP-1 activation. This signalling pathway 'cross-talk' may not exist in Jurkat. A second related discussion point is the fact that ionomycin and Tspan18 can induce weak AP-1 activation in DT40 cells, supporting the idea of pathway cross-talk. A possible mechanism for this was presented in a recent study by the Parekh group (Ng, Nelson et al. 2009) In this study, calcium entry via I_{CRAC} channels in the plasma membrane activated MAPK and c-fos, a component of the AP-1 complex, via a Syk-dependent pathway (Ng, Nelson et al. 2009). Interestingly, DT40 cells express Syk but Jurkat do not, thus providing a potential explanation for observed differences between the two cell lines.

3.5 SUMMARY

In the present chapter it has been demonstrated that:

- Tspan18 is the only tetraspanin, amongst those platelet tetraspanins tested, to be able to induce constitutive NFAT/AP-1 signalling
- Tspan18 signalling is independent of Lyn/Syk and PLCγ2
- Tspan18 mimics ionomycin in its capacity to activate NFAT/AP-1 in DT40 B and Jurkat T cells, and, in particular, synergises with PMA
- Tspan18 signalling is partially impaired in the absence of IP_3R receptors and STIM1
- Constitutive Tspan18 signalling is dependent on calcineurin and extracellular calcium

CHAPTER 4

TSPAN18 BIOINFORMATIC CHARACTERIZATION AND EXPRESSION PROFILE

4.1 INTRODUCTION

In the previous Chapter, Tspan18 was found to be the only tetraspanin protein, amongst a panel of platelet-expressed tetraspanins, to be able to induce NFAT/AP-1 signalling in the DT40 luminometry assay.

Tspan18 was firstly identified in 1999 by Perron and Bixby in the chicken embryonic spinal cord (Perron and Bixby 1999). This study reported the presence of the tetraspanins Tspan4 (previously called NAG-2) and CD9, and the presence of a third novel protein, neurospanin (later renamed Tspan18). Nothern blot experiments revealed that Tspan18 mRNA is highly expressed in the developing brain, suggesting that Tspan18 might play a role in nervous system development (Perron and Bixby 1999). The Ouwehand research group later identified Tspan18 mRNA in human megakaryocytes (Macaulay, Tijssen et al. 2007), while we reported Tspan18 mRNA expression in mouse megakaryocytes and CD4-positive helper T cells (Protty, Watkins et al. 2009). The Sickmann group, employing proteomic techniques, recently demonstrated the presence of Tspan18 in human platelets (Lewandrowski, Wortelkamp et al. 2009).

At present, a Tspan18 antibody is not available, and this limits the research work that can be performed to further study this protein. Tspan18 thus remains an uncharacterized tetraspanin protein. Before proceeding in further functional studies, this chapter aimed to perform a preliminary characterization of Tspan18 using bioinformatics and quantitative real-time polymerase chain reaction (qRT-PCR).

4.2 AIM

The aims of this Chapter were twofold. The first was to perform a preliminary Tspan18 characterization, based on the data already present in public bioinformatic databases. The second was to delineate the Tspan18 expression profile using qRT-PCR, employing both a panel of primary cells and cell lines and a panel of mouse tissues.

4.3 RESULTS

4.3.1 Tspan18 bioinformatic analysis

Using the NCBI database, it was possible to perform a preliminary Tspan18 characterization. The human Tspan18 gene was found to be located on chromosome 11, specifically in position 11p11.2. The gene was found to have 10 exons and a total length of 71,836 bp (NC_000011.8). The mRNA appeared to undergo an alternative splicing process to generate two different Tspan18 mRNAs (NM_130783 and NM_001031730). The two different mRNAs encode for proteins with distinct C-termini: Tspan18 isoform 1 (NP_001026900) and Tspan18 isoform 2 (NP_570139). These two isoforms differ only in their use of different final exons (Figure 4.1).

Tspan18 isoform 1 was found to consist of 267 amino acids with a predicted molecular weight of 29.4 KDa, whereas isoform 2 had 248 amino acids with a predicted molecular weight of 27.7 KDa. There were no cytoplasmic signalling motifs which might suggest how Tspan18 could activate the NFAT/AP-1 promoter. ClustalW2 analysis showed that the two proteins had 93% amino acid identity and differed only at the carboxy terminus (Figure 4.2).

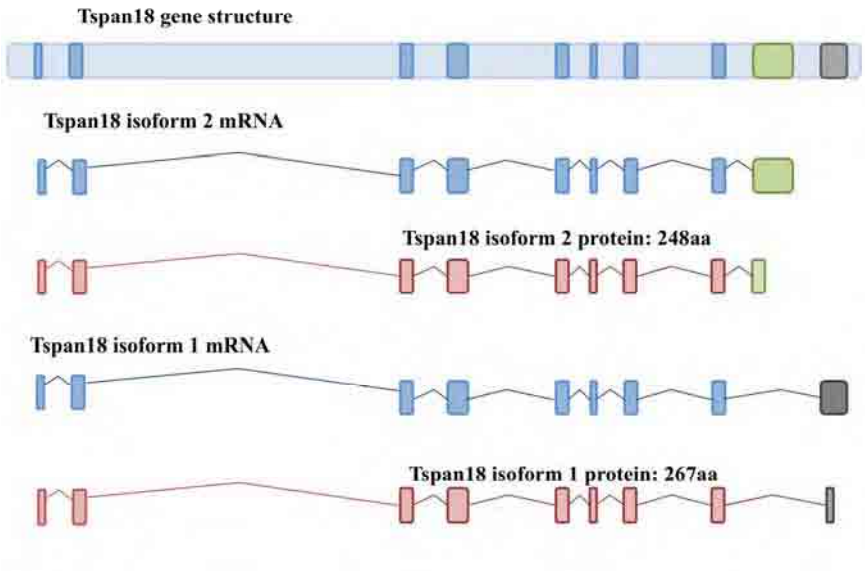


Figure 4.1 Schematic representations of Tspan18 gene structure and protein isoforms. A schematic diagram of the human Tspan18 gene structure is shown in the top panel, with exons common to the two isoforms in dark blue, the two alternatively spliced exons in green and grey, and introns in pale blue.

```
                    cytoplasmic          TM1                SEL
Tspan18 isoform 1 MEGDCLSCMKYLMFVFNFFIFLGGACLLAIGIWVMVDPTGFREIVAANPL 50
Tspan18 isoform 2 MEGDCLSCMKYLMFVFNFFIFLGGACLLAIGIWVMVDPTGFREIVAANPL 50
                  **************************************************
                         TM2          cytoplasmic      TM3
Tspan18 isoform 1 LLTGAYILLAMGGLLFLLGFLGCCGAVRENKCLLLFFFLFILIIFLAELS 100
Tspan18 isoform 2 LLTGAYILLAMGGLLFLLGFLGCCGAVRENKCLLLFFFLFILIIFLAELS 100
                  **************************************************
                                      LEL
Tspan18 isoform 1 AAILAFIFRENLTREFFTKELTKHYQGNNDTDVFSATWNSVMITFGCCGV 150
Tspan18 isoform 2 AAILAFIFRENLTREFFTKELTKHYQGNNDTDIFSATWNSVMITFGCCGV 150
                  **********************************:***************
Tspan18 isoform 1 NGPEDFKFASVFRLLTLDSEEVPEACCRREPQSRDGVLLSREECLLGRSL 200
Tspan18 isoform 2 NGPEDFKFASVFRLLTLDSEEVPEACCRREPQSRDGVLLSREECLLGRSL 200
                  **************************************************
                                                TM4
Tspan18 isoform 1 FLNKQGCYTVILNTFETYVYLAGALAIGVLAIEEERAHSVSQATGILYQL 250
Tspan18 isoform 2 FLNKQGCYTVILNTFETYVYLAGALAIGVLAIE-------------LFAM 237
                  ********************************              *: : 
                                    cytoplasmic
Tspan18 isoform 1 PASPQAFQSPGTLGATA 267
Tspan18 isoform 2 IFAMCLFR--GIQ---- 248
                   :   * :  *
```

Figure 4.2 Sequence alignments of human Tspan18 isoforms 1 and 2. The amino acid sequences of human Tspan18 isoforms 1 and 2 were aligned using the ClustalW2 program. The TMHMM program was used to predict the domain organization. Legend: TM transmembrane, SEL small extracellular loop, LEL large extracellular loop. In black are written Tspan18 isoform 1 and 2 common domains, in red are highlighted the specific Tspan18 isoform 2 domains. The amino acids are colour coded red (small or hydrophobic), blue (acidic), magenta (basic), green (hydroxyl or amine or basic) or grey (other amino acid not previously classified). Asterisks under each letter correspond to identical amino acids, colons represent conserved substitutions, and dashes represent missing amino acids.

Using four different transmembrane protein prediction programs, (TMHMM, PredictProtein, MEMSAT and DAS) it was possible to predict the location of the transmembrane regions of Tspan18 isoforms 1 and 2. The TMHMM program (accessible at http://www.cbs.dtu.dk/services/TMHMM/) predicts the location of transmembrane domains based on the hidden Markov model (Sonnhammer, von Heijne et al. 1998). After sequence analysis, the program provides a bioinformatic prediction, expressed as a confidence value between 0 and 1. Tspan18 isoform 2 appeared to contain the four transmembrane domains that are characteristic of tetraspanins (Figure 4.3A). In contrast, isoform 1 appeared to be composed of only three transmembrane domains, with an extracellular carboxy terminus, although the confidence value of this prediction was just 0.4, indicating 40% probability (Figure 4.3A). The PredictProtein program (accessible at http://www.predictprotein.org/) predicts locations of transmembrane helices based on evolutionary profiles (Rost, Fariselli et al. 1996) .Using the PredictProtein program, and taking into consideration the “best model” output, both Tspan18 isoform 2 and Tspan18 isoform 1 appeared to contain four transmembrane domains. The overall reliability of the models, both for Tspan18 isoform 1 and Tspan18 isoform 2, is 6 on a scale between 0 and 9, where 1 is the lowest value and 9 the highest value (Figure 4.3B). The MEMSAT3 program (accessible at http://bioinf4.cs.ucl.ac.uk:3000/psipred) is an improved version of the MEMSAT program, which is based on the recognition of topological models (Jones, Taylor et al. 1994; Jones 2007). The analysis with this program suggested that Tspan18 isoform 2 and Tspan18 isoform 1 consisted of four transmembrane domains (Figure 4.3C). The DAS-TMfilter program (accessible at http://www.enzim.hu/DAS/DAS.html) is based on the analysis of low-stringency dot-plots of the amino acid sequence against a collection of non-homologous membrane proteins using a previously derived, special scoring matrix (Cserzo, Eisenhaber et

al. 2002). The DAS-TMfilter program generates a graphic, and presents two cutoff values: a loose cutoff value, used to predict the exact transmembrane domain from the inserted amino acid sequence, and a strict cutoff value, used to determine how many transmembrane domains are contained in the sequence. Tspan18 isoform 2 clearly presented four tranmembrane domains, with a clear hydrophobic region in the large extracellular loop, between the amino acids 160 and 220. Tspan18 isoform 1 presented four peaks, suggesting four transmembrane domains, despite the fourth peak appearing to be thinner and of lower intensity, suggesting a lower confidence value for this peak (Figure 4.3D). In conclusion, from the data collected with four different transmembrane prediction programs, it is highly likely that Tspan18 isoform 2 has four transmembrane domains, in common with other tetraspanins. The data collected so far regarding Tspan18 isoform 1 is not so clear, but three of the four prediction programs indicate that this isoform has four transmembrane domains. Therefore Tspan18 isoform 1 has two possible structures: a more probable structure with four transmembrane domains, or a less likely structure with three transmembrane domains (Figure 4.3E).

A. TMHMM program analysis

Aminoacid sequence	prediction	confidence
1-12	Cytoplasmic	1
13-35	Transmembrane	1
36-49	Extracellular	1
50-72	Transmembrane	1
73-84	Cytoplasmic	1
85-107	Transmembrane	1
108-221	Extracellular	0.8
222-244	Transmembrane	1
245-248	Cytoplasmic	0.8

Tspan18 isoform 2

Aminoacid sequence	prediction	confidence
1-12	Cytoplasmic	1
13-35	Transmembrane	1
36-49	Extracellular	1
50-72	Transmembrane	1
73-84	Cytoplasmic	1
85-107	Transmembrane	1
108-267	Extracellular	0.4

Tspan18 isoform 1

B. PredictProtein program analysis

Tspan18 isoform 2

Aminoacid sequence	prediction	confidence
1-14	Cytoplasmic	
15-32	Transmembrane	0.65
33-51	Extracellular	
52-72	Transmembrane	0.71
73-82	Cytoplasmic	
83-103	Transmembrane	0.77
104-219	Extracellular	
220-241	Transmembrane	0.84
242-248	Cytoplasmic	

Tspan18 isoform 1

Aminoacid sequence	prediction	confidence
1-14	Cytoplasmic	
15-32	Transmembrane	0.65
33-51	Extracellular	
52-72	Transmembrane	0.71
73-82	Cytoplasmic	
83-105	Transmembrane	0.77
106-220	Extracellular	
221-245	Transmembrane	0.89
246-267	Cytoplasmic	

C. MEMSAT3 program analysis

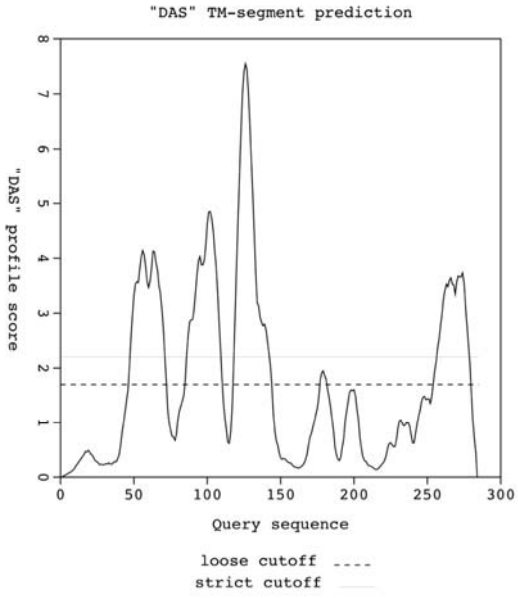
Tspan18 isoform 2

Aminoacid sequence	prediction	score
1-10	Cytoplasmic	
12-36	Transmembrane	35.45
37-52	Extracellular	
53-77	Transmembrane	33.07
78-82	Cytoplasmic	
83-106	Transmembrane	22.73
107-212	Extracellular	
213-237	Transmembrane	20.35
238-248	Cytoplasmic	

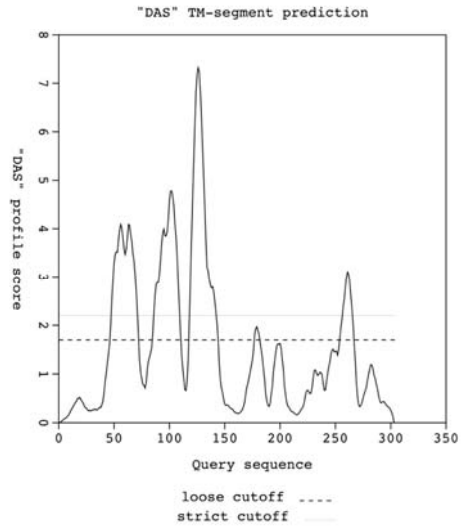
Tspan18 isoform 1

Aminoacid sequence	prediction	confidence
1-12	Cytoplasmic	
12-36	Transmembrane	35.45
37-52	Extracellular	
53-77	Transmembrane	33.07
78-82	Cytoplasmic	
83-107	Transmembrane	26.54
108-212	Extracellular	
213-233	Transmembrane	19.00
234-267	Cytoplasmic	

D. DAS-TMfilter program analysis



Tspan18 isoform 2



Tspan18 isoform 1

E.

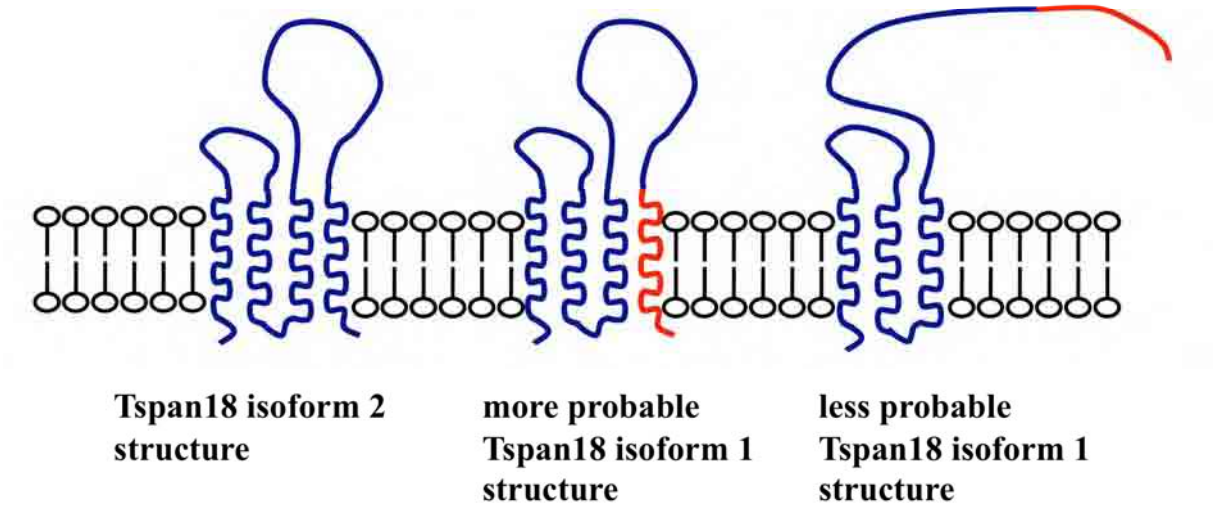


Figure 4.3 Bioinformatic prediction of Tspan18 isoform 2 and Tspan18 isoform 1 transmembrane domains. Tspan18 isoform 2 and Tspan18 isoform 1 sequences have been analyzed with the TMHMM (A), PedictProtein (B), MEMSAT (C) and DAS (D) prediction programs to identify the trasmembrane domains. In panels A, B and C, the first column represents the amino acid sequence, the second column the domain prediction and the third the degree of confidence expressed between the values 0 and 1 (A and B), or as a score (C). The DAS program produces the results as a graphic representation. On the X axis is the amino acid posistion of the analyzed sequence, while on the Y axis the degree of confidence is expressed between values of 0 and 8. The loose cut off is indicated at a value of 1.8, and the strict cut off is indicated at a value of 2.2 (D). Schematic representation of hypothetical Tspan18 conformations, based on the TMHMM prediction. Tspan18 isoform 2 amino acid sequence is represented in blue. The two alternate possibilities for the Tspan18 isoform 1 carboxy-terminus are represented in red (E).

In order to determine in which organisms the two Tspan18 isoforms are expressed, Tspan18 isoform 1 and Tspan18 isoform 2 sequences were analyzed using the Basic Local Alignment Search Tool (BLAST) tool of the NCBI database, performing a search across all the organisms available in the protein sequences database. Tspan18 isoform 1 was found to be expressed in human, chimp (*Pan troglodytes*) and macaque monkey (*Macaca mulatta*) (Figure 4.4A), while Tspan18 isoform 2 was found to be expressed in mammals, birds, amphibians and fish (Figure 4.4B). Tspan18 isoform 2 appeared to be well conserved between species, with amino acid identities varying between 99% (*Homo sapiens* versus *Pan troglodytes*) and 69% (between *Pan troglodytes* and *Danio rerio*) (Figure 4.4C).

In order to determine in which tissues Tspan18 is expressed, an analysis of human Tspan18 (Unigene number Hs.385634) was conducted using the Expression Sequence Tags (ESTs) profile of the Unigene NCBI database. Based on these data, Tspan18 appears to be expressed in most body sites, health states and developmental stages (Figure 4.4D). However, the general expression level is relatively low. For example, as a comparison, CD9 and CD81 are commonly expressed at levels of 100-500 ESTs per million in different tissues (data not shown), whereas very few tissues express Tspan18 at a level greater than 50 ESTs per million, a number which is often derived from less than five total ESTs per EST library (Figure 4.4D). Due to this relatively low level of expression, it is not possible to accurately define the Tspan18 expression profile from EST data.

A.

```
Homo sapiens     MEGDCLSCMKYLMFVFNFFIFLGGACLLAIGIWVMVDPTGFREIVAANPLLLTGAYILLA 60
Pan troglodytes  MEGDCLSCMKYLMFVFNFFIFLGGACLLAIGIWVMVDPTGFREIVAANPLLLTGAYILLA 60
Macaca mulatta   MEGDCLSCMKYLMFVFNFFIFLGGACLLAIGIWVMVDPTGFREIVAANPLLLTGAYILLA 60
                 ************************************************************

Homo sapiens     MGGLLFLLGFLGCCGAVRENKCLLLFFFLFILIIFLAELSAAILAFIFRENLTREFFTKE 120
Pan troglodytes  MGGLLFLLGFLGCCGAVRENKCLLLFFFLFILIIFLAELSAAILAFIFRENLTREFFTKE 120
Macaca mulatta   MGGLLFLLGFLGCCGAVRENKCLLLFFFLFILIIFLAELSAAILAFIFRENLTREFFTKE 120
                 ************************************************************

Homo sapiens     LTKHYQGNNDTDVFSATWNSVMITFGCCGVNGPEDFKFASVFRLLTLDSEEVPEACCRRE 180
Pan troglodytes  LTKHYQGNNDTDVFSATWNSVMITFGCCGVNGPEDFKFASVFRLLTLDSEEVPEACCRRE 180
Macaca mulatta   LTKHYQGNNDTDVFSATWNSVMITFGCCGVNGPEDFKFASVFRLLTLDSEEVPEACCRRE 180
                 ************************************************************

Homo sapiens     PQSRDGVLLSREECLLGRSLFLNKQGCYTVILNTFETYVYLAGALAIGVLAIEEERAHSV 240
Pan troglodytes  PQSRDGVLLSREECLLGRSLFLNKQGCYTVILNTFETYVYLAGALAIGVLAIEEERAHSV 240
Macaca mulatta   PQSRDGVLLSREECLLGRSLFLNKQGCYTVILNTFETYVYLAGALAIGVLAIEVRRAHSV 240
                 ************************************************ .*****

Homo sapiens     SQATGILYQLPASPQAFQSPGTLGATA 267
Pan              SQATGILYQLPASPQAFQSPGTLGATA 267
Macaca mulatta   SQATSTLYQPPASPQAFQSPGTLGVTA 267
                 ****. *** ***************.**
```

B.

```
Homo sapiens       MEGDCLSCMKYLMFVFNFFIFLGGACLLAIGIWVMVDPTGFREIVAANPLLLTGAYILLA 60
Pan troglodytes    MEGDCLSCMKYLMFVFNFFIFLGGACLLAIGIWVMVDPTGFREIVAANPLLLTGAYILLA 60
Bos taurus         MEGDCLSCMKYLMFVFNFFIFLGGACLLGIGIWVMVDPTGFREIVAANPLLITGAYILLA 60
Mus musculus       MEGDCLSCMKYLMFVFNFFVFLGGACLLGVGIWVLVDPTGFREIVATNPLLTTGAYIVLA 60
Rattus norvegicus  MEGDCLSCMKYLMFVFNFFVFLGGACLLGVGIWVLVDPTGFREIVATNPLLTTGAYVVLA 60
Gallus gallus      MEGDCLSCMKYLMFLFNFFIFLGGACLLGVGIWVIVDPTGFREIVAANPLLFTGAYIMLA 60
Xenopus tropicalis MEGDNLSCIKYLMFIFNFFIFLGGATLLGLGVWVFVDPTGFREIIATTPLLFMGAYLVLA 60
Danio rerio        MEGDCLSCIKYLMFIFNFFIFLGGSFLLGVGIWVLVDPTGFREIVAANSLLFTGVYAILI 60
                   **** ***:*****:*****:*****: **.:*:**:*********:*:..** .* :*

Homo sapiens       MGGLLFLLGFLGCCGAVRENKCLLLFFFLFILIIFLAELSAAILAFIFRENLTREFFTKE 120
Pan troglodytes    MGGLLFLLGFLGCCGAVRENKCLLLFFFLFILIIFLAELSAAILAFIFRENLTREFFTKE 120
Bos taurus         MGGLLFLLGFLGCCGAVRENKCLLLFFFLFILIIFLAELSAAILAFIFRGNLTREFFTKE 120
Mus musculus       MGGLLFLLGFLGCCGAVRENRCLLLFFFLFILIIFLVELSAAILAFIFREHLTREFFTKE 120
Rattus norvegicus  MGGLLFLLGFLGCCGAVRENRCLLLFFFLFILIIFLVELSAAILAFIFREHLTREFFTKE 120
Gallus gallus      MGAMLFLLGFLGCCGAIRENKCLLLFFFMFILLIFLAELSAAILAFIFRENLTKEFFTKE 120
Xenopus tropicalis MGGMLFLLGFLGCCGAIRENKCLLVFFFMFILVIFLAELSAAILAFLFRENLSKDFFARE 120
Danio rerio        MGGMLFLLGFLGCCGAIRENKCLLLFFFMLILVIFLAELAVAILAFIFREHLTRDYFTKE 120
                   **.:************:***:***:***::**:***.**:.*****:** :*::::*::*

Homo sapiens       LTKHYQGNNDTDIFSATWNSVMITFGCCGVNGPEDFKFASVFRLLTLDSEEVPEACCRRE 180
Pan troglodytes    LTKHYQGNNDTDVFSATWNSVMITFGCCGVNGPEDFKFASVFRLLTLDSEEVPEACCRRE 180
Bos taurus         LTKHYQGSNDTDVFSATWNSVMITFGCCGVNGPEDFEYASVFRLLTLDSDEVPEACCRRE 180
Mus musculus       LTKHYQGDNDTDVFSATWNSVMITFGCCGVNGPEDFKLASVFRLLTLDTEEVPKACCRRE 180
Rattus norvegicus  LTKHYQGDNDTDVFSATWNSVMITFGCCGVNGPEDFKLASVFRLLTLDTEEVPKACCRRE 180
Gallus gallus      LEKHYVRNNDTHVFSSTWNSVMITFACCGVNGPEDFEAVPPLSHLPLE-ETTPEACCQRN 179
Xenopus tropicalis VEKHYHGDNSTEVFSSTWNSIMITFGCCGVNGPEDFNDAHRFRAMHFF-APVPEACCRRE 179
Danio rerio        LETHYQGTNSTDVFTSTWNAIMTTFNCCGVNSAEDFDDQSLFRRLNPS-RIVPEVCCQR- 178
                   :..**  *.*.:*::***::* ** *****.:***.    : :      .*:.**:*

Homo sapiens       PQSRDGVLLSREECLLGRSLFLNKQGCYTVILNTFETYVYLAGALAIGVLAIELFAMIFA 240
Pan troglodytes    PQSRDGVLLSREECLLGRSLFLNKQGCYTVILNTFETYVYLAGALAIGVLAIELFAMIFA 240
Bos taurus         PQSRDGVLLSREECLLGRDLFLNKQGCYTVILNAFETYVYLAGALAIGVLAIELFAMIFA 240
Mus musculus       PQTRDGVVLSREECQLGRNPFINKQGCYTVILNTFETYVYLAGAFAIGVLAIELFLMVFA 240
Rattus norvegicus  PQTRDGVVLSREECQLGRNPFINKQGCYTVILNTFETYVYLAGAFAIGVLAIELFLMVFA 240
Gallus gallus      VQSREGMFVNRKACLEGDERFQNRQGCYTVILNSFETYVYLAGALAIGVLAIELFAMIFA 239
Xenopus tropicalis VQSRAGKIVSRAECLTGGENYQNRQGCYSVIVNSVEPYVYIAGALAIGVLAIELLSMVFA 239
Danio rerio        ----TDLMMSKEECLRGIMPIRNP-GGYSAVVDYFETYIYMAGALAIVVLTIELFAMVFA 233
                       . .:.:  *  *     *: ***:.::: .*.*:*:***:** **:***: *:**

Homo sapiens       MCLFRGIQ- 248
Pan troglodytes    MCLFRGIQ- 248
Bos taurus         MCLFRGIIQ 249
Mus musculus       MCLFRGIQ- 248
Rattus norvegicus  MCLFRGIQ- 248
Gallus gallus      MCLFRGIQ- 247
Xenopus tropicalis MCLFRGIQ- 247
Danio rerio        MCLFRGIQ- 241
                   *******
```

C.

SeqA	Name	Len(aa)	SeqB	Name	Len(aa)	Score
1	Homo	248	2	Pan	248	99
1	Homo	248	3	Bos	249	95
1	Homo	248	4	Gallus	247	80
1	Homo	248	5	Xenopus	247	72
1	Homo	248	6	Mus	248	90
1	Homo	248	7	Danio	241	70
1	Homo	248	8	Rattus	248	89
2	Pan	248	3	Bos	249	96
2	Pan	248	4	Gallus	247	81
2	Pan	248	5	Xenopus	247	72
2	Pan	248	6	Mus	248	90
2	Pan	248	7	Danio	241	70
2	Pan	248	8	Rattus	248	90
3	Bos	249	4	Gallus	247	80
3	Bos	249	5	Xenopus	247	72
3	Bos	249	6	Mus	248	89
3	Bos	249	7	Danio	241	70
3	Bos	249	8	Rattus	248	89
4	Gallus	247	5	Xenopus	247	73
4	Gallus	247	6	Mus	248	77
4	Gallus	247	7	Danio	241	69
4	Gallus	247	8	Rattus	248	76
5	Xenopus	247	6	Mus	248	71
5	Xenopus	247	7	Danio	241	68
5	Xenopus	247	8	Rattus	248	71
6	Mus	248	7	Danio	241	69
6	Mus	248	8	Rattus	248	99
7	Danio	241	8	Rattus	248	69

D. **EST Profile**

Hs.385634 - TSPAN18: Tetraspanin 18

Breakdown by Body Sites

	Hs.385634	
adipose tissue	0	0 / 13157
adrenal gland	89	3 / 33345
ascites	0	0 / 40058
bladder	33	1 / 30132
blood	0	0 / 124118
bone	13	1 / 71802
bone marrow	0	0 / 49117
brain	50	56 / 1104762
cervix	0	0 / 48493
connective tissue	33	5 / 149587
ear	61	1 / 16342
embryonic tissue	50	11 / 215834
esophagus	0	0 / 20212
eye	56	12 / 211510
heart	99	9 / 90306
intestine	59	14 / 235742
kidney	51	11 / 212573
larynx	81	2 / 24485
liver	0	0 / 208425
lung	50	17 / 338196
lymph	22	1 / 44402
lymph node	0	0 / 91914
mammary gland	0	0 / 154518
mouth	44	3 / 67217
muscle	36	4 / 108182
nerve	0	0 / 15823
ovary	29	3 / 102639
pancreas	41	9 / 215274
parathyroid	48	1 / 20648
pharynx	0	0 / 41510
pituitary gland	0	0 / 16730
placenta	28	8 / 284180
prostate	15	3 / 190687
salivary gland	49	1 / 20272
skin	0	0 / 211665
spleen	18	1 / 54043
stomach	20	2 / 97180
testis	66	22 / 331398
thymus	0	0 / 81184
thyroid	83	4 / 47950
tonsil	0	0 / 17042

trachea	0	0 / 52429
umbilical cord	290	4 / 13761
uterus	51	12 / 233967
vascular	38	2 / 51943

Breakdown by Health State

	Hs.385634	
adrenal tumor	0	0 / 12864
bladder carcinoma	56	1 / 17761
breast (mammary gland) tumor	0	0 / 94664
cervical tumor	0	0 / 34589
chondrosarcoma	12	1 / 82864
colorectal tumor	17	2 / 115116
esophageal tumor	0	0 / 17293
gastrointestinal tumor	0	0 / 119843
germ cell tumor	105	28 / 264768
glioma	37	4 / 107556
head and neck tumor	43	6 / 137382
kidney tumor	28	2 / 69393
leukemia	10	1 / 96639
liver tumor	0	0 / 96673
lung tumor	19	2 / 103503
lymphoma	13	1 / 72058
non-neoplasia	41	4 / 97517
normal	48	163 / 3376109
ovarian tumor	25	2 / 77205
pancreatic tumor	76	8 / 104951
primitive neuroectodermal tumor...	55	7 / 126444
prostate cancer	0	0 / 103703
retinoblastoma	85	4 / 46514
skin tumor	0	0 / 125583
soft tissue/muscle tissue tumor	31	4 / 125858
uterine tumor	11	1 / 90821

Breakdown by Developmental Stage

		Hs.385634
embryoid body	70	5 / 70770
blastocyst	64	4 / 62335
fetus	95	54 / 567918
neonate	96	3 / 31174
infant	295	7 / 23654
juvenile	0	0 / 55759
adult	38	75 / 1953448

Figure 4.4 Tspan18 bioinformatics analysis. Amino acid lineup of Tspan18 isoform 1 from human (*Homo sapiens*), chimp (*Pan troglodytes*) and macaque (*Macaca mulatta*) (A.). Amino acid lineup of Tspan18 isoform 2 in primates and cow (*Bos taurus*), mouse (*Mus musculus*), rat (*Rattus norvegicus*), chicken (*Gallus gallus*), frog (*Xenopus tropicalis*) and zebrafish (*Danio rerio*) (B.). The amino acids are colour coded red (small or hydrophobic), blue (acidic), magenta (basic), green (hydroxyl or amine or basic) or grey (other amino acid not previously classified). Asterisks under each letter correspond to identical amino acids, colons represent conserved substitutions, full stops represent semi-conserved substitutions, and dashes represent missing amino acids. Tspan18 isoform 2 is highly conserved among species, with amino acid identities between 99% (*Homo sapiens* versus *Pan troglodytes*) and 69% (*Pan troglodytes* versus *Daino rerio*), as indicated in the final 'Score' column (C.). Human Tspan18 EST profile by body sites, health states and developmental stages. The first column represents the source, the second the transcripts per million (TPM), the third the predicted intensity of the spot based on TPM, and the fourth represents the number of Tspan18 ESTs compared to the total ESTs in the pool (D.).

4.3.2 Tspan18 qRT-PCR expression profile

In order to determine the Tspan18 expression profile, the qRT-PCR technique was employed. For each sample of cells or tissue, total RNA was extracted, RNA was converted into cDNA, and cDNA was analyzed by qRT-PCR.

Tspan18 isoform 2 expression was firstly assessed in a panel of human cell lines and primary cells. The result showed that Tspan18 isoform 2 was expressed most strongly in HMEC-1 and HUVEC endothelial cells and in peripheral blood leucocytes, more weakly in DG75 B cells, Jurkat T cells, hepatocytes and U937 monocytes, but not at all in the others tested (Figure 4.5A). In contrast, isoform 1 was not detected at substantial levels in any cell type (Figure 4.5B), suggesting that this isoform may not be expressed. However, this does not rule out the possibility that this isoform is expressed in a subpopulation of cells such as peripheral blood leukocytes, which appeared to have the highest level of those tested (Figure 4.5B).

The expression profile of the mouse homologue of Tspan18 was further examined by qRT-PCR from a panel of mouse tissues. The result showed that Tspan18 isoform 2 was relatively highly expressed in mouse lung, while expression in the other analyzed tissues was approximately 10-fold lower (Figure 4.6). This is consistent with the relatively strong endothelial cell expression observed for human Tspan18 (Figure 4.5), since lung tissue contains a relatively large proportion of endothelial cells. Indeed, qRT-PCR analysis of RhoJ, a *bone fide* endothelial-specific gene, yielded similar data to Tspan18 from this panel of tissues (S Kaur and V Heath, unpublished data).

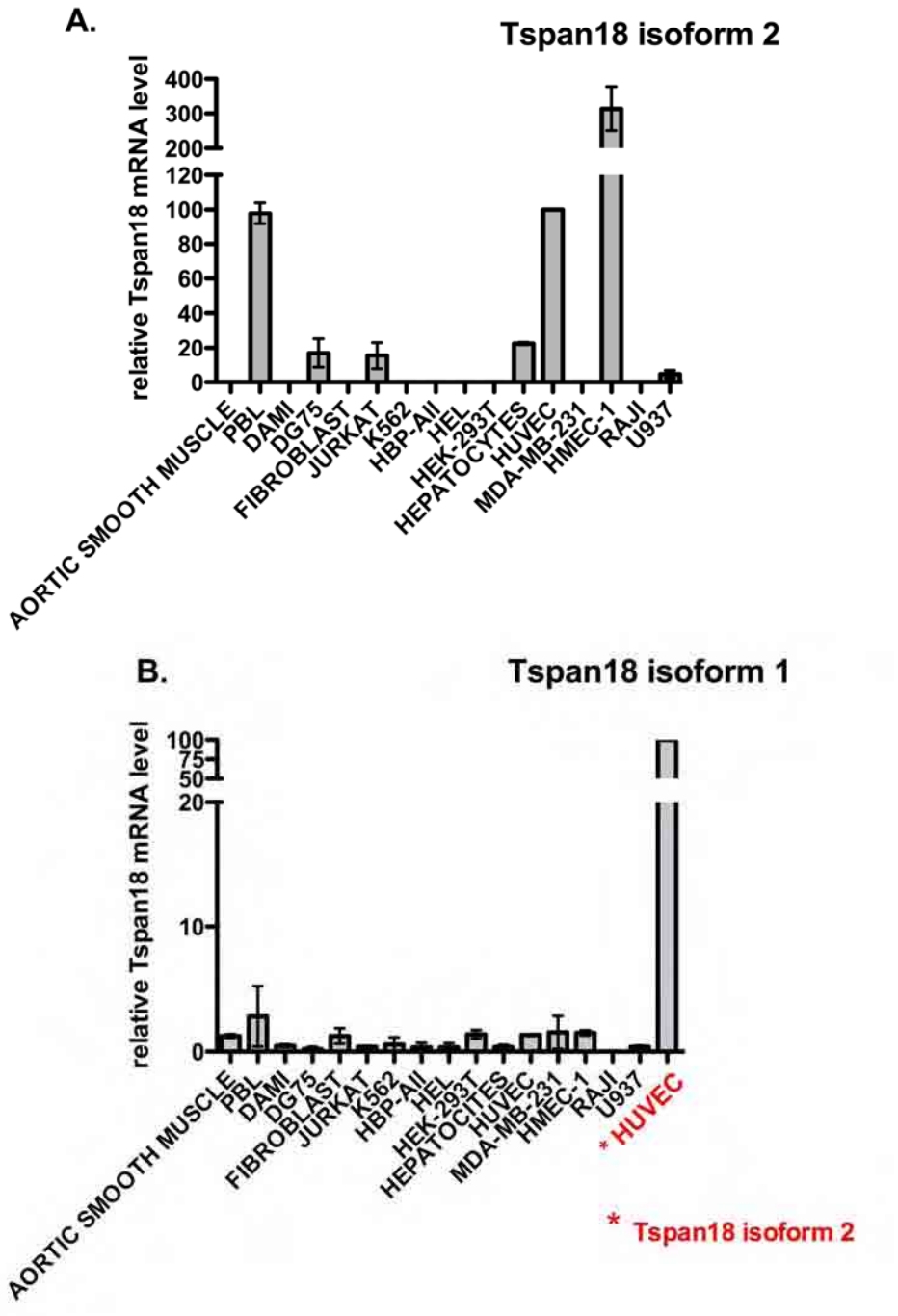


Figure 4.5 Human Tspan18 qRT-PCR expression profile in different primary cells and cell lines. Each sample was normalized for the relative qRT-PCR actin value. Data were adjusted such that Tspan18 isoform 2 expression in HUVEC was 100 (A.). qRT-PCR expression profile of Tspan18 isoform 1 in different cell lines. Data were adjusted such that Tspan18 isoform 2 expression in HUVEC was 100 (in red) (B.) Error bars represent the standard error of two independent experiments. The cell types were PBL (peripheral blood leukocytes), DAMI (megakaryocytic cell line), DG75 (B cell line), Jurkat (T cell line), K562 (erthythroleukaemia cell line), HBP-ALL (T cell line), HEL (erythroleukaemia cell line), HEK-293T (embryonic kidney cell line), HUVEC (umbilical vein endothelial cell) MDA-MB-231 (epithelial cell line), HMEC-1 (microvascular endothelial cell line), RAJI (B cell line), U937 (monocyte cell line).

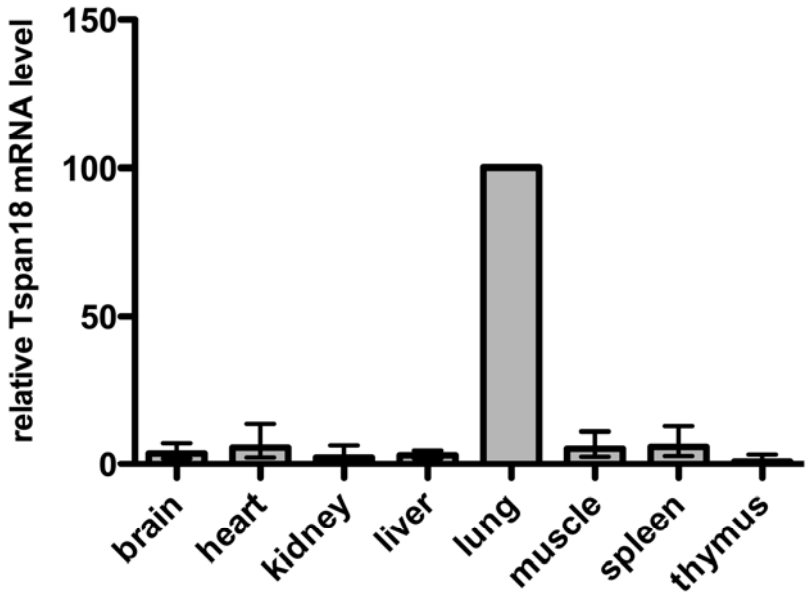


Figure 4.6 Mouse Tspan18 qRT-PCR expression profile in different mouse tissues. Each sample was normalized for the relative qRT-PCR HPRT (hypoxanthine phosphoribosyl transferase) value. Data were adjusted such that mouse Tspan18 isofom 2 expression in lung tissue was 100. Error bars represent the standard error of three independent experiments.

4.4 DISCUSSION

In this Chapter, the Tspan18 gene and protein sequence were characterized using data in public sequence databases, and the Tspan18 expression profile was evaluated using the qRT-PCR technique. The bioinformatics analysis revealed two Tspan18 protein isoforms expressed in humans, *Pan troglodytes* and *Macaca mulatta*, generated by the alternative splicing of the last exon. The qRT-PCR data suggested that only one of these isoforms was expressed at substantial levels (Tspan18 isoform 2), and further suggested relatively strong endothelial expression and weaker expression in leukocytes.

The alternative Tspan18 splicing form, Tspan18 isoform 1, appears to be a relatively recent development in evolution, since this does not occur in more primitive mammals, chicken, frog or fish. While Tspan18 isoform 2 contains the canonical four transmembrane regions typical of tetraspanins, the bioinformatics domain prediction did not unequivocally reveal if the last exon of isoform 1 is a transmembrane domain or an extracellular domain, as three of the four employed programs to predict the location of the transembrane indicated that this isoform present four transmembrane domains. Nevertheless, it is possible that, this less likely and atypical isoform 1, may have a distinct function from the more classical isoform 2, or might regulate the function of isoform 2 by acting as a dominant negative. A key function of the carboxy-terminus of tetraspanins is not without precedent. For example, the carboxy-terminus of CD63 contains intracellular targetting motifs and PDZ-binding motifs (Berditchevski and Odintsova 2007). In addition, the carboxy-terminus of CD151 is essential for the regulation of integrin function (Lammerding, Kazarov et al. 2003). However, the qRT-PCR data suggested that this atypical isoform is either not expressed or is expressed at very low levels compared to isoform 2. It is nevertheless possible that isoform 1 is expressed in a subpopulation of cells such as leukocytes. The future generation of an isoform 1-specific antibody would address

this issue at the protein level.

Bioinformatics of Tspan18 species homologues further revealed that Tspan18 protein sequence is relatively highly conserved and expressed in a broad range of healthy and pathological tiessues. However, Tspan18 sequence conservation, at least at the level of human versus mouse, is quite typical of other tetraspanins (Tomlinson, JTH, 2009).

The qRT-PCR data revealed that Tspan18 has a restricted expression profile. The mouse homologue was relatively highly expressed in lung, which is suggestive of strong endothelial cell expression, since the endothelial-specific gene RhoJ showed the same expression pattern in mouse tissues (S Kaur and V Heath, unpublished data). Moreover, human Tspan18 isoform 2 was relatively highly expressed in the endothelial cells tested. However, Tspan18 is clearly not restricted to this cell type. Expression was also detected in human peripheral blood leucocytes, consistent with our earlier identification of expression in CD4-positive helper T cells, with weak expression in some lymphocyte cell lines and hepatocytes. In addition, Tspan18 has been identified in human platelets using proteomics (Lewandrowski, Wortelkamp et al. 2009). The future generation of an antibody is essential to determine in precisely which cell types Tspan18 protein is expressed.

The qRT-PCR technique is well established for the quantitation of gene expression (VanGuilder, Vrana et al. 2008). The qRT-PCR technique employed in this study uses a Corbett Rotorgene 6000 qRT-PCR machine, which has some technical advantages over a conventional block qRT-PCR. For instance, as the amplification reaction is performed on a spinning rotor, the samples are exposed to less thermal variation than the conventional qRT-PCR, resulting in increased reproducibility (Kim, Yang et al. 2008).

An intrinsic necessity of the qRT-PCR technique employed in this Chapter is tissue

homogenization. This step inevitably destroys the tissue morphology, and it is not possible to identify where in the organ the mRNA is expressed. A possible strategy that could complement the data collected so far is *in situ* hybridisation. This technique involves the hybridization of a labeled complementary nucleic acid to allow the visualization of a specific mRNA on a tissue section, so preserving the tissue morphology (Jin and Lloyd 1997). The employment of this technique in future would address whether Tspan18 is relatively highly expressed on endothelial cells, particularly if a mouse embryo was used, in which the vasculature can be readily detected.

4.5 SUMMARY

In the present Chapter it has been demonstrated that:

- In humans, Tspan18 protein consists of two isoforms, Tspan18 isoform 1 and Tspan18 isoform 2, deriving from the alternative splicing of the last gene exon.
- Bioinformatic analysis revealed that it is lickely Tspan18 isoform 1 contains four transmembrane domains, and is expressed, in addition to humans, in *Macaca mulatta* and *Pan troglodytes*.
- qRT-PCR analysis revealed that Tspan18 isoform 1 is expressed at 5- to 20-fold lower than isoform 2, if at all. isoform 2 appears to be relatively highly expressed in endothelial cells, with weaker expression in leukocytes and hepatocytes but not other cell types tested.
- Tspan18 isoform 2 is highly expressed in mouse lung and is expressed 10-fold lower in the other tested tissues, consistent with strong endothelial cell expression.

CHAPTER 5

SUBCELLULAR LOCALISATION AND STRUCTURE-FUNCTION ANALYSES OF TSPAN18

5.1 INTRODUCTION

5.1.1 Overview

In Chapter 3, Tspan18, a novel and previously uncharacterized tetraspanin protein, was found to be able to induce NFAT/AP-1 activation when over-expressed in the DT40 B cell line. Moreover, a preliminary bioinformatic characterization, performed in Chapter 4, revealed that in humans Tspan18 exists as two different isoforms, Tspan18 isoform 1 and Tspan18 isoform 2. The qRT-PCR expression profile demonstrated that Tspan18 isoform 2 is expressed in both cell lines and primary cells (particularly HMEC-1 and HUVEC endothelial cells), while a mouse tissue analysis revealed that Tspan18 isoform 2 is highly expressed in mouse lung tissues, consistent with relatively strong endothelial expression. Tspan18 isoform 1, however, was not readily detectable in the cell types analyzed.

At present, a Tspan18 antibody is not available, and it is not possible to study Tspan18 protein in tissue sections or perform biochemical assays assessing expression levels of endogenous Tspan18. In order to overcome this technical problem and perform a preliminary Tspan18 characterization using biochemical approaches, a human FLAG-tagged Tspan18 isoform 2 protein construct was employed in this chapter.

5.1.2 Lipid rafts

Lipid rafts are defined as “small (10–200 nm) heterogeneous, highly dynamic, sterol- and sphingolipid-enriched domains that compartmentalize cellular processes. Small rafts can sometimes be stabilized to form larger platforms through protein–protein and protein–lipid interactions” (Pike 2009). Lipid raft membrane microdomains are enriched in glycosphingolipids, cholesterol, glycosylphosphatidylinositol-anchored proteins and Src

family kinases; they appear to play many roles, especially in immune-receptor signalling. Lipid rafts play a critical role in the recruitment and the formation of multi-protein signalling complexes. The concept of lipid rafts as signalling platforms was proposed in 1997 (Simons and Ikonen 1997).

Tspan18 contains a conserved amino-terminal CXXC motif. Such a motif, in LAT family adapters and when palmitoylated, targets these proteins to lipid rafts (Lin, Weiss et al. 1999). In this chapter, the raft localization of Tspan18 will be investigated, alongside two other tetraspanins, Tspan9 and CD9, because of the presence and absence of CXXC motifs in these proteins, respectively.

5.1.3 Glycosylation and palmitoylation

Proteins can undergo several post-translational modifications, some of which include the addition of lipid molecules (Folch, Arsove et al. 1951). S-palmitoylation is the reversible addition of a fatty acid (palmitate) to proteins on cysteine residues via a thioester bond (Joyoti 2004). This post-translational modification is reversible, in contrast to myristoylation. S-palmitoylation can regulate several protein functions, including trafficking, localization, partitioning into microdomains, and protein-protein interactions (Joyoti 2004).

Palmitoylation is a key protein modification that supports the assembly of tetraspanin complexes. Removal of palmitoylation sites from tetraspanin proteins does not disrupt primary tetraspanin associations, such as CD9-CD9, CD151-$\alpha_3\beta_1$ or CD151-$\alpha_6\beta_4$ associations (Berditchevski, Odintsova et al. 2002; Kovalenko, Yang et al. 2004; Yang, Kovalenko et al. 2004), but does reduce secondary tetraspanin associations, resulting in impaired cell signalling and altered cell morphology (Hemler 2008). Moreover, CD81

palmitoylation is implicated in regulating the B cell receptor complex. The Pierce research group showed that CD81 palmitoylation is necessary for sustained localization of the CD19/CD21/CD81 B cell co-receptor complex to lipid rafts and for sustained BCR signalling (Cherukuri, Shoham et al. 2004), although this has recently been challenged (Sanyal, Fernandez et al. 2009).

Glycosylation is the enzymatic process that links complex heteropolymeric sugars to proteins or lipids (Taylor and Drickamer 2006). Sugars can be attached to proteins either through a nitrogen atom (N-linked) or an oxygen atom (O-linked). The function of glycosylation can be categorized in the following five broad categories: providing structural components (cell walls and extracellular matrix), modifying protein properties (solubility and stability), directing trafficking of glycoconjugates, mediating and modulating cell adhesion and cell signalling (Taylor and Drickamer 2006).

Most tetraspanins contain at least one N-glycosylation site, while several contain two, three or even four sites (Tarrant, Robb et al. 2003). Although the precise role of tetraspanin glycosylation is not known, tetraspanins can affect the glycosylation of their molecular partners. CD151, for example, regulates $\alpha_3\beta_1$ integrin glycosylation (Baldwin, Novitskaya et al. 2008).

5.2 AIM

The aim of this chapter was to investigate the sub-cellular localisation and to perform a preliminary biochemical characterization of Tspan18 using amino-terminal FLAG- and GFP-tagged human Tspan18 constructs, transfected into HEK-293T or DT40 cell lines.

5.3 RESULTS

5.3.1 Study of Tspan18 subcellular localization

In order to determine the subcellular localization of Tspan18 in the absence of a Tspan18 antibody, an amino-terminal GFP-tagged Tspan18 construct was generated, transfected into DT40 cells, HEK-293T cells, and C_2C_{12}, and analysed by confocal microscopy two days post-transfection. DT40 were chosen because of the observed Tspan18 signalling phenotype in these cells (Chapter 3). In DT40 cells, the non-receptor protein tyrosine kinase Btk was used as a cytoplasmic control, alongside Tspan18, while the platelet collagen receptor GPVI/FcRγ complex was employed as a cell surface control. DT40 cells have a relatively small amount of cytoplasm relative to the size of the nucleus (Figure 5.1A). HEK-293T cells were transfected with GFP, used as negative control or Tspan18-GFP tagged, but the result is inconclusive, and the cell morphology does not appear to be round and not spread (Figure 5.2B). The mouse fibroblast C_2C_{12} cells were transfected with GFP and Tspan18-GFP tagged. In this case, cells appeared to be speeded on the glass coverslip, and Tspan18 appears to localize in the plasma membrane and in the cytoplasm. In conclusion, the data collected so far are inconclusive and do not clearly distinguish if Tspan18 is localized to the plasma membrame or if Tspan18 is localised to intracellular membranes, such as the ER and golgi during biosynthesis.

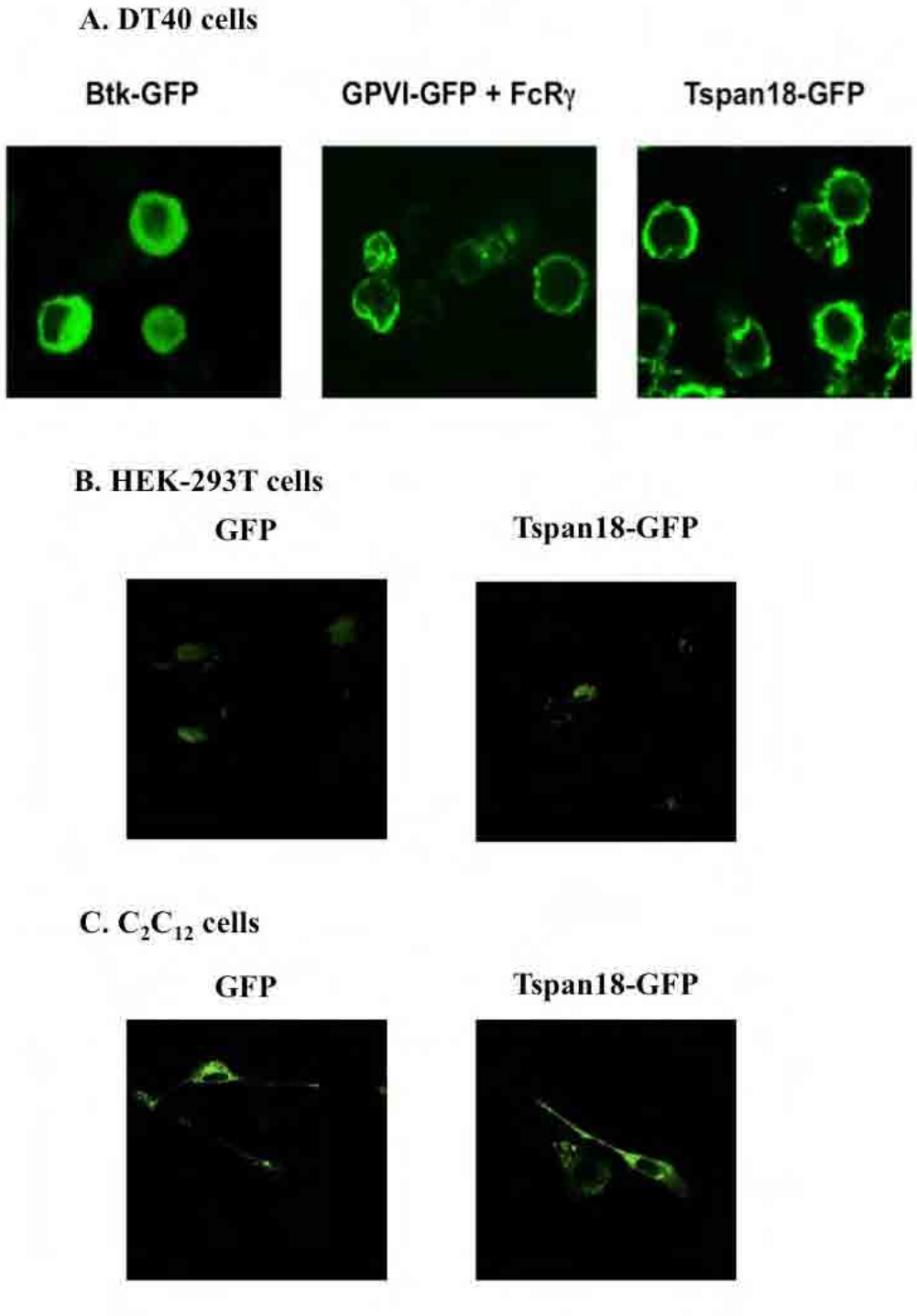


Figure 5.1 Study of Tspan18 subcellular localization. DT40 cells were transfected with constructs encoding GFP-tagged Btk, GFP-tagged GPVI and FcRγ, or GFP-tagged Tspan18. This experiment was performed in collaboration with Majd Protty.(A). HEK-293T cells were transfected with constructs encoding GFP or GFP-tagged Tspan18 (B). C_2C_{12} cells were transfected with constructs encoding GFP or GFP-tagged Tspan18 (C). Two days post-transfection, cells were analysed by confocal microscopy.

5.3.1 Analysis of lipid rafts for Tspan18

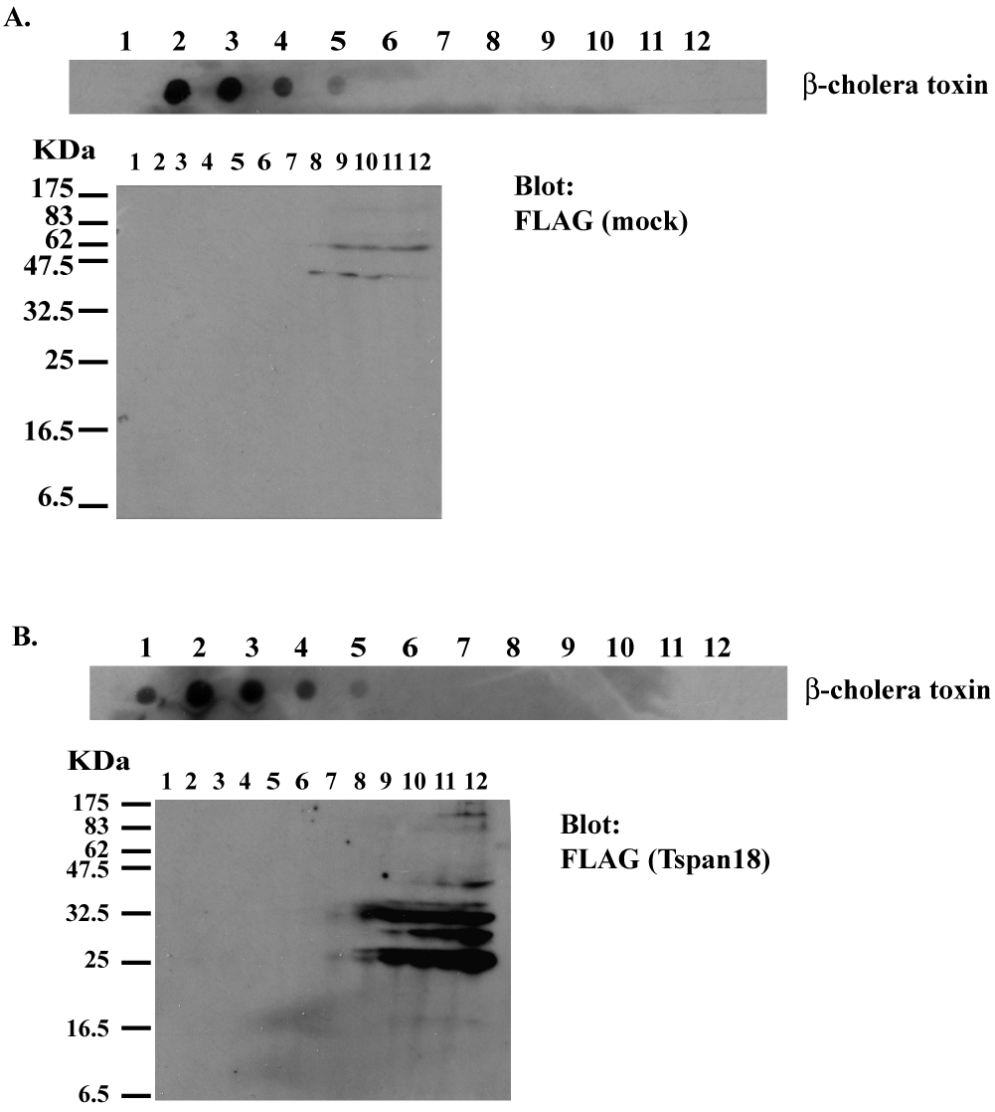
Tetraspanin microdomains are thought to be distinct from lipid rafts (Le Naour, Andre et al. 2006). However, Tspan18 has an N-terminal CXXC motif, which, in LAT family adapters, directs these proteins to lipid rafts when palmitoylated (Lin, Weiss et al. 1999). This raises the possibility that Tspan18 is localised to lipid rafts, which could explain the unique capacity of this tetraspanin to activate a signalling pathway leading to NFAT activation (Chapter 3).

In order to determine if Tspan18 is a lipid raft-associated protein, HEK-293T cells were transfected with FLAG-tagged human Tspan18. Tspan9 was also tested in parallel, as it contains a CXXC motif, while CD9 was tested as it lacks a CXXC motif. Following tetraspanin transfection, cells were lysed in 1% Triton lysis buffer and separated by ultracentrifugation over a sucrose gradient. After collection of 12 fractions, each fraction was analysed by dot-blot using the β-cholera toxin, which interacts with the lipid raft-specific lipid GM1. The result showed that in all the tested samples the lipid rafts mostly localized in fractions 2, 3, 4 and 5 (Figure 5.2). This lipid raft localization was consistent with data already published in the literature (Canobbio, Trionfini et al. 2008). The fractions resulting from the separation of each sample were analyzed by SDS-PAGE electrophoresis and blotted using a FLAG antibody, to identify the transfected tetraspanin proteins. The result demonstrated that Tspan18 was not localised to lipid rafts, but was present in the soluble fractions (fractions 9, 10, 11 and 12) (Figure 5.2A-B). Moreover, the majority of both Tspan9 and CD9 were similarly not present in the lipid rafts (Figure 5.2C and Figure 5.2D). As the isolation of lipid rafts is detergent-sensitive and the choice of the detergent is critical, the same approach was employed to evaluate Tspan18 presence in lipid rafts using the milder detergent Brij 58. The data were comparable to samples lysed with Triton X-100 (data not

shown), suggesting that Tspan18, Tspan9 and CD9 are not lipid-raft associated tetraspanin proteins.

5.3.2 Analyses of Tspan18 glycosylation and palmitoylation mutants

In order to determine whether glycosylation or palmitoylation are required for Tspan18-induced NFAT/AP-1 activation, two Tspan18 isoform 2 mutants were generated: a glycosylation site mutant for which the two key asparagine residues were mutated to alanine (Tspan18 GLY), and a palmitoylation mutant in which the six putative cysteine palmitoylation sites were mutated to serine (Tspan18 CS). The capacity of the two mutants to activate NFAT/AP-1 was tested using the luciferase reporter assay described in Chapter 3. Over-expression of the glycosylation-deficient mutant activated NFAT/AP-1 to a degree comparable to the wild-type Tspan18 form, while the palmitoylation-deficient mutant induced only 50% of the wild type signal (Figure 5.3A). Interpretation of these data was complicated by the fact that the mutant-transfected cells responded to positive control PMA plus ionomycin stimulation at only 50% of the level of wild-type transfectants (Figure 5.3B), and that the expression levels of the proteins were different (Figure 5.3C). Indeed, the palmitoylation mutant expressed at a higher level than wild-type, and the glycosylation mutant was higher still (Figure 5.3C). Interestingly, the latter showed only a single lower band at 25 kDa, close to the predicted molecular weight of the protein backbone (27.7 kDa), while the wild-type and palmitoylation-deficient forms showed the typical doublet (Figure 5.3C). This suggests that the lower band represents non-glycosylated Tspan18 at a relatively early stage in the biosynthetic pathway. Taken together, these results demonstrate that neither


A.
1 2 3 4 5 6 7 8 9 10 11 12
β-cholera toxin
KDa
1 2 3 4 5 6 7 8 9 10 11 12
175
83
62
47.5
32.5
25
16.5
6.5
Blot:
FLAG (mock)
B.
1 2 3 4 5 6 7 8 9 10 11 12
β-cholera toxin
KDa
1 2 3 4 5 6 7 8 9 10 11 12
175
83
62
47.5
32.5
25
16.5
6.5
Blot:
FLAG (Tspan18)

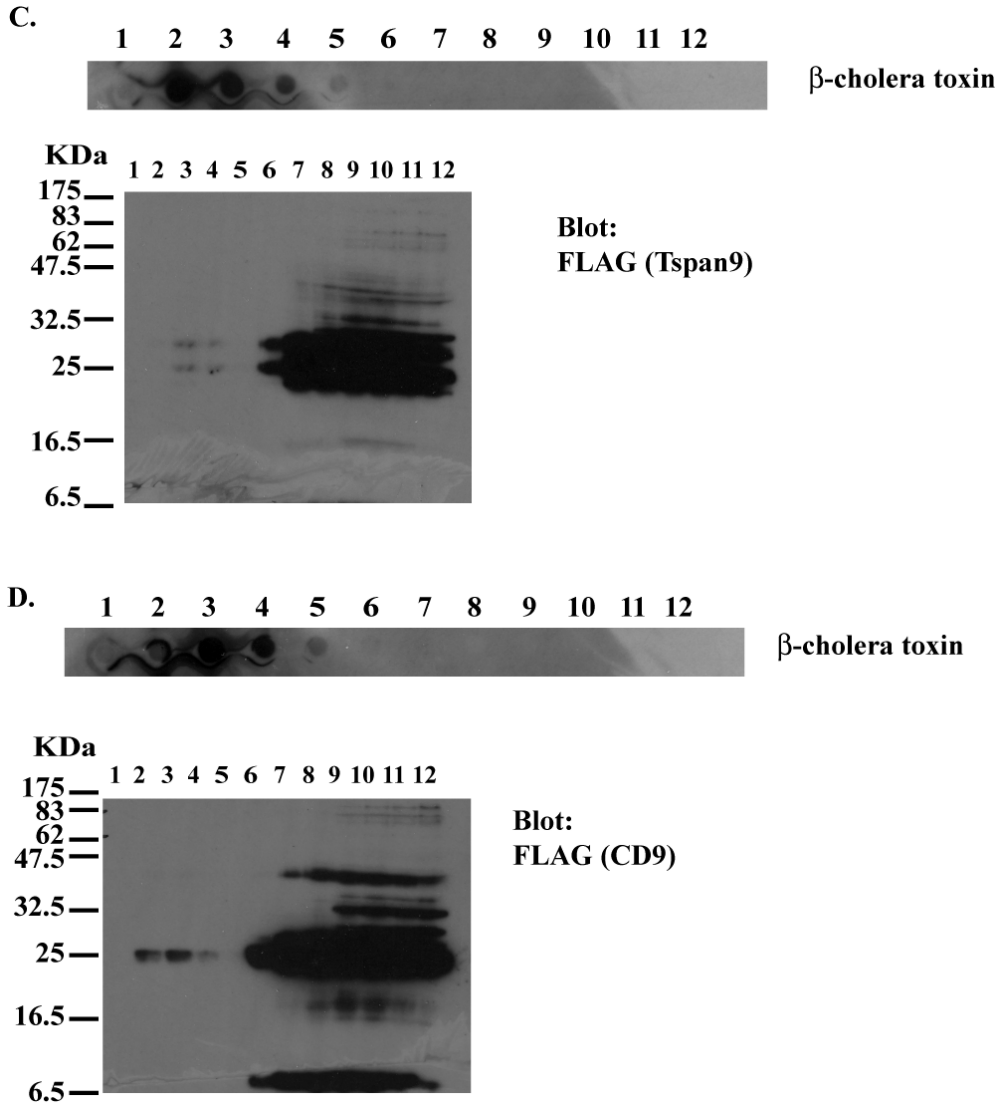
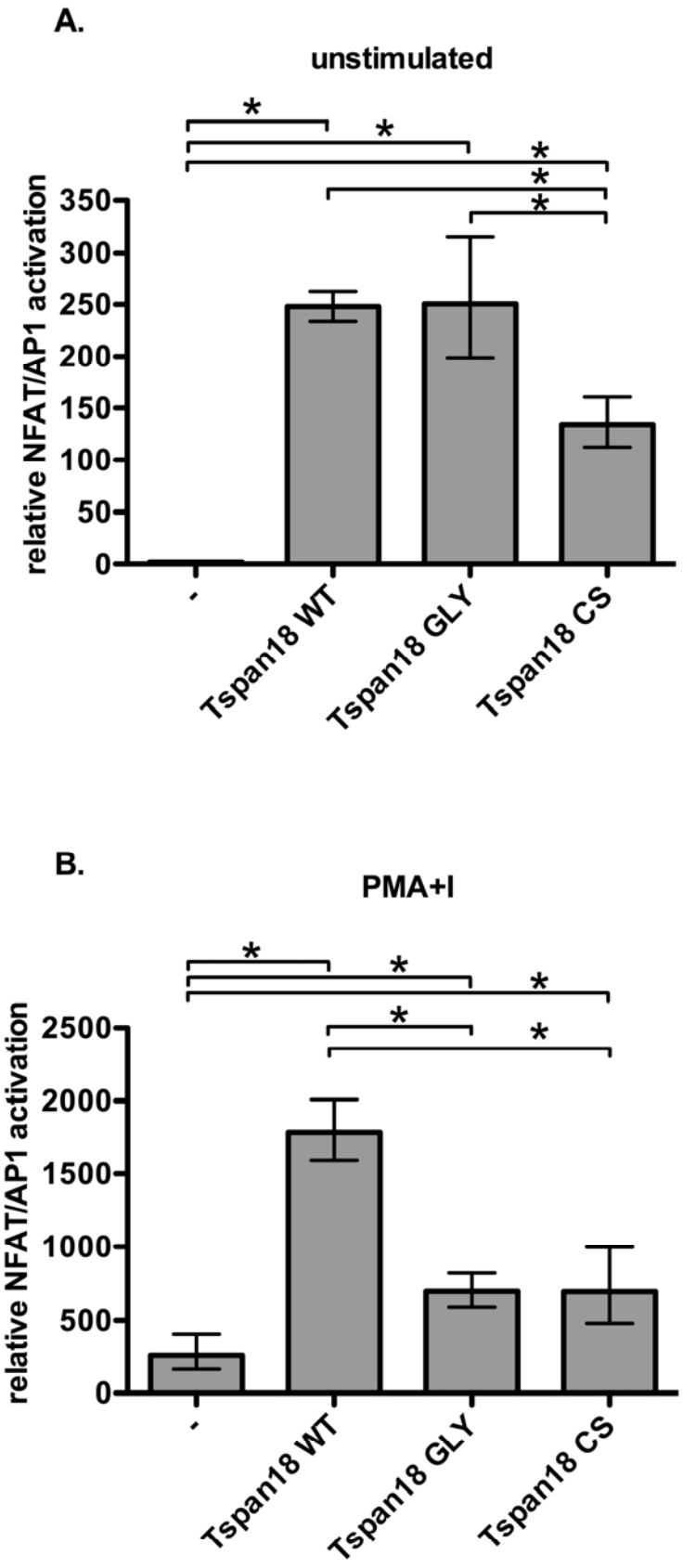


Figure 5.2 Transfected Tspan18, CD9 and Tspan9 do not localize to HEK-293T cell lipid rafts. HEK-293T cells were transfected with control vector (A.), FLAG-tagged Tspan18 isoform 2 (B.), FLAG-Tspan9 (C) or FLAG-CD9 (D). Two days after the transfection, cells were lysed in 1% Triton X-100 lysis buffer and lysates were ultracentrifuged at 200,000 x*g* for 16 hours. Samples were divided into 12 fractions and analysed by dot-blot with the β-cholera toxin, which detects the lipid raft-specific lipid GM1 (upper panels). An aliquot of each fraction was analyzed by western blotting with the FLAG antibody (lower panels). Data are representative of five independent experiments.


A.
unstimulated
relative NFAT/AP1 activation
350
300
250
200
150
100
50
0
*
*
*
*
*
*
-
Tspan18 WT
Tspan18 GLY
Tspan18 CS
B.
PMA+I
relative NFAT/AP1 activation
2500
2000
1500
1000
500
0
*
*
*
*
*
-
Tspan18 WT
Tspan18 GLY
Tspan18 CS

C.

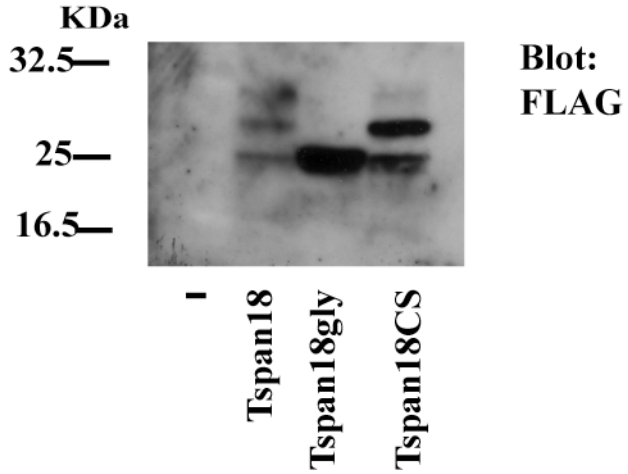


Figure 5.3 Neither glycosylation nor palmitoylation are essential for Tspan18-induced NFAT/AP-1 activation. DT40 cells were transfected with the NFAT/AP-1-luciferase and β-gal constructs, together with control vector, 20 μg of wild-type FLAG-Tspan18, 20 μg of glycosylation-deficient FLAG-Tspan18 with two mutated N-linked glycosylation sites (Tspan18gly), or 20 μg of palmitoylation-deficient FLAG-Tspan18 with six cysteine residues replaced with serine residues (Tspan18CS). Transfected cells were left unstimulated (A.) or were stimulated with PMA and ionomycin (B.). Data were normalized for the relative β-gal values and adjusted such that Tspan18 signalling in unstimulated cells was 100. Error bars represent the standard error of three independent experiments. Aliquots of transfected cells were separated by SDS-PAGE and blotted with the FLAG antibody (C.).

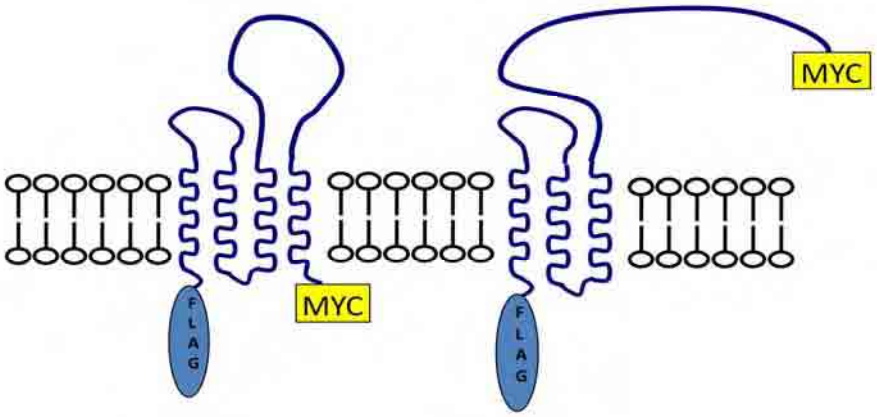
glycosylation nor palmitoylation are essential for Tspan18 signalling, but palmitoylation appears to be important for maximal signalling.

5.3.3 Biochemical analysis of Tspan18 isoform 1

The bioinformatic characterization of Tspan18 conducted in Chapter 4 identified two alternatively spliced forms of the gene, in primates but not in other mammals, which differed in their carboxy-terminal regions. Isoform 2 showed the classical four-transmembrane topology that is typical of tetraspanins, but the analysis conducted on the carboxy tail of Tspan18 isoform 1 demonstrated that is is lickely this region might contain fourth transmemrbane domain, but this was predicted with a lower confidence level that the other three transmembrane domains (Figure 4.3).

In order to biochemically test whether Tspan18 isoform 1 has three or four transmembranes, or in other words an extracellular or intracellular carboxy-terminus, a Tspan18 isoform 1 expression construct was generated with a FLAG tag at the amino terminus and a MYC tag at the carboxy-terminus (Figure 5.4A). Tspan18 isoform 1 was then transfected into DT40 cells and analyzed by flow cytometry and western blotting. In these experiments, extracellular MYC tagged G6f was used as a positive control, since this platelet membrane protein is known to be surface-expressed (Craig Hughes, unpublished data). In flow cytometry using the anti-MYC antibody, the Tspan18 isoform 1-transfected cells were comparable to mock-transfected cells and thus were negative (Figure 5.3B). This was in contrast to the G6f transfectants, in which a proportion was positive for MYC staining (Figure 5.3B). This failure to detect surface isoform 1 was not due to a lack of expression, since the protein was readily detected in whole cell lysates by anti-MYC blotting (Figure 5.4C). These finding confirm the

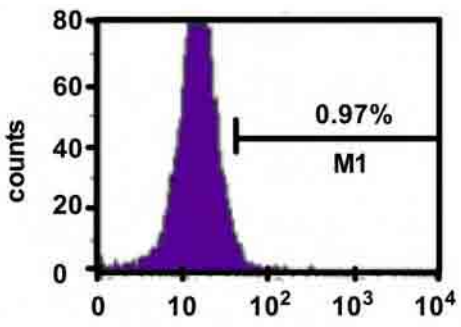
A.

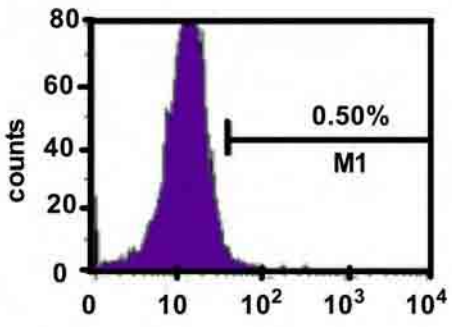


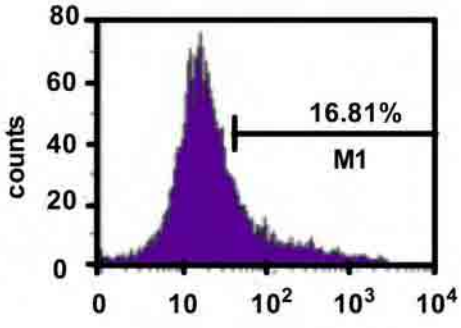
hypothetical tetraspanin Tspan18 isoform 1

hypothetical trispanin Tspan18 isoform 1

B.







C.

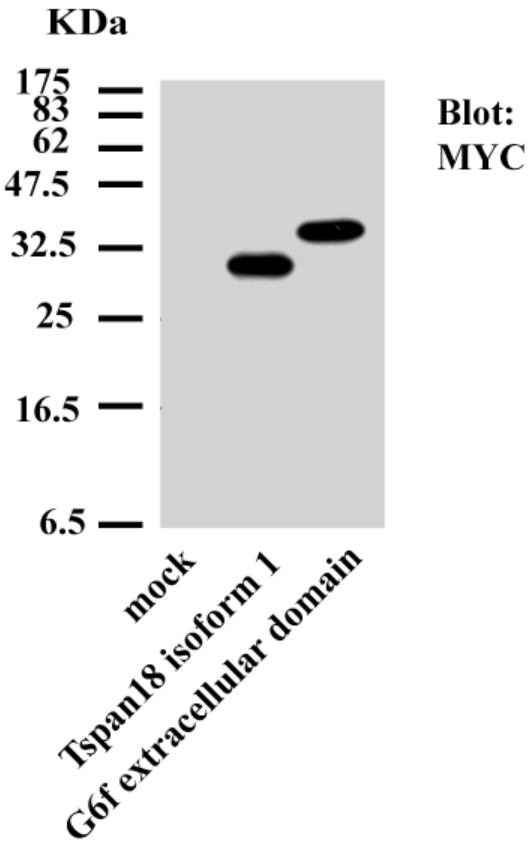
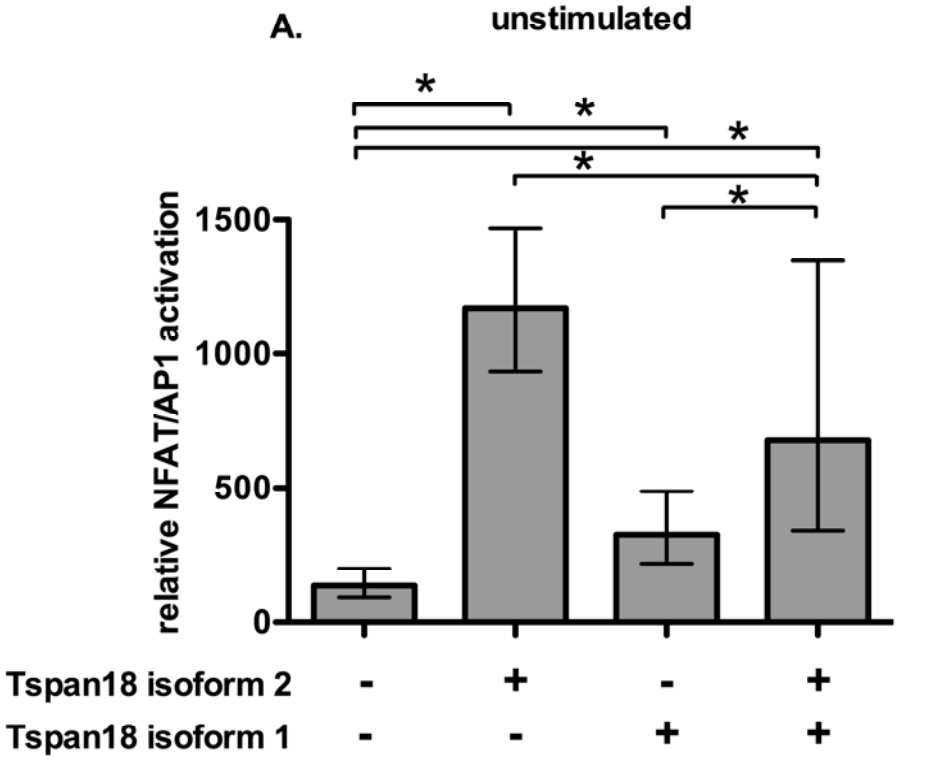


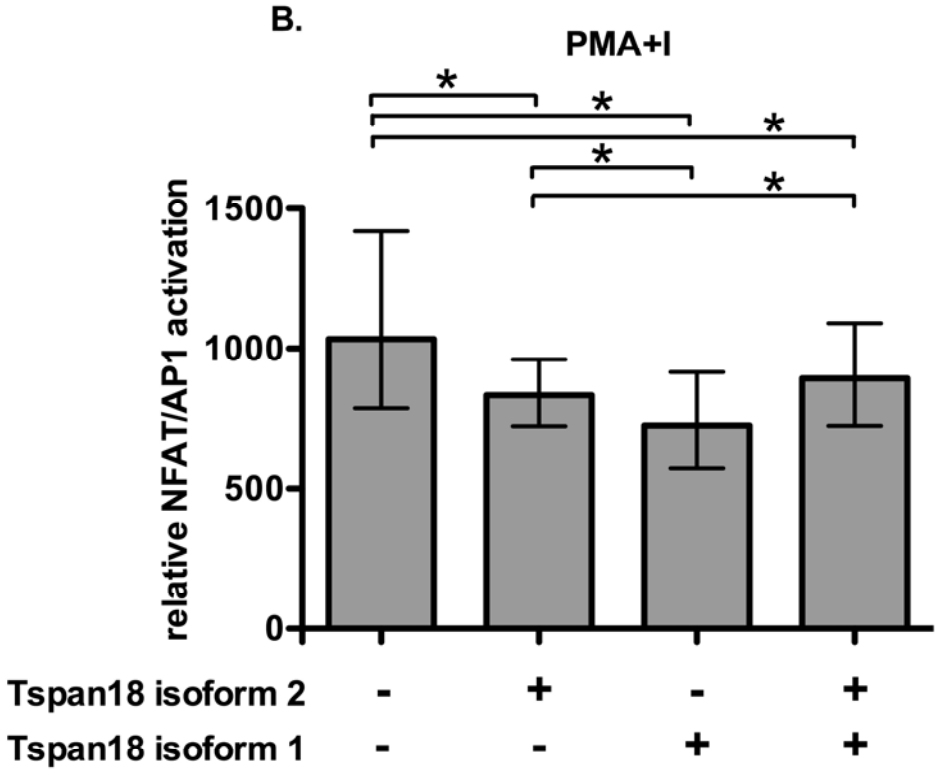
Figure 5.4 Two distinct topologies for a Tspan18 isoform 1 protein tagged with FLAG and MYC epitopes. The Tspan18 isoform 1 construct was cloned into the pEF6 vector with a FLAG tag at the amino-terminus and a MYC tag at the carboxy-terminus (A.). DT40 cells were transfected with empty vector (mock), the Tspan18 isoform 1 construct or a G6f positive control with an extracellular MYC tag. Twenty-four hours post transfection, cells were stained with mouse anti-MYC primary antibody, followed by anti-mouse FITC-conjugated secondary antibody. Cells were analyzed by flow cytometry (B.). Aliquots of samples were lysed in 1% Triton X-100 lysis buffer, separated by SDS-PAGE electrophoresis and western blotted with the MYC antibody, to evaluate Tspan18 isoform 1 and G6f expression (C.).

bioinformaitc finding that Tspan18 isoform 1 present four transmembrane domains. Despite this result, in theory this protein could possess only three transmembrane regions but be expressed on only intracellular membranes. Future surface biotinylation and immunofluorescence experiments could be used to address this issue. Nevertheless, the western blotting results have proved that isoform 1 can be expressed as a stable protein.

5.3.4 Tspan18 isoform1 capability of activating NFAT/AP-1

To determine whether Tspan18 isoform 1 could activate NFAT/AP-1, in common with isoform 2, DT40 cells were transfected with the NFAT/AP-1 luciferase construct and either Tspan18 isoform 1, Tspan18 isoform 2, or a combination of both isoforms. In contrast to isoform 2, which activated NFAT/AP-1 by approximately ten-fold over basal, isoform 1 only induced two-fold activation, showing a significant reduction in capability of NFAT/AP-1 activation (Figure 5.5A). Moreover, when the two Tspan18 isoforms were co-transfected in the same sample, the combined NFAT/AP-1 activation was only four-fold over basal, again showing a significant reduction in signalling (Figure 5.5A). All of the transfectants were able to respond robustly to the PMA and ionomycin stimulation, used as a positive control, albeit to slightly different degrees (Figure 5.5B). An aliquot of each sample was analyzed by SDS-PAGE and blotted with the FLAG antibody, to monitor the expression level of the different transfected constructs. Isoform 1 appeared to migrate at a lower molecular weight than isoform 2, but was expressed at a much lower level, despite the fact that four times more DNA was used in the transfections (Figure 5.5C; see legend). However, isoform 1 expression was greater in the presence of isoform 2 (Figure 5.5C). Several experiments were conducted in an attempt to achieve similar expression levels of the two isoforms, by varying the amount


A.
unstimulated
relative NFAT/AP1 activation
1500
1000
500
0
Tspan18 isoform 2 - + - +
Tspan18 isoform 1 - - + +


B.
PMA+I
relative NFAT/AP1 activation
1500
1000
500
0
Tspan18 isoform 2 - + - +
Tspan18 isoform 1 - - + +

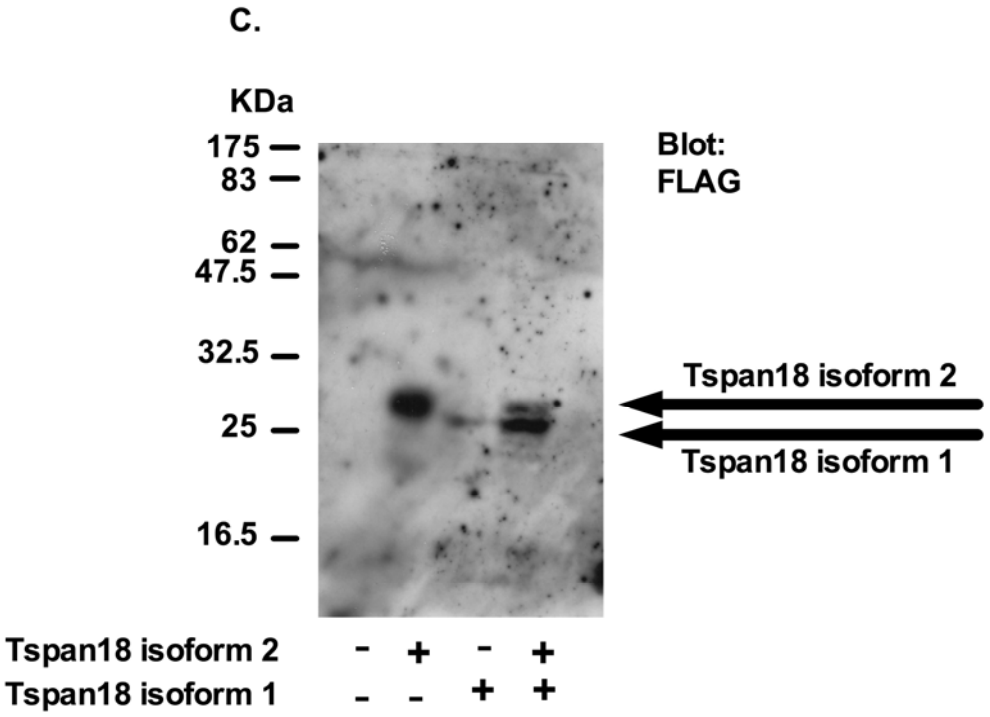


Figure 5.5 Analysis of Tspan18 isoform 1 capability of activating NFAT/AP-1. DT40 cells were transfected with NFAT/AP-1-luciferase and β-gal constructs, together with either 5 μg of FLAG-Tspan18 isoform 2, 20 μg of FLAG-Tspan18 isoform 1, or a combination of 5 μg of FLAG-Tspan18 isoform 2 and 20 μg of FLAG-Tspan18 isoform 1. Cells were left unstimulated (A.) or were stimulated with PMA and ionomycin (B.). An aliquot of whole cell lysate was separated by SDS-PAGE and blotted with the FLAG antibody, in order to check Tspan18 expression levels (C.). Data were normalized for β-gal values and analyzed by ANOVA. Statistical significance is indicated by Tukey's test (* $P < 0.05$). Error bars represent the standard deviation of three independent experiments.

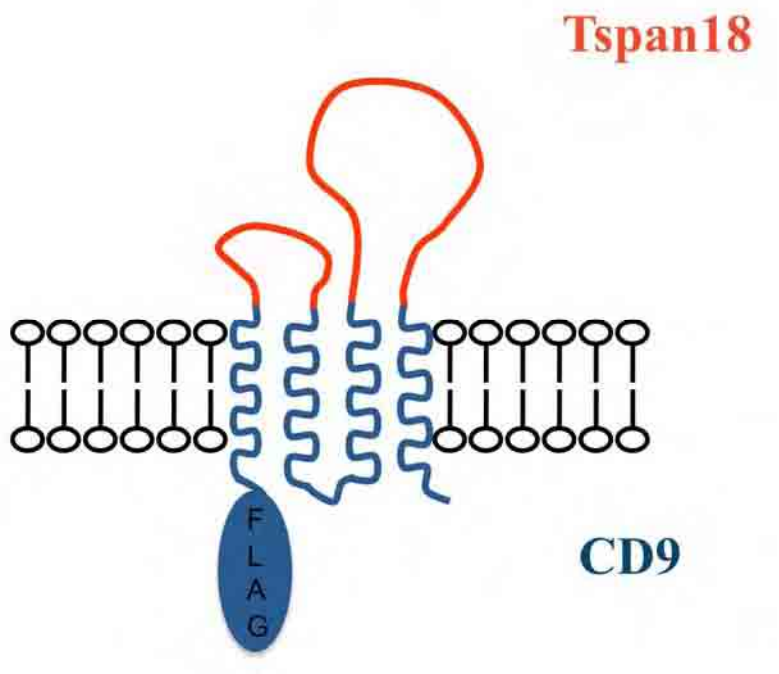
of transfected DNA, but these were not successful (data not shown). However, in agreement with Figure 5.5C, it was clear that DT40 cells expressed Tspan18 isoform 1 with more difficulty, but the reason for this remains unclear at present. In conclusion, isoform 1 did not induce substantial NFAT/AP-1 activation, but this could be due to a relatively low expression level. In addition, isoform 1 appeared to impair isoform 2-induced NFAT/AP-1 activation, although again this could be because of an effect on isoform 2 expression level.

5.3.5 The Tspan18 extracellular loops in a chimeric protein are sufficient to induce NFAT/AP-1 signalling

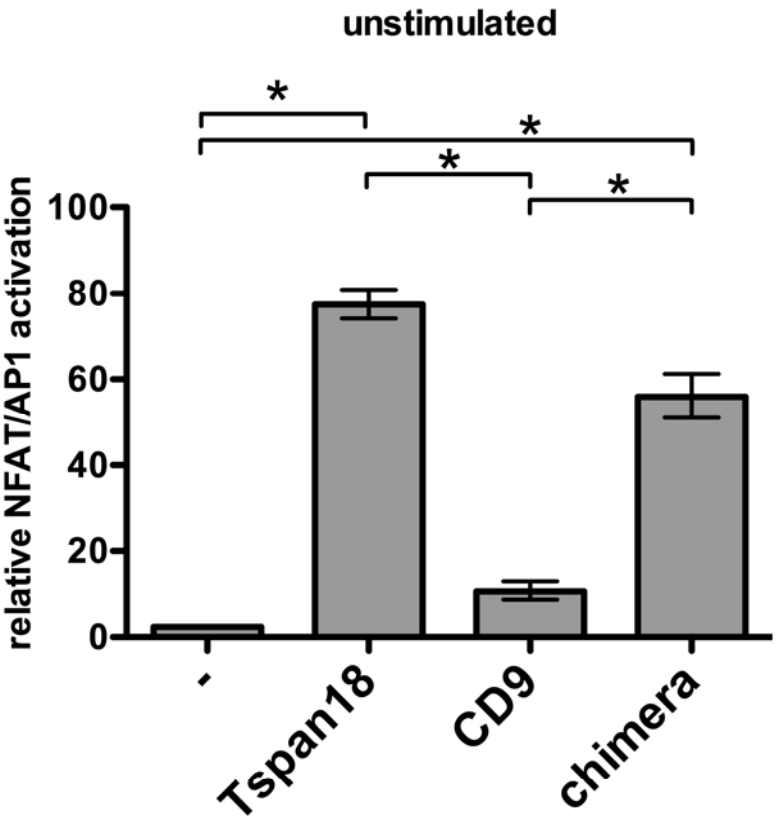
In order to begin to understand which region of Tspan18 is crucial in inducing NFAT/AP-1 signalling, a CD9/Tspan18 chimeric construct was generated in which the transmembrane and intracellular regions of human Tspan18 were replaced with those of CD9 (Figure 5.6A), a tetraspanin that was unable to induce NFAT/AP-1 activation (Figure 3.2).

In order to assess whether the CD9/Tspan18 chimera could induce NFAT/AP-1 activation, DT40 cells were transfected with the NFAT/AP-1-luciferase reporter in combination with 20 μg FLAG-Tspan18 (isoform 2), 20 μg FLAG-CD9 or 20 μg FLAG-chimera constructs. The CD9/Tspan18 chimera significantly induced NFAT/AP-1 activation to a level that was 70% of that induced by Tspan18 (Figure 5.6B). In contrast the CD9 control did not induce significant NFAT/AP-1 activation (Figure 5.6B). All samples responded robustly to the PMA and ionomycin stimulation, used as a positive control, although with somewhat different degrees of activation (Figure 5.6C.). Finally, the expression level of each construct was assessed by anti-FLAG western blotting, and the result confirmed expression of each construct (Figure 5.6D). A slightly lower expression of the chimera, relative to Tspan18, might explain the partial reduction in signalling response observed in Figure 5.6B. In

A.


Tspan18
F
L
A
G
CD9

B.


unstimulated
relative NFAT/AP1 activation
100
80
60
40
20
0
*
*
*
*
-
Tspan18
CD9
chimera

]

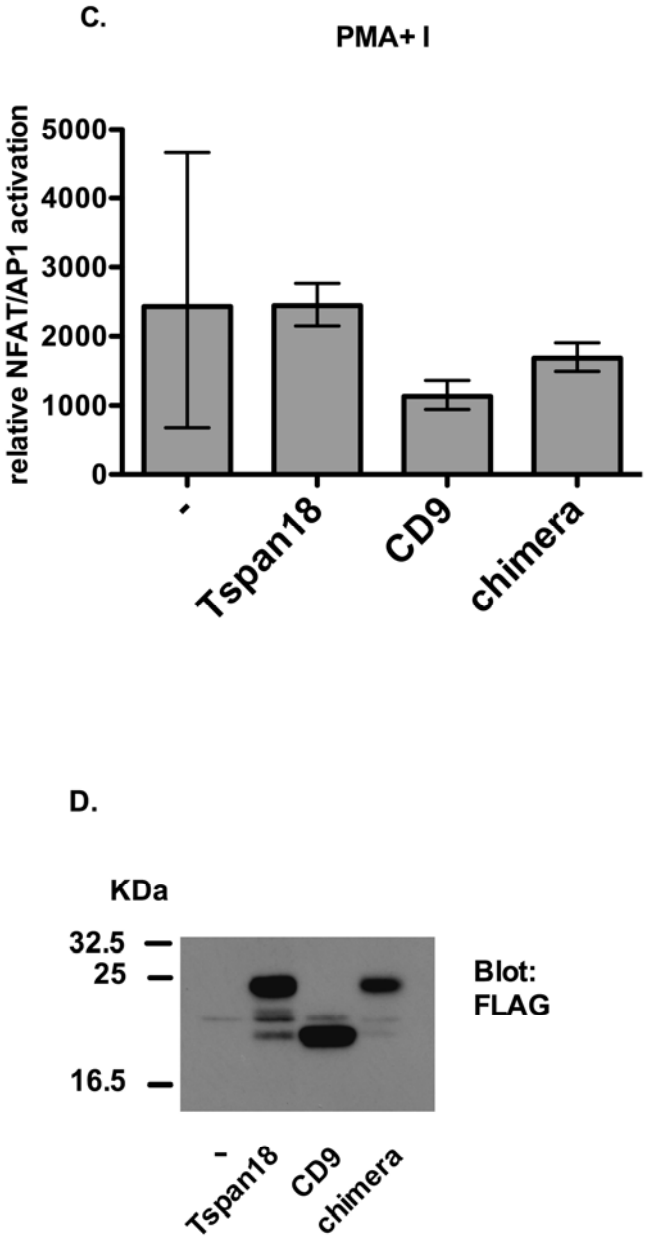


Figure 5.6 The Tspan18 extracellular loops are sufficient to induce NFAT/AP-1 signalling in the context of a chimeric protein. Diagrammatic representation of the human CD9/Tspan18 chimeric construct (A.). DT40 cells were transfected with vector control, 20 μg of FLAG-Tspan18, 20 μg of FLAG-CD9 or 20 μg of the FLAG-CD9/Tspan18 chimera. Cells were left unstimulated (B.) or stimulated with PMA and ionomycin (C.). Data were normalized for β-gal values and analyzed by ANOVA test. Statistical significance is indicated by Tukey's test (**P*<0.05). Error bars represent the standard deviation of three independent experiments. An aliquot of whole cell lysate was separated by SDS-PAGE and blotted with the FLAG antibody, in order to check expression levels of the different constructs (of a Tspan18 antibody, which has yet to be made, or transfected GFP- and FLAG-tagged Tspan18 constructs.

conclusion these data show that the extracellular regions of Tspan18, in the context of a chimeric protein, are sufficient to promote NFAT/AP-1 activation and conversely that the transmembrane and cytoplasmic regions are not essential.

5.4 DISCUSSION

In this chapter, a number of experiments have been performed to address the subcellular localisation and structure/function of Tspan18. The key findings are as follows: (1) Tspan18 is localised to the cell surface and/or a cytoplasmic region close to the cell surface; (2) Tspan18, like other tetraspanins such as CD9 and Tspan9, is not localised to lipid raft membrane microdomains; (3) the two N-glycosylation and six putative palmitoylation sites are not essential for Tspan18-induced NFAT/AP-1 activation; (4) isoform 1, the atypical form of Tspan18 described in the previous chapter, transfects relatively poorly and does not induce substantial NFAT/AP-1 activation; and (5) the extracellular region of Tspan18 is sufficient to induce NFAT/AP-1 activation in the context of a Tspan18/CD9 chimera.

In order to study the Tspan18 subcellular localization, an amino-terminal GFP-tagged Tspan18 construct was generated and transfected into DT40, HEK-293T and C_2C_{12} cells. Confocal microscopy showed Tspan18 localisation to be similar to that of a GFP-tagged form of GPVI, a known plasma membrane protein, and distinct from GFP-tagged Btk, a cytoplasmic tyrosine kinase. However, the experiment conducted in HEK-293T cells is inconclusive, and cells observed in transmitted light appeared to have a round shape (data not shown). In C_2C_{12} Tspan18 appeared to be distributed similary to the GFP, which present a cytoplasmic distribution. Form the esperiments here presented, it is not possible to conclude that Tspan18 is expressed only at the plasma membrane. It is also possible that Tspan18 is

expressed on intracellular membranes that lie close to the plasma membrane. Consistent with the idea that at least some Tspan18 is localised to the plasma membrane, FLAG-Tspan18 stably expressed in Jurkat T cells was found to be plasma membrane localised because of its capacity to be detected by streptavidin blotting following cell surface biotinylation (M.G. Tomlinson, unpublished data). To resolve this, future experiments will utilise a cell type such as HUVEC, in which Tspan18 is normally expressed and which has a relatively large cytoplasm, thus more clearly allowing the distinction between plasma membrane and cytoplasmic staining. Such experiments would ideally involve the use of a Tspan18 antibody, which has yet to be made, or transfected GFP- and FLAG-tagged Tspan18 constructs.

Since the lipid raft concept was introduced in 1997 (Simons and Ikonen 1997), several research groups have attempted to biochemically and functionally characterize these membrane microdomains and their associated proteins. Despite this extensive research work, the notion of lipid rafts has always been controversial. Some major topics of controversy include the necessity to perform the separation at low temperatures (4^{o}C) and the critical choice of the optimal detergent and its concentration (Jacobson and Dietrich 1999). The Israels research group investigated the possible localisation of the tetraspanins CD9 and CD63 in lipid rafts of resting human platelets. This group used the detergents Triton X-100 or Brij 35 (at 1%) and showed that neither of these two tetraspanins was lipid raft-associated (Israels and McMillan-Ward 2007). In this chapter, only a minimal fraction of transfected Tspan18 was present in the lipid raft fractions of HEK-293T cells, in either the relatively strong Triton X-100 or the weaker Brij 58 detergents. These data suggest that the unique signalling capacity of over-expressed Tspan18 is unlikely to be due to a specific association with lipid raft microdomains and their associated signalling machinery. However, future experiments should

repeat these experiments in transfected DT40 cells, because these were the cells in which the signalling phenomenon has been observed.

In this chapter, the effects of glycosylation and palmitoylation on Tspan18 signalling have been preliminarily evaluated employing glycosylation and palmitoylation mutants. Palmitoylation has been shown to reduce secondary tetraspanin associations, resulting in impaired cell signalling and altered cell morphology (Hemler 2008), while the precise molecular role of tetraspanin glycosylation has not been clarified yet (Tarrant, Robb et al. 2003). Neither the prevention of glycosylation or palmitoylation prevented Tspan18 signalling, although the latter reduced the magnitude by 50%. It is possible to speculate that the reduction in signalling is a result of a reduced ability for Tspan18 to assemble into tetraspanin complexes and, consequently, reducing the interaction with its molecular partners. From the comparative analysis of the the western blot films presented so far, it seems Tspan18 isoform 1 presents only one western blot band, while Tspan18 isoform 2 presents three western blot bands (Figure 5.3, Figure 5.4 or Figure 3.8). It is possible to speculate that the diffent Tspan18 bands might be different Tspan18 glycosilation forms, as suggested from the analysis of the Figure 5.3. In this figure, a Tspan18 mutant form lacking of the glycosilation site was generated, and this was displayed as a single band. Despite this hypothesis, this does not explain why Tspan18 isoform 1, which contain a glycosilation site, does not present multiple western blotting bands. The second hyoithesis might be that the observed bands are different Tspan18 breackdown products, despite this is unlikely, as they seem to be recurrent. In conclusion, the specific reason behind this observation is at present not known. The second non-consitency that needs to be addressed regards Tspan18 western blotting molecular weight. Tspan18 isoform 2 has a molecular weight of 27.7KDa (aminoacid sequence only). In the presented western blotting, Tspan18 isoform 2 present its major bands

in the range of 32.5-25 KDa. This minor difference might be given by the fact that the western blotting separation time might not be consistant among experiment. The other possibility might be an error in reporting the markers from the gel to the film. In anycase, this registered variablility is minor, as the western blots appear to be clear and almost free from background noise or unspecific bands, confirming the visualized proiten is Tspan18, which is FLAG tagged.

In the previous chapter, an alternative isoform of Tspan18 (isoform 1) was identified in human, chimp and monkey, whereas the more typical isoform 2 was present in mammals and lower vertebrates. Quantitative PCR demonstrated that isoform 1 was expressed at relatively low levels, or was absent, from the human cell types analysed. Consistent with this, the present chapter found that transfected isoform 1 was expressed at substantially lower levels than isoform 2, suggesting that the former may be a relatively unstable protein. This could in part be due to the lack of the fourth transmembrane in isoform 1, although the flow cytometry experiments used in this chapter could not conclusively prove this; they merely demonstrated that isoform 1 was not a plasma membrane ‘trispanin’ in transfected cells. Future experiments should use flow cytometry experiments in combination with confocal microscopy to address this issue. Consistent with the low levels of expression of isoform 1, there was no substantial activation of NFAT/AP-1. Interestingly, isoform 1 appeared to be stabilised by the co-expression of isoform 2, which again complicated the interpretation of the NFAT/AP-1 data. In summary, it was not possible to conclusively conclude whether isoform 1 could induce signalling or, if not, whether it could inhibit isoform 2. Future experiments could be directed at raising an isoform 1-specific antibody to address these issues. However, addressing the role of the more typical isoform 2, for which mRNA clearly exists in cells such as endothelial cells (Chapter 4), should be a higher priority.

Finally, in this chapter, the role of Tspan18 extracellular loops have been evaluated, employing a chimeric construct composed of Tspan18 small and large extracellular loops and CD9 cytoplasmic and transmembrane domains. The construct has been raised on the basis that CD9 was not able to induce NFAT-AP1 signalling (Chapter 3). The result demonstrated that the chimera retains 70% of Tspan18 signalling capability. If the Tspan18 signalling mechanism involves an interaction with a signalling partner protein, these data suggest that the interaction is mediated by the extracellular region of the tetraspanin. This would be similar to the interaction of tetraspanin CD151 with the laminin-binding integrin $\alpha 3\beta 1$, for which the large extracellular loop of CD151 mediates the interaction (Yauch, Kazarov et al. 2000).

5.5 SUMMARY

In the present Chapter it has been demonstrated that:

- Tspan18 is localised to the plasma membrane and/or cytoplasm in transfected DT40 cells
- Tspan18 is not present in lipid rafts of transfected HEK-293T cells
- Tspan18-induced NFAT/AP-1 activation does not require N-glycosylation or palmitoylation of Tspan18, although the latter has a partial effect
- Transfected Tspan18 isoform 1 is expressed poorly and does not induce substantial NFAT/AP-1 activation

- Tspan18 large and small extracellular loops are sufficient for NFAT/AP-1 activation in the context of a chimeric form of Tspan18

CHAPTER 6

TETRASPANIN OVER-EXPRESSION DOES NOT AFFECT GPVI SIGNALLING

6.1 INTRODUCTION

Earlier chapters of this thesis have shown that over-expression of Tspan18, but not other tetraspanins, could induce NFAT/AP-1 activation. Since tetraspanins lack intrinsic enzymatic activity, it is possible that Tspan18 signals indirectly, through a specific interaction with a signalling receptor. For Tspan18 this could be a calcium channel or a protein that regulates calcium channels, such as a STIM1. A platelet receptor that could be regulated by other tetraspanins is the collagen receptor GPVI. Indeed, before the start of this thesis, our group identified GPVI as a component of platelet tetraspanin microdomains, a finding that was recently published (Protty, Watkins et al. 2009). GPVI was found to associate with CD9 and CD151 in the weak detergent 1% Brij97, but its interaction was lost in the more stringent 1% Triton X-100 detergent. This suggests that GPVI might specifically interact with one of the several other platelet tetraspanins, and might be regulated within tetraspanin microdomains. This would not be without precedent for an ITAM-containing receptor, because T cells from several tetraspanin-deficient mice hyperproliferate in response to TCR cross-linking (Tarrant, Groom et al. 2002; Tarrant, Robb et al. 2003; van Spriel, Puls et al. 2004), suggesting negative regulation of this receptor by tetraspanin microdomains. This chapter will take advantage of the DT40 cell line model, used to study Tspan18 signalling earlier in this thesis, to address the hypothesis that one or more platelet tetraspanins regulates GPVI signalling.

6.2 AIM

The aim of this chapter was to determine whether over-expression of a panel of platelet tetraspanins affects GPVI/FcRγ-induced NFAT/AP-1 activation in the DT40 B cell line model.

6.3 RESULTS

6.1 GPVI/FcRγ can induce NFAT/AP-1 signalling in the DT40 B cell line

In order to establish the NFAT/AP-1 luciferase reporter assay to study collagen-induced GPVI/FcRγ signalling, DT40 cells were transfected with constructs encoding GPVI/FcRγ and the NFAT/AP-1 reporter. Cells were unstimulated, stimulated with 10 μg/ml of collagen, or stimulated with PMA and ionomycin as a positive control (Figure 6.1A). The result demonstrated that GPVI/FcRγ was able to activate the NFAT/AP-1 promoter upon collagen stimulation inducing a statistically significant response of approximately 100-fold over basal. In addition, the PMA and ionomycin positive control induced similar robust NFAT/AP-1 activation in both control and GPVI/FcRγ transfections, despite the presence of Tspan18 is not statistically significant (Figure 6.1A). In order to monitor the expression level of GPVI at the cell surface, an aliquot of each sample was analyzed by flow cytometry, after staining with the monoclonal GPVI antibody. The result showed that GPVI was expressed at the cell surface of approximately 18% of the cells (Figure 6.1B), a value that is typical for transient transfection of this cell line.

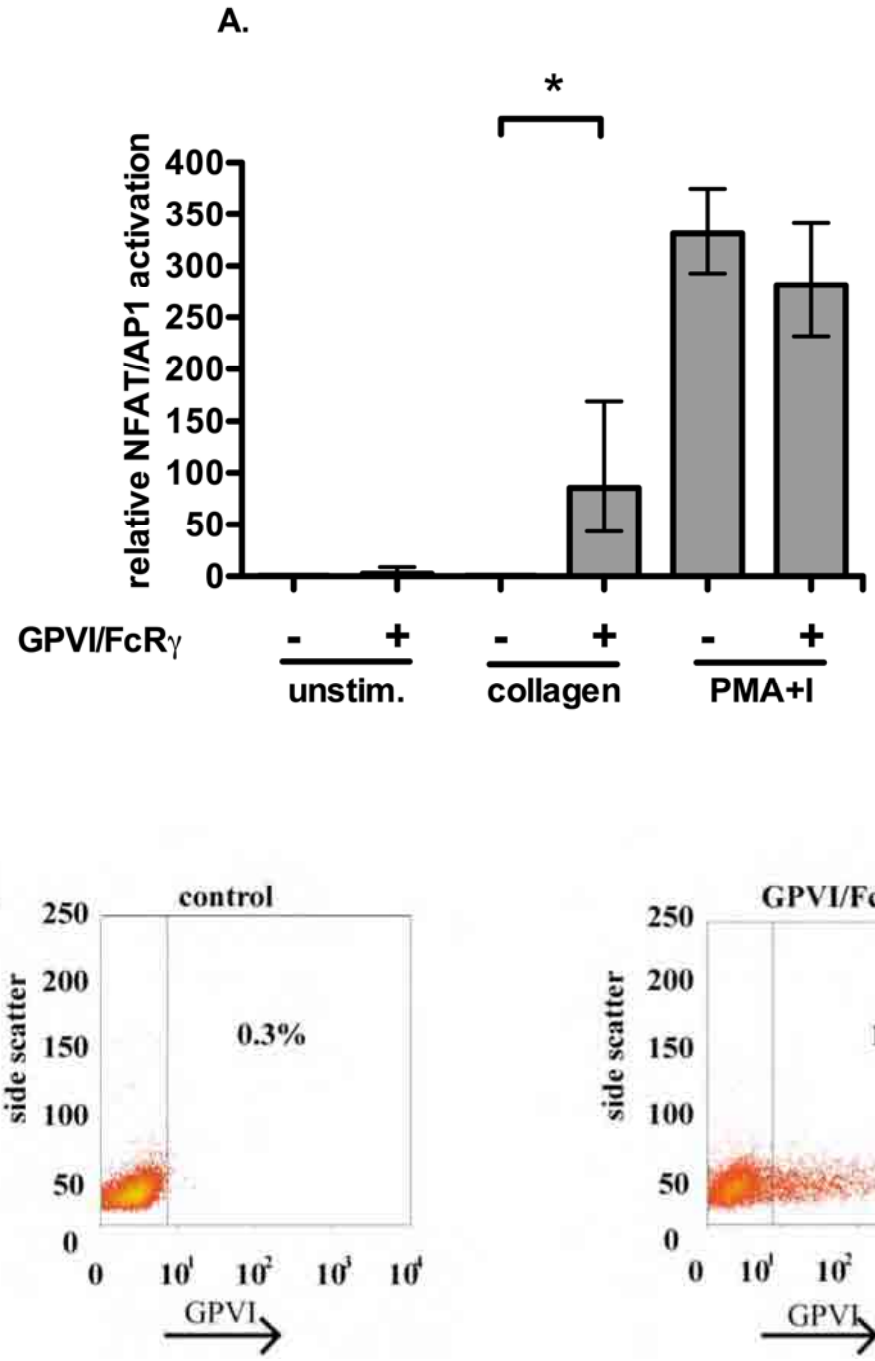


Figure 6.1: GPVI/FcRγ can induce NFAT/AP-1 activation in DT40 cells. (A.) DT40 cells were transfected with 20 μg of the NFAT/AP-1-luciferase reporter construct, 2 μg each of GPVI, FcRγ and β-galactosidase expression constructs, the latter as a control for transfection efficiency. Cells were unstimulated (unstim) or stimulated with 10 μg/ml of collagen or PMA (50 ng/ml) and ionomycin (1 μM) as a positive control. Data were divided for the relative β-gal value and analyzed by ANOVA followed by a Tukey's test. Error bars represent the standard deviation of eight independent experiments (B.) GPVI surface expression level was measured by FACS analysis and representative dot plots are shown.

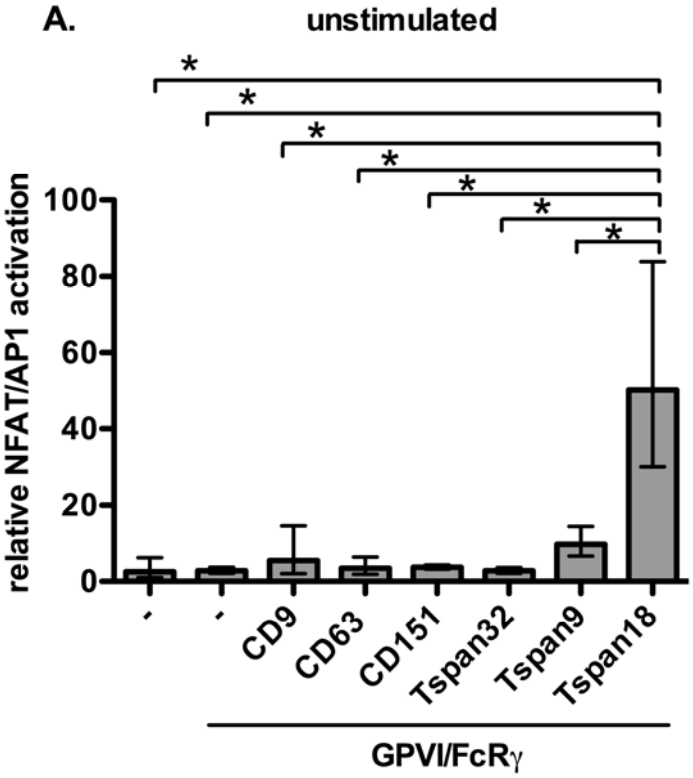
6.2 The effect of tetraspanin over-expression on GPVI/FcRγ signalling

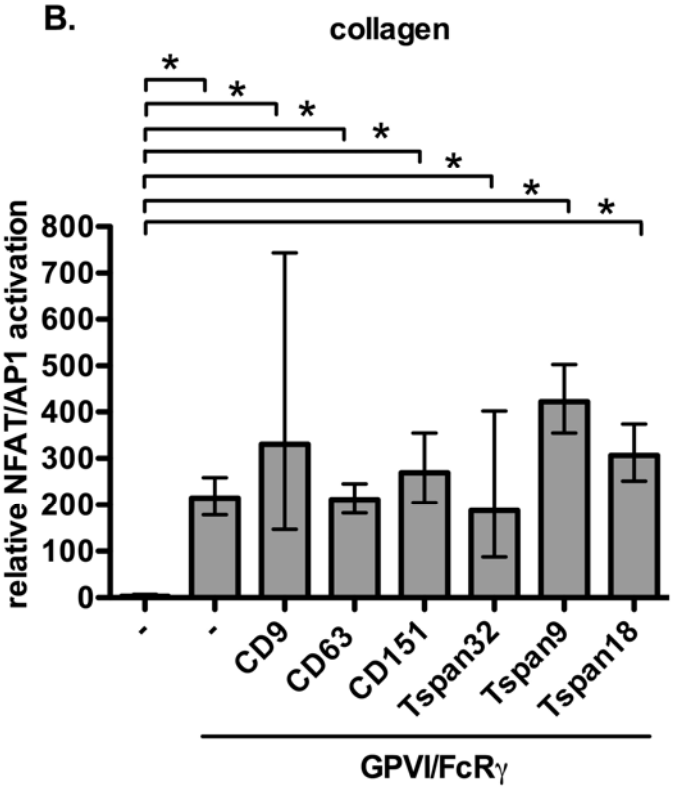
To determine if some of the known platelet tetraspanins have an effect on GPVI/FcRγ signalling, the panel of FLAG-tagged tetraspanin proteins, used in Figure 3.2, was individually co-transfected with constructs for GPVI, FcRγ and the NFAT/AP-1 reporter in DT40 cells, and left unstimulated (Figure 6.2A), or stimulated with collagen (Figure 6.2B) or PMA and ionomycin (Figure 6.2C) The result demonstrated that, among the tested tetraspanin proteins, only Tspan18 can significally enhance basal GPVI/FcRγ signalling. Interestingly, despite from the Figure 6.2A might appear that CD9 and Tspan9 might have an effect on GPVI/FcRγ signalling, this did not appeared to be statistically significative. Of all the tested tetraspanin proteins, none appeared to enhance GPVI/FcRγ collagen signalling (Figure 6.3B). PMA and ionomycin stimulation was performed to check cell response (Figure 6.3C), showing all samples responded to this positive control, despite with a different degree of activation. Despte Tspan18 and CD9 and Tspan18 PMA and ionomcin response appeared to be statistically significative, this is not believed to be of relevance in this context, as this positive control showed sample variation.

6.3 Tspan18 synergises with GPVI/FcRγ to activate NFAT/AP-1

The remaining experiments in this chapter were designed to address the basal signal in unstimulated cells transfected with GPVI/FcRγ and Tspan18 (Figure 6.3A). GPVI/FcRγ basal signalling in this model has been observed before (Mori, Pearce et al. 2008), but the current data does not distinguish between the two possibilities that either Tspan18 alone, or Tspan18 in synergy with GPVI/FcRγ, is responsible for the NFAT/AP-1 activation. Therefore DT40

cells were transfected with Tspan18 in the presence or absence of the GPVI/FcRγ complex. In unstimulated cells, Tspan18 alone induced an 11-fold NFAT/AP-1 activation, GPVI/FcRγ alone induced a 7-fold activation, whereas the combination of GPVI/FcRγ and Tspan18 produced a synergistic response of 62-fold activation (Figure 6.3A). Such synergy suggests that Tspan18 and GPVI/FcRγ activate the NFAT/AP-1 promoter through at least partially different mechanisms.


A.
unstimulated
relative NFAT/AP1 activation
100
80
60
40
20
0
*
*
*
*
*
*
*
-
-
CD9
CD63
CD151
Tspan32
Tspan9
Tspan18
GPVI/FcRγ


B.
collagen
relative NFAT/AP1 activation
800
700
600
500
400
300
200
100
0
*
*
*
*
*
*
*
-
-
CD9
CD63
CD151
Tspan32
Tspan9
Tspan18
GPVI/FcRγ

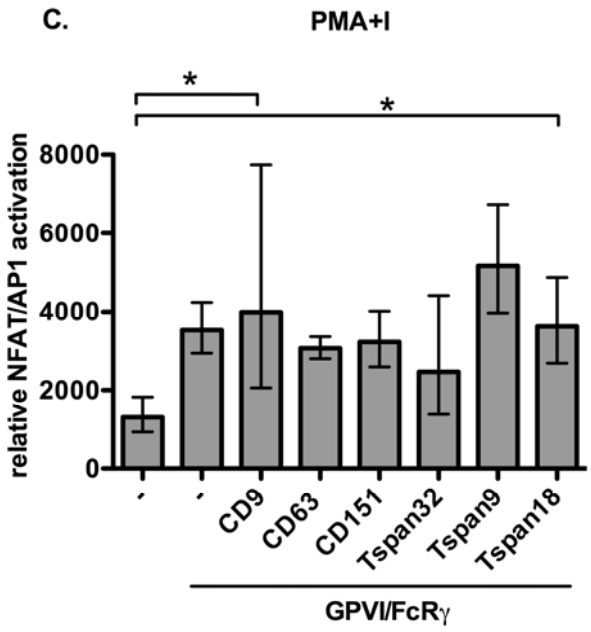
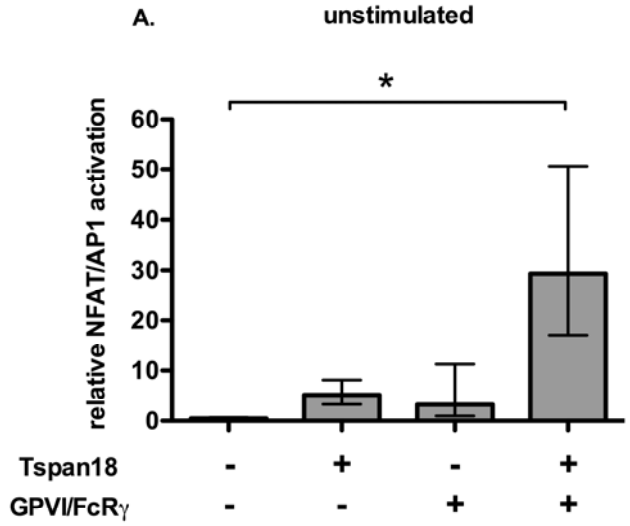
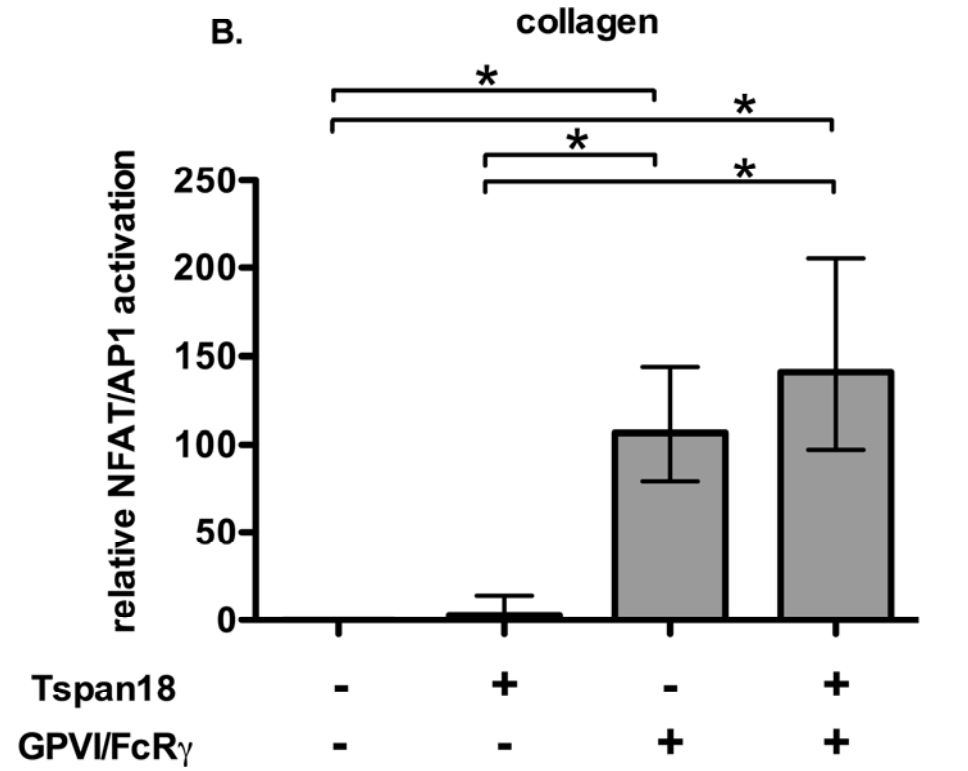
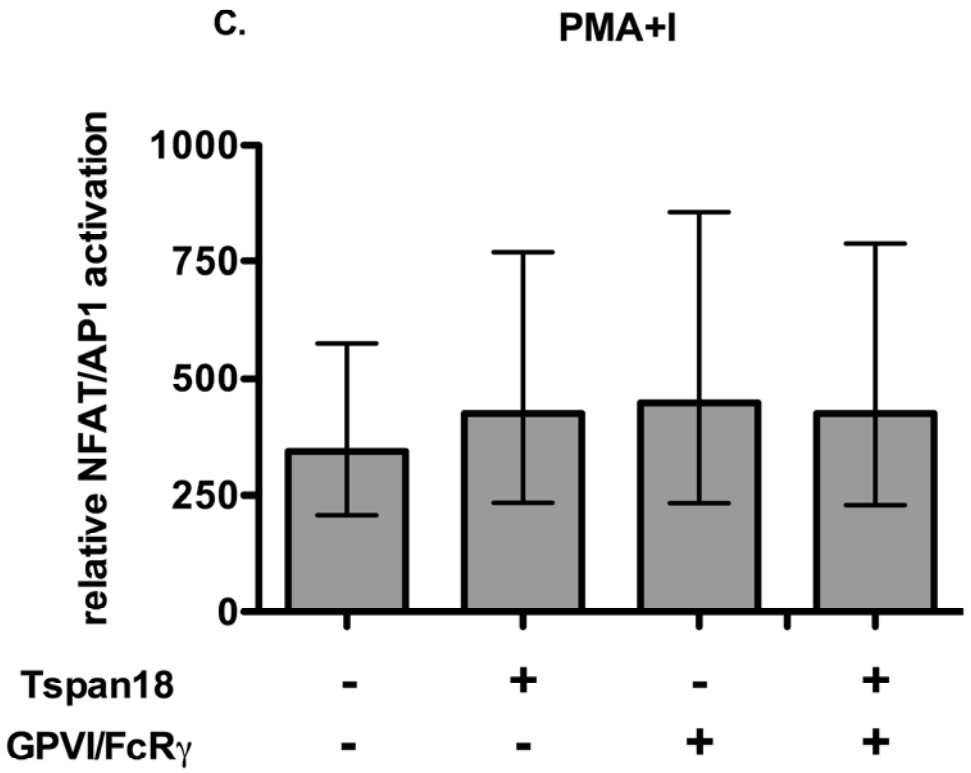


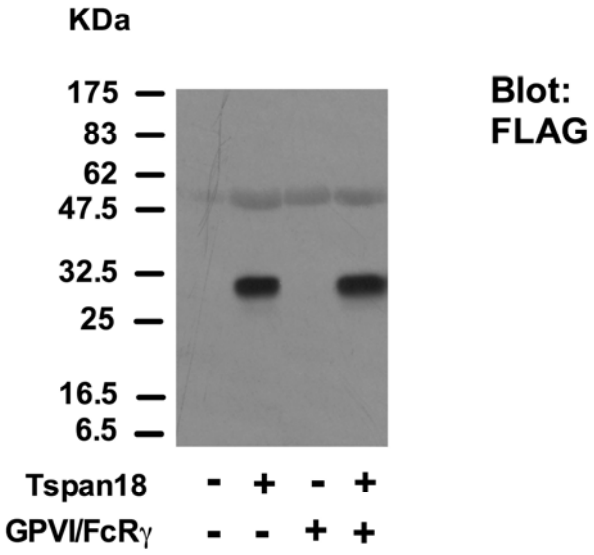
Figure 6.2: Tetraspanin over-expression does not affect collagen-induced GPVI/FcRγ activation of NFAT/AP-1. DT40 cells were transfected with 20 μg of the NFAT/AP-1-luciferase reporter construct, 2μg each of GPVI, FcRγ and β-galactosidase expression constructs and 5 μg of the indicated tetraspanin proteins. Cells were unstimulated (A), stimulated with collagen (B) or stimulated with PMA and ionomycin as a positive control (C). Data were divided for the relative β-gal value and moltiplied for 100. Error bars represent the standard deviation of two independent experiments. Data were analyzed by ANOVA and Tukey's test. Significantdifference among samples has been indicated ($P<0.05$).



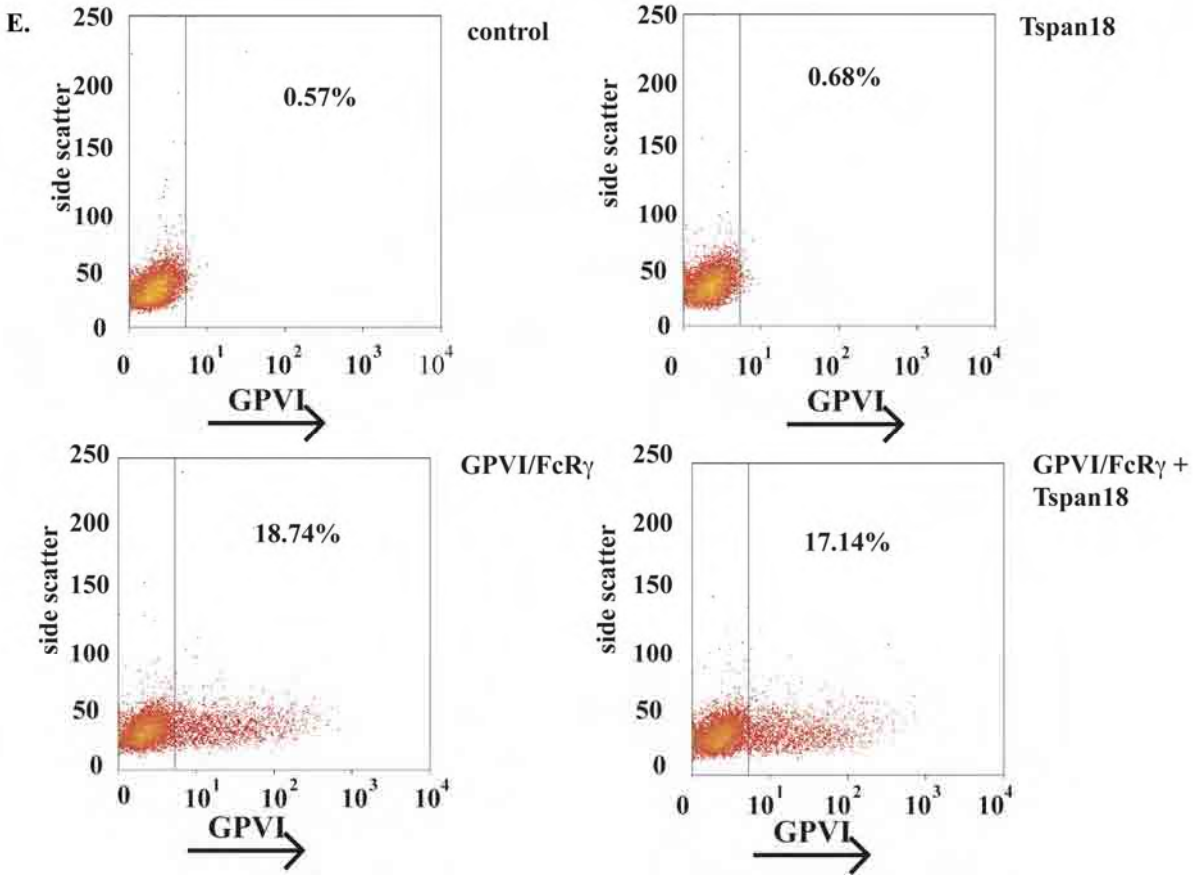

B.
collagen
*
*
*
*
relative NFAT/AP1 activation
250
200
150
100
50
0
Tspan18
GPVI/FcRγ
-
+
-
+
-
-
+
+


C.
PMA+I
relative NFAT/AP1 activation
1000
750
500
250
0
Tspan18
GPVI/FcRγ
-
+
-
+
-
-
+
+

D.


KDa
175
83
62
47.5
32.5
25
16.5
6.5
Blot:
FLAG
Tspan18 - + - +
GPVI/FcRγ - - + +

E.


control
0.57%
Tspan18
0.68%
GPVI/FcRγ
18.74%
GPVI/FcRγ +
Tspan18
17.14%
side scatter
GPVI
0
50
100
150
200
250
10^1
10^2
10^3
10^4

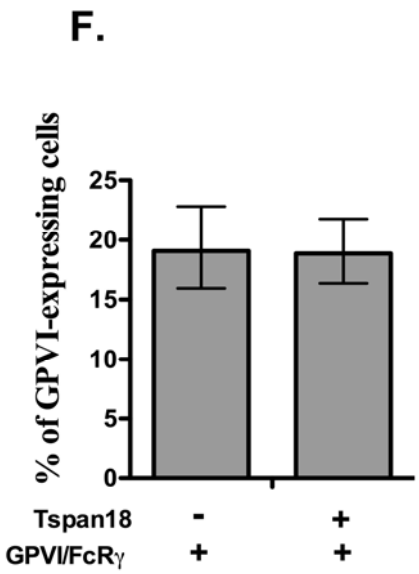


Figure 6.3: Tspan18 over-expression is sufficient to activate NFAT/AP-1. DT40 cells were transfected with the NFAT/AP-1-luciferase reporter, a β-galactosidase construct and either a control vector, 5μg of Tspan18, 2μg each of GPVI and FcRγ, or with the GPVI/FcRγ complex in conjunction with Tspan18. Samples were left unstimulated (A.), stimulated with collagen (B.) or with PMA and ionomycin (C.). Data were divided for the relative β-galactosidase value. Error bars represent the standard deviation of three independent experiments. Data were analyzed by ANOVA and Tukey's test. Significant difference among samples has been indicated ($P<0.05$). Aliquots of whole cell lysates were separated by SDS-PAGE and western blotted with a FLAG antibody, to evaluate Tspan18 expression (D.). Cells were stained with the GPVI monoclonal antibody and analyzed by flow cytometry (E.). The percentage of GPVI-expressing cells in the presence or absence of Tspan18 was calculated from three experiments and the error bars represent standard errors. Data were analysed by Student T-test and did not presented a significant difference (F).

In contrast to unstimulated cells, Tspan18 did not significantly affect NFAT/AP-1 activation in response to collagen stimulation of GPVI/FcRγ or the PMA and ionomycin positive control (Figure 6.3C and Figure 6.3D, respectively), as observed previously. Different doses of collagen and expression constructs were tested, but in all cases Tspan18 did not affect collagen-induced GPVI/FcRγ signalling (data not shown).

Two additional controls were performed, to verify that the GPVI/FcRγ expression did not affect Tspan18 expression (and vice versa). As the Tspan18 construct has an intracellular FLAG tag, Tspan18 expression was evaluated by SDS-PAGE followed by western blotting using a FLAG antibody. The result showed that Tspan18 expression was not affected by the presence of GPVI (Figure 6.3D). GPVI surface expression was analyzed by flow cyotometry, using a GPVI monoclonal antibody, and expression was similar in the presence or absence of Tspan18, suggesting that the tetraspanin did not affect GPVI expression (Figures 6.3E-F).

These data show that Tspan18 synergises with GPVI/FcRγ to activate NFAT/AP-1 in resting cells, but does not affect collagen-induced GPVI/FcRγ signalling.

6.4 DISCUSSION

In this chapter it was demonstrated that in the DT40 model B cell line, using the NFAT/AP-1 transcriptional reporter, a potent readout for combined calcium and MAPK signalling, over-expression of a panel of tetraspanin proteins did not affect GPVI/FcRγ signalling in response to collagen. However, Tspan18 was found to synergize with GPVI/FcRγ to activate NFAT/AP-1 under unstimulated conditions.

The aim of this chapter was to determine whether tetraspanins affect GPVI/FcRγ signalling, because of our finding before the start of this thesis that these proteins can be co-immunoprecipitated from human platelet lysates (Protty, Watkins et al. 2009), and because other ITAM-containing receptors are known to be regulated by tetraspanins (Tarrant, Groom et al. 2002; van Spriel, Puls et al. 2004). The first hypothesis was that a platelet tetraspanin might specifically interact with GPVI and/or FcRγ, and might enhance GPVI/FcRγ signalling by promoting its sustained localisation in lipid rafts, in a manner similar to that reported for the tetraspanin CD81 and the CD19/CD21/B cell receptor complex (Cherukuri, Shoham et al. 2004), although this finding has recently been questioned (Sanyal, Fernandez et al. 2009). However, none of the six platelet tetraspanins affected collagen-induced GPVI signalling when over-expressed in DT40 B cells. Moreover, of the six platelet tetraspanins, only Tsoan18 could significantly enhance basal GPVI/FcRγ signalling. From the analysis of the Figure 6.2A, it seemed that CD9 and Tspan9 could slightly enhance basal GPVI/FcRγ signalling, but this difference did not appeared to be significant at a Tukey's test analysis. It is possible that GPVI/FcRγ signalling is regulated by some other platelet tetraspanin protein that was not included in the tested panel. Indeed, several other platelet tetraspanins have recently been identified by proteomics (Lewandrowski, Wortelkamp et al. 2009). It is also possible that tetraspanin over-expression is not the best technique to determine whether these proteins affect GPVI/FcRγ signalling. An alternative approach would be to use RNA interference (RNAi) to study GPVI/FcRγ signalling in cells lacking one or more tetraspanins.

At present, it is still unclear whether tetraspanins functionally regulate GPVI/FcRγ, or indeed whether a substantial proportion of platelet GPVI/FcRγ is localised within tetraspanin microdomains. The signalling studies performed by the Jackson group using tetraspanin

knockout mice demonstrated that GPVI/FcRγ signalling is not affected by the absence of CD151 or Tspan32 (Lau, Wee et al. 2004; Goschnick, Lau et al. 2006). Taking together these findings and the data in this chapter, it is possible to speculate that GPVI/FcRγ association with tetraspanin proteins might be a way of regulating other GPVI/FcRγ functions distinct from signalling. For example, since the platelet laminin-binding integrin α6β1 is recruited to tetraspanin microdomains via a well-characterised interaction with CD151 (Sterk, Geuijen et al. 2002), and since GPVI is also a laminin receptor (Inoue, Suzuki-Inoue et al. 2006), it is possible that platelet tetraspanin microdomains act as a platform for efficient binding to laminin, a major component of the sub-endothelial matrix. Indeed, the close proximity of the two laminin receptors within the microdomains would allow 'inside-out' signals, generated by GPVI/FcRγ engagement, to rapidly activate the integrin to induce strong adhesion and enable the platelets to plug the wound. A second possibility is that tetraspanin microdomains regulate GPVI shedding by the metalloprotease ADAM10, which can cleave off the GPVI extracellular region so rendering the platelet non-responsive to collagen (Gardiner, Karunakaran et al. 2007), and which is strongly associated with tetraspanin microdomains (Arduise, Abache et al. 2008).

6.5 SUMMARY

In the present chapter it has been demonstrated that:

- Tspan18, but not the other platelet tetraspanins tested, synergizes with GPVI/FcRγ to induce NFAT/AP-1 activation in unstimulated cells
- Of the tested platelet tetraspanins, none appear to affect collagen-induced GPVI/FcRγ signalling when over-expressed

CHAPTER 7

GENERAL DISCUSSION

7.1 SUMMARY OF MAJOR FINDINGS

The signalling experiments performed in this thesis utilised a highly sensitive NFAT/AP-1 luciferase transcriptional reporter assay in model cell lines. This assay was previously used by our group to study the platelet collagen receptor GPVI (Tomlinson, Calaminus et al. 2007), and allowed an investigation of the effects of tetraspanin proteins on NFAT/AP-1 activation. Indeed, the first chapter of this thesis addressed the question as to whether over-expression of platelet-expressed tetraspanin proteins could induce NFAT/AP-1 signalling. The result demonstrated that one of the six tested tetraspanins could affect NFAT/AP-1 signalling: Tspan18. This suggested a unique signalling role for Tspan18, and its signalling mechanism was studied using mutant cell lines and specific signalling inhibitors. The results demonstrated that Tspan18 signalling was independent of Syk, Lyn, PLCγ, IP_3Rs and STIM1. However, Tspan18 signalling was calcineurin- and calcium-dependent, strongly indicating this tetraspanin protein might be involved in calcium signalling. This was supported by studies that showed Tspan18 signalling to mimic the calcium ionophore ionomycin. In conclusion, it was proposed that Tspan18 might interact with and regulate a calcium channel or a protein involved in calcium entry.

7.2 MODELS FOR TSPAN18 REGULATION OF NFAT ACTIVATION

The mechanistic studies in Chapter 3 showed that Tspan18-induced NFAT/AP-1 activation did not require IP_3 receptors, which release calcium from intracellular stores to initiate store-operated calcium entry into the cell (Taylor, Rahman et al. 2009). In contrast, Tspan18 signalling was dependent on calcineurin, the phosphatase which dephosphorylates NFAT proteins, allowing their translocation to the nucleus where they can activate target genes

(Macian 2005). These key findings suggest that Tspan18 exerts its effect at a point on the NFAT signalling pathway between IP_3 receptors and calcineurin. Possible Tspan18-regulated proteins will be discussed later in this section, but how could Tspan18 over-expression induce their activation? Given that tetraspanins are thought to organise other transmembrane and signalling proteins in cell membranes, through specific tetraspanin-partner protein interactions (Hemler 2008; Charrin, le Naour et al. 2009), it seems likely that Tspan18 over-expression induces signalling by clustering or conformationally activating a signalling partner. However, although the data in this thesis suggest that Tspan18 is a positive regulator of NFAT activation, this may not necessarily be the case. For example, relatively low level, endogenous Tspan18 might normally negatively regulate a component of the NFAT signalling pathway, but over-expression might perturb the system to induce artefactual pathway activation. Such 'informative artefacts' are a feature of previous over-expression studies on cytoplasmic adapters, for example (Burack and Shaw 2000).

A number of possible models involving different candidate Tspan18 partner proteins can be proposed to explain the Tspan18 signalling phenomenon. Firstly, Since STIM1-induced Orai1 opening represents the major mechanism for store-operated calcium entry in lymphocytes (Wang, Deng et al. 2009), it is possible that Tspan18 interacts with either of these proteins. The proposed working model, which illustrates this scenario, is presented in Figure 7.1. The figure, also takes into consideration all the other players considered in this thesis, and other possible working hypotheses.

ER-resident STIM1 senses depletion of calcium stores and then reorganises itself in the membrane to form plasma membrane-proximal clusters, which then induce Orai1 tetramerisation and calcium channel opening (Wang, Deng et al. 2009). It is not difficult to imagine that a tetraspanin might be involved in such a process. For example, tetraspanins are

known to regulate ICAM-1 and VCAM-1 endothelial cell adhesion molecules by promoting their clustering (Barreiro, Zamai et al. 2008). A possible interaction of Tspan18 with STIM1 and Orai1 could be tested in future by co-immunoprecipitation in cells transfected with epitope-tagged proteins. The cells could be first stimulated with thapsigargin, an inhibitor of the sarco/endoplasmic reticulum calcium ATPase (SERCA) pumps in the ER, to activate store-operated calcium channel entry and induce STIM1/Orai1 clustering (Park, Hoover et al. 2009). The cells could also be treated with a membrane-permeable cross-linker such as DSP (Dithiobis[succinimidyl propionate]), which captures potentially low affinity interactions and is the 'gold standard' method in the field for confirming direct tetraspanin-partner protein interactions (Charrin, le Naour et al. 2009).

An involvement of STIM1 and Orai1 in Tspan18 signalling could also be tested functionally, through NFAT/AP-1 reporter assays either in combination with inhibitors of store-operated calcium entry, or RNAi to knock down STIM1 and/or Orai1, or DT40 mutants. Possible pharmacological inhibitors include 50 μM 2-aminoethoxydiphenyl borate (2-APB) or 0.3 μM lanthanum (La^{3+}), which both completely inhibit store-operated calcium entry in DT40 cells at these concentrations (Broad, Braun et al. 2001; Prakriya and Lewis 2001; Morita, Tanimura et al. 2009) The 2-APB could also be used at 5 μM, because at low concentration this drug enhances store-operated calcium entry by coupling STIM and Orai proteins (Wang, Deng et al. 2009) and thus may mimic the Tspan18 effect. RNAi knockdown of STIM1 and Orai1 and would best be used in human Jurkat T cells (in combination with PMA to yield a robust NFAT/AP-1 response, as shown in Figure 3.6), because knockdown of these proteins is well established in human cells but has not been done in chicken. Finally, in the current absence of Orai1-deficient DT40 cells, Tspan18-induced NFAT/AP-1 activation could be measured in DT40 cells doubly deficient in STIM1 and STIM2 (Wang, Deng et al. 2009). This would be useful because residual BCR- and Tspan18-induced NFAT/AP-1 activation was observed in

STIM1-deficient cells (Figure 3.8). This is possibly due to compensation by STIM2 and made it impossible to determine whether STIM proteins are essential for Tspan18 signalling.

The above discussion has focussed on STIM1 and Orai1, because of their major role in lymphocyte calcium entry (Park, Hoover et al. 2009). However, it is certainly impossible to rule out other possibilities. Indeed, Tspan18 might interact with and regulate a calcium channel that does not involve Orai1. Examples include the two DT40 ryanodine receptors (RyRs), RyR1 and RyR3 (Kiselyov, Shin et al. 2001), which are calcium release channels in the ER, distinct from IP_3 receptors, and which can be inhibited with the pharmacological inhibitor Ruthenium Red at 100 μM in these cells (Kiselyov, Shin et al. 2001) (Figure 7.1). Another possibility is the ER-resident SERCA pump which, if inhibited by Tspan18 over-expression, would be predicted to activate NFAT signalling in a manner that is IP_3 receptor-independent (Sugawara, Kurosaki et al. 1997), but calcineurin-dependent, consistent with the Tspan18 mechanistic data. Finally, Tspan18 could interact with and regulate lymphocyte-expressed store-operated calcium channels on the plasma membrane such as TRPC1 or TRPC3 (Philipp, Strauss et al. 2003), or the non-store-operated plasma membrane TRPV or TRPM calcium channels. Investigation of the non-store-operated channels would be complicated by the large number of family members, namely six and eight, respectively (Inada, Iida et al. 2006). Nevertheless, each possibility in this section could be studied by the co-immunoprecipitation or RNAi studies outlined for STIM1 and Orai1 in the previous paragraph (Figure 7.1).

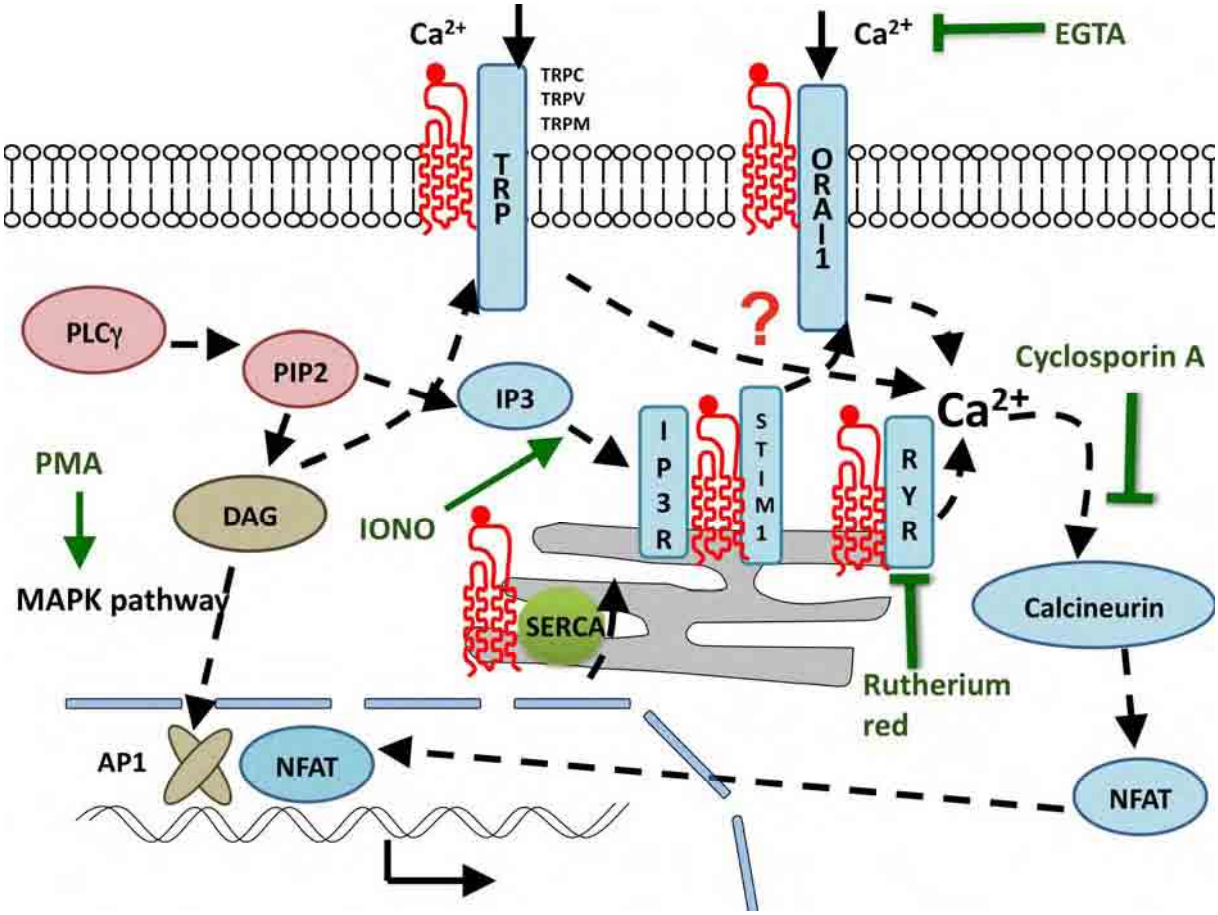


Figure 7.1: Working hypotheses of Tspan18 mechanism of function in DT40 cells. Tspan18 (in red) might inderact and regulate the IP_3 receptor, or STIM1, or might interact with and regulate the rynodine receptors. Tspan18 might interact with and regulate the ORAI1 plasma membrane calcium channel, or the TRP calcium channels. In green are indicated stimulatory or blockage agents.

7.3 DO TETRASPANINS REGULATE GPVI?

We have recently reported that the platelet collagen receptor GPVI is a component of tetraspanin membrane microdomains on human platelets (Protty, Watkins et al. 2009). The evidence for this was initially derived from immunoprecipitation experiments using surface-biotinylated human platelets lysed in the relatively weak detergent 1% Brij97, which maintains tetraspanin-tetraspanin interactions and the integrity of tetraspanin microdomains (Hemler 2008). The pattern of co-immunoprecipitated surface proteins, as detected following SDS-PAGE and streptavidin blotting, was very similar for GPVI and CD151 immunoprecipitations. Moreover, CD9 and CD151 were detected in GPVI immunoprecipitations. As controls for specificity, the platelet von Willebrand receptor GPIb and the integrins αIIbβ3 and α2β1, each of which are generally expressed at much higher levels than GPVI, were not found to be tetraspanin-associated (Protty, Watkins et al. 2008).

These findings were the starting point for the final GPVI chapter of this thesis, which aimed to test the effect of tetraspanin over-expression on collagen-induced GPVI signalling using a newly-developed cell line model (Tomlinson, Calaminus et al. 2007). No evidence was obtained to suggest that GPVI collagen signalling was regulated by tetraspanins. However, recent developments suggest that the possibility of GPVI regulation by tetraspanins should be re-visited. Firstly, a proteomic analysis of the platelet surface has identified ten tetraspanins that are likely to be expressed on platelets (Lewandrowski, Wortelkamp et al. 2009). These are, in estimated order of abundance, CD9, Tspan9, Tspan33, CD151, CD63, Tspan14, Tspan32, Tspan15, Tspan2 and Tspan18 (Tomlinson 2009). Our lab has human and mouse FLAG-tagged expression constructs for each of these. In a relatively simple experiment, each could be co-transfected with GPVI/FcRγ constructs in an easily-transfectable cell line such as DT40 or HEK293T. The cells could be surface-cross-linked with a membrane impermeable chemical cross-linker such as BS3, considered as the ‘gold standard’ in the field for

covalently capturing potentially low affinity tetraspanin interactions with partner proteins. FLAG immunoprecipitations in highly-stringent conditions would then be followed by SDS-PAGE and GPVI blotting to determine whether any platelet tetraspanin specifically interacts with the collagen receptor. Identification of a GPVI-binding tetraspanin would prompt further GPVI functional experiments using tetraspanin RNAi knockdown in GPVI-expressing megakaryocyte-like cell lines and potentially tetraspanin-deficient platelets; our lab has recently generated Tspan9-knockout mice (unpublished) and has obtained Tspan33-knockouts from the Tsai group (Heikens, Cao et al. 2007).

Additional recent developments that suggest re-visiting the potential tetraspanin regulation of GPVI are the capacity for this collagen receptor to also bind laminin (Inoue, Suzuki-Inoue et al. 2006), the identification of ADAM10 as the ectodomain sheddase responsible for cleaving off the extracellular domain of GPVI following activation (Gardiner, Karunakaran et al. 2007), and the finding that ADAM10 is a major component of tetraspanin microdomains on various cell types (Arduise, Abache et al. 2008) including platelets (Tomlinson MG, unpublished). These developments direct future experiments aimed at determining how tetraspanins might regulate GPVI. For example, both platelet laminin receptors, GPVI and the integrin $\alpha 6\beta 1$, are components of tetraspanin microdomains (Sterk, Geuijen et al. 2002; Protty, Watkins et al. 2008). Could tetraspanin microdomains act as platforms for the optimal regulation of binding to laminin, a major component of the subendothelial matrix (Lammerding, Kazarov et al. 2003)? In addition could tetraspanin microdomains be platforms for the positive or negative regulation of ADAM10-induced GPVI shedding? Answers to these questions await further experiments.

7.4 FUTURE DIRECTIONS

The work in this thesis represents the first characterisation of the tetraspanin protein Tspan18. The preliminary sub-cellular localisation and expression profiling performed in Chapters 4 and 5, together with the functional data in Chapter 3, has raised a number of issues for future work. These include (1) the generation of Tspan18 antibodies, (2) studies to determine the expression profile and sub-cellular localisation of Tspan18, (3) over-expression and RNAi knockdown in a more relevant cell type such as endothelial cells, (4) experiments to determine whether Tspan18 induces calcium mobilisation, and (5) identification of a Tspan18 partner protein or proteins.

An anti-Tspan18 antibody would be of great use to characterise the Tspan18 expression profile, subcellular localisation and associated proteins. However, the generation of anti-tetraspanin antibodies is a major problem in the field and such reagents do not exist for most tetraspanins, including Tspan18. Despite this, we have recently made the first antibodies to the tetraspanin Tspan9 using an anti-peptide approach in chicken and rabbit, by targeting the 12 amino acid carboxy-terminal cytoplasmic tail (Protty, Watkins et al. 2009). The equivalent approach for Tspan18 is not possible because the carboxy-terminal tail is predicted to have only four amino acid residues. In addition, the amino-terminus is not considered to be a good immunogen (Eurogentec, personal communication to Tomlinson MG). Therefore a peptide corresponding to the carboxy-terminal region of the major extracellular region could be used, because this was successful for the generation of antibodies to Tspan2 by the Brophy group (Birling, Tait et al. 1999). Based on the Brophy Tspan2 antibody, the Tspan18 antibody might only recognise denatured protein, and thus may only be useful for western blotting. An alternative immunogen could be a recombinant GST fusion protein for the major extracellular region of Tspan18. However, such an approach is not favoured because it was not successful for tetraspanins CD37 and CD53 (Tomlinson MG, 1995, unpublished data).

In parallel with the polyclonal approach described above, monoclonal anti-Tspan18 antibodies could be generated using recombinant full-length human Tspan18 as an immunogen. This material would be generated in the yeast *Pichia pastoris*, as has proved successful for tetraspanin CD81 (Jamshad, Rajesh et al. 2008). More importantly, Prof Jane McKeating's group has recently generated seventeen monoclonal antibodies to CD81 using this methodology (McKeating JA personal communication to Tomlinson MG). An alternative immunogen for monoclonal antibody generation in mice is a mouse cell line expressing human Tspan18. Indeed, immunisation with cells was the method that generated most of the antibodies to tetraspanins such as CD9, CD37, CD53, CD63, CD81, CD82 and CD151 in the 1980s. However, this method is not efficient (Tomlinson MG, personal communication), most likely because of the high degree of sequence conservation between tetraspanins, and their small size and tendency to associate with larger proteins, which together may impair their visibility to the immune system. A novel approach which was initiated, but not completed, in Chapter 5 of this thesis, was to generate a non-palmitoylated chimeric construct of the CD9 cytoplasmic and intracellular regions and the Tspan18 extracellular loops. In transfected cells this construct might fail to interact efficiently with other tetraspanins or CD9/Tspan18 partner proteins, and might be more immunogenic, but this has not been tested. In summary, one of these approaches is likely to generate monoclonal antibodies for flow cytometry, immunoprecipitation and potentially western blotting and immunohistochemistry. The antibodies could also be tested in functional studies to examine the effect of Tspan18 cross-linking on intracellular calcium or NFAT activation, for example.

The experiments in Chapter 5 identified Tspan18 mRNA in endothelial cells and peripheral blood leukocytes using qRT-PCR. If a Tspan18 antibody can be generated in future, the expression profile could be extensively studied by immunostaining of tissue sections, an ideal method to examine Tspan18 expression on different endothelial cell types. Alternatively, flow

cytometry could be performed for sub-populations of leukocytes. The sub-cellular localisation could also be investigated by confocal fluorescence microscopy, following labelling of endogenous Tspan18 in an endothelial cell type such as HUVEC. In the present absence of an antibody, endothelial expression could best be studied by *in situ* hybridisation of day 9.5 mouse embryos, at which stage the vasculature has just developed and the mice are small enough to allow full penetration of the labelled RNA probe. The sub-cellular localisation of Tspan18 could be examined by confocal fluorescence microscopy in HUVEC transfected with GFP- or FLAG-tagged Tspan18, the latter of which could avoid potential mis-loacalisation due to the relatively large GFP tag.

The NFAT activation experiments could also be extended to HUVEC, to examine the consequence of Tspan18 over-expression in a cell that has endogenous Tspan18. Although some publications have used NFAT/AP-1-luciferase assays in HUVEC (Qin, Zhao et al. 2006), this has not proved successful in our group because of low transfection efficiencies and the need to serum-starve the cells to prevent constitutive growth factor-induced NFAT activation during the assay (Tomlinson MG, unpublished data). To overcome this problem, several groups have employed a construct encoding a GFP-tagged NFAT protein, which is localised to the cytoplasm in resting cells but rapidly translocates to the nucleus upon activation (Schabbauer, Schweighofer et al. 2007). This construct has been recently obtained by our group and could be used to determine whether Tspan18 over-expression can activate NFAT in HUVEC by fluorescence microscopy on a single cell level. Such experiments would be facilitated by a red-fluorescent version of Tspan18, which has recently been generated in our group (Yang J and Tomlinson MG, unpublished). Transfection of this red-Tspan18 into HUVEC or DT40 cells could also be used to determine whether Tspan18 induces elevated levels of intracellular calcium, using a green fluorescent calcium indicator and single cell imaging. This could be done in both resting cells and upon thapsigargin induction of store-

operated calcium entry. Similar experiments were attempted in this thesis using fluorimetry to detect calcium elevation in populations of transfected cells, but none was detected, perhaps due to relatively low transfection efficiencies (data not shown).

An additional type of experiment that would be ideally suited to HUVEC is knockdown of Tspan18 using RNAi duplexes. Indeed, our group has recently achieved 95-100% knockdown of tetraspanins CD81 and CD151 in these cells, as detected by flow cytometry (Tomlinson MG, unpublished). Moreover, we have achieved over 90% knockdown of Tspan18 mRNA, as detected by qRT-PCR (Figure Appendix 3). Single cell calcium imaging or fluorimetry could be performed on the knockdown cells, using growth factor agonists or thapsigargin, to determine whether Tspan18 is necessary for normal calcium mobilisation in HUVEC. In addition, a variety of functional assays could be performed to determine whether Tspan18 is necessary for processes such as HUVEC tube formation on the basement membrane extract Matrigel, migration in a scratch-wound assay, chemotaxis, proliferation and survival. If these experiments indicated an important role for Tspan18 in endothelial cells, an important next step would be to address whether this is true *in vivo*. To answer this question in a relatively quick and inexpensive way, Tspan18 could be studied in the widely-used zebrafish model of endothelial cell angiogenesis (Staton, Reed et al. 2009). Firstly, *in situ* hybridisations would be performed on zebrafish embryos to determine whether either of the two zebrafish Tspan18 homologues is expressed in the vasculature. Secondly, antisense morpholinos would be used to knock down Tspan18 expression in embryos, to determine whether this tetraspanin is necessary for blood vessel development through angiogenesis. Ultimately, Tspan18 function could be addressed in mammals through the generation of knockout mice.

Finally, a key future experiment will be to identify a Tspan18-interacting protein, or proteins, that could explain the mechanism by which Tspan18 over-expression induces NFAT

activation. Our group has previously used tetraspanin immunoprecipitations from biotinylated cells, followed by streptavidin western blotting, to visualise tetraspanin-associated proteins (Varga-Szabo, Braun et al. 2008; Protty, Watkins et al. 2009). Similar experiments could be performed for endothelial cell Tspan18, using specific antibodies to immunoprecipitate endogenous Tspan18, or FLAG antibody to immunoprecipitate transfected FLAG-tagged Tspan18. Immunoprecipitations would be performed in 1% Brij97 lysis buffer, which maintains tetraspanin microdomains, or the more stringent 1% Triton X-100 lysis buffer, which disrupts tetraspanin microdomains but not relatively strong tetraspanin-partner protein interactions (Hemler 2005). If candidate Tspan18-associated proteins are visualised in Triton, immunoprecipitations would be scaled up to visualise the band on coomassie gels, and the bands excised to obtain peptide sequence by mass spectrometry. Candidate Tspan18 binding partners would be tested by co-immunoprecipitation from cells treated with a chemical cross-linker. An alternative approach would be to target candidate Tspan18-interacting proteins, such as STIM1 or Orai1, in direct co-immunoprecipitation experiments.

7.5 SPECULATIONS ON THE ROLE OF TSPAN18 ON PLATELETS, ENDOTHELIAL CELLS AND LEUKOCYTES

Tspan18 protein was recently shown to be expressed on human platelets using proteomics (Lewandrowski, Wortelkamp et al. 2009; Tomlinson 2009), and the data in Chapter 5 of this thesis has demonstrated mRNA expression in endothelial cells and peripheral blood leukocytes. It is possible that Tspan18 regulates calcium entry in each of these cell types, either through regulation of store-operated calcium entry involving STIM and Orai proteins, or through an alternative mechanism such as regulation of a plasma membrane calcium channel or channel regulator. Importantly, and as discussed earlier, it is not possible from the

current data to determine whether this regulation is positive or negative, although the NFAT activation data suggests that an activatory role is more likely.

An increase in intracellular calcium following platelet activation is essential for integrin activation, secretion of positive feedback mediators such as ADP and thromboxane A_2, and pro-coagulant activity (Bergmeier and Stefanini 2009). Studies in STIM1- and Orai1-deficient mice have shown these two proteins to be essential for store-operated calcium entry in platelets (Varga-Szabo, Braun et al. 2008; Braun, Varga-Szabo et al. 2009). Mutant platelets exhibited defective aggregation *in vitro*, which was most clearly seen in response to collagen through GPVI, and defective thrombus formation *in vivo* (Varga-Szabo, Braun et al. 2008; Braun, Varga-Szabo et al. 2009). It is possible that Tspan18 regulates such platelet functions through regulation of store-operated calcium entry.

Endothelial cells are also thought to utilise STIM1 and Orai1 as the primary means of store-operated calcium entry, which is essential for important processes such as cell proliferation (Abdullaev, Bisaillon et al. 2008). Calcium, through activation of calmodulin and protein kinase C, activates several intracellular enzymes including myosin light chain kinase, which results in cytoskeletal re-organisation and increased endothelial cell permeability. Calcium/calmodulin also activates endothelial nitric oxide synthase, which produces the nitric oxide signalling molecule. Nitric oxide is a potent vasodilator and is atheroprotective through inhibition of platelet activation and smooth muscle cell proliferation (Rudolph and Freeman 2009). Unlike platelets, endothelial cells are nucleated and are regulated by NFAT-induced transcriptional responses. Indeed, vascular development and angiogenesis are dependent on NFAT transcription factors (Kulkarni, Greenberg et al. 2009). Tspan18 could regulate these processes through regulation of calcium and/or NFAT signalling.

Calcium/calcineurin/NFAT signalling is essential for normal leukocyte activation, as evidenced by the widespread clinical use of the immunosuppressive drug cyclosporin A, which inhibits calcineurin, to prevent rejection following organ transplants (Schulz and Yutzey 2004). Indeed, NFAT was first identified as the nuclear factor of activated T cells which activates the transcription of a number of genes involved in immunity, including interleukin-2 (Liu, Masuda et al. 1999). It is possible that Tspan18 plays a regulatory role in the immune response via regulation of NFAT activation. However, since Tspan18 mRNA was detected in peripheral blood leukocytes, it is currently unclear precisely which type of leukocyte expresses this tetraspanin. Interestingly, our group recently reported relatively strong Tspan18 mRNA expression on CD4-positive helper T cells (Protty, Watkins et al. 2008) , suggesting a role for Tspan18 on this cell type that is central to the immune response.

In summary, this thesis has characterised Tspan18, a novel tetraspanin expressed on platelets, endothelial cells and leukocytes with a potential role in calcium and NFAT signalling. Future knockdown and knockout studies are now required to determine whether Tspan18 is essential for key processes such as haemostasis and thrombosis, blood vessel development and function, and immunity.

APPENDICES

Appendix 1 ClustalW sequence alignment of all human tetraspanin proteins

```
Tspan9   ------MARGCLC--------CLKYMMFLFNLIFWLCGCGLLGVGIWLSVSQGNFATFSP 46
Tspan4   ------------------------------------------------------------ 
CD53     ------MGMSSLK--------LLKYVLFFFNLLFWICGCCILGFGIYLLIHN-NFGVLFH 45
CD9      ----MP-VKGGTK--------CIKYLLFGFNFIFWLAGIAVLAIGLWLRFDSQTKSIFEQ 47
Tspan2   ----MGRFRGGLR--------CIKYLLLGFNLLFWLAGSAVIAFGLWFRFGGAIKELSSE 48
CD81     ----MG-VEGCTK--------CIKYLLFVFNFVFWLAGGVILGVALWLRHDPQTTNLLYL 47
Tspan8   -------MAGVSA--------CIKYSMFTFNFLFWLCGILILALAIWVRVSNDSQAIFGS 45
CD82     ------MGSACIK--------VTKYFLFLFNLIFFILGAVILGFGVWILADKSSFISVLQ 46
CD37     ----MSAQESCLS--------LIKYFLFVFNLFFFVLGSLIFCFGIWILIDKTSFVSFVG 48
Tspan7   -MAS--RRMETKP-----VITCLKTLLIIYSFVFWITGVILLAVGVWGKLTLGTYISLIA 52
Tspan6   -MASPSRRLQTKP-----VITCFKSVLLIYTFIFWITGVILLAVGIWGKVSLENYFSLLN 54
CD151    --MGEFNEKKTTC-----GTVCLKYLLFTYNCCFWLAGLAVMAVGIWTLALKSDYISLLA 53
Tspan11  ---------------------------------------------LVEKSGYLSVLA 12
Tspan5   -MSGK--HYKGPE-----VSCCIKYFIFGFNVIFWFLGITFLGIGLWAWNEKGVLSNISS 52
Tspan17  -MPGKHQHFQEPE-----VGCCGKYFLFGFNIVFWVLGALFLAIGLWAWGEKGVLSNISA 54
Tspan14  -MHYY--RYSNAK-----VSCWYKYLLFSYNIIFWLAGVVFLGVGLWAWSEKGVLSDLTK 52
Tspan33  -MARRPRAPAASGEEFSFVSPLVKYLLFFFNMLFWVISMVMVAVGVYARLMKHAEAALAC 59
Tspan15  -MPRG--DSEQVRYCARFSYLWLKFSLIIYSTVFWLIGALVLSVGIYAEVERQKYKTLES 57
ROM1     MAPVLPLVLPLQP------RIRLAQGLWLLSWLLALAGGVILLCSGHLLVQLRHLGTFLA 54
RDS      -MALLKVKFDQKK------RVKLAQGLWLMNWFSVLAGIIIFSLGLFLKIELRKRSDVMN 53
Tspan18  ---MEGDCLS-----------CMKYLMFVFNFFIFLGGACLLAIGIWVMVDPTGFREIVA 46
Tspan1   -----MQCFS-----------FIKTMMILFNLLIFLCGAALLAVGIWVSIDGASFLKIFG 44
UP1A     -MASAAAAEAEKG------SPVVVGLLVVGNIIILLSGLSLFAETIWVTADQYRVYPLMG 53
UP1B     -MAKDNSTVR-----------CFQGLLIFGNVIIGCCGIALTAECIFFVSDQHSLYPLLE 48
Tspan31  ------MVCGGFA--------CSKNALCALNVVYMLVSLLLIGVAAWGKGLGLVSSIHII 46
Tspan13  ------MVCGGFA--------CSKNCLCALNLLYTLVSLLLIGIAAWGIGFGLISSLRVV 46
Tspan3   -----MGQCGITS---------SKTVLVFLNLIFWGAAGILCYVGAYVFITYDDYDHFFE 46
Tspan19  -------MLRNNK------TIIIKYFLNLINGAFLVLGLLFMGFGAWLLLDRNNFLTAFD 47
CD63     -----MAVEGGMK--------CVKFLLYVLLLAFCACAVGLIAVGVGAQLVLSQTIIQGA 47
Tspan12  -----MAREDSVK--------CLRCLLYALNLLFWLMSISVLAVSAWMRDYLNNVLTLTA 47
Tspan16  ----MAEIHTPYS--------SLKKLLSLLNGFVAVSGIILVGLGIGGKCGGASLTNVLG 48
Tspan10  ------MEEGERS-------PLLSQETAGQKPLSVHRPPTSGCLGPVPREDQAEAWGCSC 47
Tspan32  -MGPWSRVRVAKB--------CQMLVTCFFILLLGLSVATMVTLTYFGAHFAVIRRASLE 51


Tspan9   SFP---------SLSAANLVIAIGTIVMVTGFLGCLGAIKENKCLLLSFFIVLLVILLAE 97
Tspan4   --------------------------MAIGFVGCLGAIKENKCLLLTFFLLLLLVFLLE 33
CD53     NLP---------SLTLGNVFVIVGSIIMVVAFLGCMGSIKENKCLLMSFFILLLIILLAE 96
CD9      ET--NN--NNSSFYTGVYILIGAGALMMLVGFLGCCGAVQESQCMLGLFFGFLLVIFAIE 103
Tspan2   DK--SP--E--YFYVGLYVLVGAGALMMAVGFFGCCGAMRESQCVLG------------ 89
CD81     ELGDKP--APNTFYVGIYILIAVGAVMMFVGFLGCYGAIQESQCLLGTFFTCLVILFACE 105
Tspan8   ED------VGSSSYVAVDILIAVGAIIMILGFLGCCGAIKESRCMLLLFFIGLLLILLLQ 99
CD82     TSS-------SSLRMGAYVFIGVGAVTMLMGFLGCIGAVNEVRCLLGLYFAFLLLILIAQ 99
CD37     LAF-------VPLQIWSKVLAISGIFTMGIALLGCVGALKELRCLLGLYFGMLLLLFATQ 101
Tspan7   ENS---------TNAP-YVLIGTGTTIVVFGLFGCFATCRGSPWMLKLYAMFLSLVFLAE 102
Tspan6   EKA---------TNVP-FVLIATGTVIILLGTFGCFATCRASAWMLKLYAMFLTLVFLVE 104
CD151    SGT---------YLATAYILVVAGTVVMVTGVLGCCATFKERRNLLRLYFILLLIIFLLE 104
Tspan11  SST---------FAASAYILIFAGVLVMVTGFLGFGAILWERKGCLSTYFCLLLVIFLVE 63
Tspan5   ITDLG-------GFDPVWLFLVVGGVMFILGFAGCIGALRENTFLLKFFSVFLGIIFFLE 105
Tspan17  LTDLG-------GLDPVWLFVVVGGVMSVLGFAGCIGALRENTFLLKFFSVFLGLIFFLE 107
Tspan14  VTRMH-------GIDPVVLVLMVGVVMFTLGFAGCVGALRENICLLNFFCGTIVLIFFLE 105
```

```
Tspan33  LA----------VDPAILLIVVGVLMFLLTFCGCIGSLRENICLLQTFSLCLTAVFLLQ 108
Tspan15  AF----------LAPAIILILLGVVMFMVSFIGVLASLRDNLYLLQAFMYILGICLIME 106
ROM1     PSCQF-------PVLPQAALAAGAVALGTGLVGVGASRASLNAALYPPWRGVLGPLLVAG 107
RDS      -NSES-------HFVPNSLIGMGVLSCVFNSLAGKICYDALDPAKYARWKPWLKPYLAIC 105
Tspan18  ANP--------LLLTGAYILLAMGGLLFLLGFLGCCGAVRENKCLLLFFFLFILIIFLAE 98
Tspan1   PLSSSA--M--QFVNVGYFLIAAGVVVFALGFLGCYGAKTESKCALVTFFFILLLIFIAE 100
UP1A     VSGKD------DVFAGAWIAIFCGFSFFMVASFGVGAALCRRRSMVLTYLVLMLIVYIFE 107
UP1B     ATDND------DIYGAAWIGIFVGICLFCLSVLGIVGIMKSSRKILLAYFILMFIVYAFE 102
Tspan31  G-----------------GVIAVGVFLLLIAVAGLVGAVNHHQVLLFFYMIILGLVFIFQ 89
Tspan13  G-----------------VVIAVGIFLFLIALVGLIGAVKHHQVLLFFYMIILLLVFIVQ 89
Tspan3   DVY---------TLIPAVVIIAVGALLFIIGLIGCCATIRESRCGLAT------------ 85
Tspan19  ENN-------HFIVPISQILIGMGSSTVLFCLLGYIGIHNEIRWLLIVYAVLITWTFAVQ 100
CD63     TPG----------SLLPVVIIAVGVFLFLVAFVGCCGACKENYCLMITFAIFLSLIMLVE 97
Tspan12  ETRVEEAVILTYFPVVHPVMIAVCCFLIIVGMLGYCGTVKRNLLLLAWYFGTLLVIFCVE 107
Tspan16  LSS-------AYLLHVGNLCLVMGCITVLLGCAGWYGATKESRGTLLFCILSMVIVLIME 101
Tspan10  CPP----------ETKHQALSGTPKKGPAPSLSPGSSCVKYLIFLSNFPFSLLGLLALAI 97
Tspan32  KNP-----YQAVHQWAFSAGLSLVGLLTLGAVLSAAATVREAQGLMAGGFLCFSLAFCAQ 106

Tspan9   LILL--------ILFFVYMDKVNENAKKDLKEGLLLYHT--ENNVG-------LKNAWNI 140
Tspan4   ATIA--------ILFFAYTDKIDRYAQQDLKKGLHLYGT--QGNVG-------LTNAWSI 76
CD53     VTLA--------ILLFVYEQKLNEYVAKGLTDSIHRYHS--DNST---------KAAWDS 137
CD9      IAAA--------IWGYSHKDEVIKEV-QEFYKDTYNKLKTKDEPQ---------RETLKA 145
Tspan2   -------------------SAIRHV-QTMYEEAYNDYLKDRGKG---------NGTLIT 119
CD81     VAAG--------IWGFVNKDQIAKDV-KQFYDQALQQAVVDDDANN-------AKAVVKT 149
Tspan8   VATG--------ILGAVFKSKSDRIVNETLYENTKLLSATGESEKQ-------FQEAIIV 144
CD82     VTAG--------ALFYFNMGKLKQEMGGIVTELIRDYNS-SRED-S-------LQDAWDY 142
CD37     ITLG--------ILISTQRAQLERSLRDVVEKTIQKYGT-NPEETA-------AEESWDY 145
Tspan7   LVAG--------ISGFVFRHEIKDTFLRTYTDAMQTYNGN----------DERSRAVDH 143
Tspan6   LVAA--------IVGFVFRHEIKNSFKNNYEKALKQYNSTG----------DYRSHAVDK 146
CD151    IIAG--------ILAYAYYQQLNTELKENLKDTMTKRYHQPGH--------EAVTSAVDQ 148
Tspan11  LVAG--------VLAHVYYQRLSDELKQHLNRTLAENYGQPGA--------TQITASVDR 107
Tspan5   LTAG--------VLAFVFKDWIKDQLYFFINNNIRAYRDDID----------LQNLIDF 146
Tspan17  LATG--------ILAFVFKDWIRDQLNLFINNNVKAYRDDID----------LQNLIDF 148
Tspan14  LAVA--------VLAFLFQDWVRDRFREFFESNIKSYRDDID----------LQNLIDS 146
Tspan33  LAAG--------ILGFVFSDKARGKVSEIINNAIVHYRDDLD----------LQNLIDF 149
Tspan15  LIGG--------VVALTFRNQTIDFLNDNIRRGIENYYDDLD----------FKNIMDF 147
ROM1     TAGGGGLLVVALGLALALPGSLDEALEEGLVTALAHYKDTEVPGHCQ------AKRLVDE 161
RDS      VLFNIILFLVAL-CCFLLRGSLENTLGQGLKNGMKYYRDTDTPGRCF------MKKTIDM 158
Tspan18  LSAA--------ILAFIFRENLTRE---FFTKELTKHYQGNNDTDI-------FSATWNS 140
Tspan1   VAAA--------VVALVYTTMAEHF---LTLLVVPAIKKDYGSQED-------FTQVWNT 142
UP1A     CASC--------ITSYTHRDYMVSNPSLITKQMLTFYSADTDQGQE-------LTRLWDR 152
UP1B     VASC--------ITAATQQDFFTPN--LFLKQMLERYQNNSPPNNDDQWKNNGVTKTWDR 152
Tspan31  FVIS----------CSCLAINRSKQTDVINASWWVMSNK---------------TRDE 122
Tspan13  FSVS----------CACLALNQEQQGQLLEVGWNNTAS----------------ARND 121
Tspan3   --------------------VENEVDRSIQKVYKTYNGTNPDAAS---------RAIDY 115
Tspan19  VVLS--------AFIITKKEEVQQLWHDKIDFVISEYGSKDKPEDIT------KWTILNA 146
CD63     VAAA--------IAGYVFRDKVMSEFNNNFRQQMENYPKNNHT----------ASILDR 138
Tspan12  LACG----------VWTYEQEVMVPVQWSDMVTLKARMTNYGLPRYR-----WLTHAWNY 152
Tspan16  VTAA----------------------------------------------------TV 107
Tspan10  GLWG----------LAVKGSLGSDLGGPLPADPMLGLALGG----------LVVSAVSL 136
Tspan32  VQVV--------FWRLHSPTQVEDAMLDTYDLVYEQAMKGTSHVR---------RQELAA 149

Tspan9   IQAEMRCCGVTDYTDWY----------------------------PVLGENTVPDRC 169
Tspan4   IQTDFRCCGVSNYTDWF----------------------------EVYNATRVPDSC 105
CD53     IQSFLQCCGINGTSDW-----------------------------------TSGPPAS 160
CD9      IHYALNCCG-------------------------------------LAGGVEQ 161
```

```
Tspan2   FHSTFQCCG-------------------------------------------KES--SE 133
CD81     FHETLDCCGSST-------------------------------------LTALTTSV 169
Tspan8   FQEEFKCCGLVNGAADW-----------------------------GNNFQHYPELCA 173
CD82     VQAQVKCCGWVSFYNWT-----------------------------DNAELMNRPEVT 171
CD37     VQFQLRCCGWHYPQDWF-----------------------------QVLILRGNGSEA 174
Tspan7   VQRSLSCCGVQNYTNWS-----TSPYFL-----------------------EHGIPPSC 174
Tspan6   IQNTLHCCGVTDYRDWT-----DTNYYS-----------------------EKGFPKSC 177
CD151    LQQEFHCCGSNNSQDWR-----DSEWIRS-------------------QEAGGRVVPDSC 184
Tspan11  LQQDFKCCGSNSSADWQ-----HSTYILL-------------------REAEGRQVPDSC 143
Tspan5   TQEYWQCCGAFGADDWN-----LNIYFN--------------CTDSNASRERCGVPFSC 186
Tspan17  AQEYWSCCGARGPNDWN-----LNIYFN--------------CTDLNPSRERCGVPFSC 188
Tspan14  LQKANQCCGAYGPEDWD-----LNVYFN--------------CSGASYSREKCGVPFSC 186
Tspan33  GQKKFSCCGGISYKDWS-----QNMYFN--------------CSEDNPSRERCSVPYSC 189
Tspan15  VQKKFKCCGGEDYRDWS-----KNQYHD--------------CSAPGP--LACGVPYTC 185
ROM1     LQLRYHCCGRHGYKDWF-----GVQWVSSRYLDPGDRDVADRIQSNVEGLYLTDGVPFSC 216
RDS      LQIEFKCCGNNGFRDWF-----EIQWISNRYLDFSSKEVKDRIKSNVDGRYLVDGVPFSC 213
Tspan18  VMITFGCCGVNGPEDFK-FASVFRL----------------------LTL-DSEEVPEAC 176
Tspan1   TMKGLKCCGFTNYTDFE-DSPYFK--------------------------ENSAFPPFC 174
UP1A     VMIEQECCGTSGPMDWVNFTSAFRA----------------------ATPEVVFPWPPLC 190
UP1B     LMLQDNCCGVNGPSDWQKYTSAFRT----------------------ENNDADYPWPRQC 190
Tspan31  LERSFDCCGLFNLTTLY-------------------------------------QQDYDF 145
Tspan13  IQRNLNCCGFRSVN-----------------------------------------PNDT 139
Tspan3   VQRQLHCCGIHNYSDWEN-------------------------TDWFKETKNQSVPLSC 149
Tspan19  LQKTLQCCGQHNYTDWIK---------------------------NKNKENSGQVPCSC 178
CD63     MQADFKCCGAANYTDWEK-----------------------------IPSMSKNRVPDSC 169
Tspan12  FQREFKCCGVVYFTDWL-------------------------------EMTEMDWPPDSC 181
Tspan16  VLLFFPIVG------------------------------------------------- 116
Tspan10  AGYLGALCENTCLLRGFS-----------------------------------GGILAFL 161
Tspan32  IQDVFLCCGKKSPFSRLG-------------------------STEADLCQGEEAARED 183


Tspan9   CMENSQGCGR-----------------------------------NATTPLWRTGCYEKV 194
Tspan4   CLEFSESCGL-----------------------------------HAPGTWWKAPCYETV 130
CD53     CPSDRK-------------------------------------------VEGCYAKA 174
CD9      FISDICPKK----------------------------------DVLETFTVKSCPDAI 185
Tspan2   QVQPTCPK---------------------------------------ELLGHKNCIDEI 153
CD81     LKNNLCPSGS---------------------------------NIISNLFKEDCHQKI 194
Tspan8   CLDKQRPCQS---------------------------------YNGKQVYKETCISFI 198
CD82     Y--PCSCEVKGEEDNSLSVRKGFCEAP---GNRTQSGN------HPEDWPVYQEGCMEKV 220
CD37     HRVPCSCYNLSATNDSTILDKVILPQLSRLGHLARSRHSADICAVPAESHIYREGCAQGL 234
Tspan7   CMNETDCNPQD----------------------------LHNLTVAATKVNQKGCYDLV 205
Tspan6   CKLE-DCTPQ----------------------------------RDADKVNNEGCFIKV 201
CD151    CKTVVALCGQ----------------------------RDHASNIYKVEG-GCITKL 212
Tspan11  CKTVVARCGQ----------------------------RAHPSNIYKVEG-GCLTKL 171
Tspan5   CTKDPAEDVINTQCG--------------------YDARQKPEVDQQIVIYTKGCVPQF 225
Tspan17  CVRDPAEDVLNTQCG--------------------YDVRLKLELEQQGFIHTKGCVGQF 227
Tspan14  CVPDPAQKVVNTQCG--------------------YDVRIQLKSKWDESIFTKGCIQAL 225
Tspan33  CLPTPDQAVINTMCG--------------------QGMQAFDYLEASKVIYTNGCIDKL 228
Tspan15  CIRN-TTEVVNTMCG--------------------YKTIDKERFSVQDVIYVRGCTNAV 223
ROM1     CNPHSPRPCLQNRLSD------------------SYAHPLFDPRQPNQNLWAQGCHEVL 257
RDS      CNPSSPRPCIQYQITN------------------NSAHYSYDHQTEELNLWVRGCRAAL 254
Tspan18  CRREPQSRDG------------------------VLLSREECLLGRSLFLNKQGCYTVI 211
Tspan1   CNDNVTN----------------------------TANETCTKQKAHDQKVEGCFNQL 204
UP1A     CRRTGNFIP--------------------------LNEEGCRLGHMDYLFTKGCFEHI 222
UP1B     CVMNNLKEP--------------------------LNLEACKLGVPGFYHNQGCYELI 222
Tspan31  CTAICKSQSP-----------------------------------------TCQMCGEKF 164
Tspan13  CLASCVKSDH-------------------------------------------SCSPCAPII 158
Tspan3   CRETASNCNG---------------------------------SLAHPSDLYAEGCEALV 176
Tspan19  TKSTLRKWFCD--------------------------------EPLNATYLEGCENKI 204
```

```
CD63     CINVTVGCGIN---------------------------------FNEKAIHKEGCVEKI 195
Tspan12  CVREFPGCSK-----------------------------QAHQEDLSDLYQEGCGKKM 210
Tspan16  ------------------------------------------------DVALEHTFVTL 127
Tspan10  VLEAVAGALVVALWG-----------------------------PLQDSLEHTLRVAI 190
Tspan32  CLQGIRSFLR-------------------------------THQQVASSLTSIGLALTL 211


Tspan9   KMWFD--DNKHVLGTVGMCILIMQILGMAFSMTLFQHIHRTGKKYDA------------ 239
Tspan4   KVWLQ--ENLLAVGIFGLCTALVQILGLTFAMTMYCQVVKA-DTYCA------------ 174
CD53     RLWFH--SNFLYIGIITICVCVIEVLGMSFALTLNCQIDKTSQTIGL------------ 219
CD9      KEVFD--NKFHIIGAVGIGIAVVMIFGMIFSMILCCAIRRNREMV-------------- 228
Tspan2   ETIIS--VKLQLIGIVGIGIAGLTIFGMIFSMVLCCAIRNSRDVI-------------- 196
CD81     DDLFS--GKLYLIGIAAIVVAVIMIFEMILSMVLCCGIRNSSVY---------------- 236
Tspan8   KDFLA--KNLIIVIGISFGLAVIEILGLVFSMVLYCQIGNK------------------ 237
CD82     QAWLQ--ENLGIILGVGVGVAIIELLGMVLSICLCRHVHSEDYSKVPKY----------- 267
CD37     QKWLH--NNLISIVGICLGVGLLELGFMTLSIFLCRNLD-HVYNRLARYR---------- 281
Tspan7   TSFME--TNMGIIAGVAFGIAFSQLIGMLLACCLSRFITANQYEMV-------------- 249
Tspan6   MTIIE--SEMGVVAGISFGVACFQLIGIFLAYCLSRAITNNQYEIV-------------- 245
CD151    ETFIQ--EHLRVIGAVGIGIACVQVFGMIFTCCLYRSLKLEHY----------------- 253
Tspan11  EQFLA--DHLLLMGAVGIGVACLQICGMVLTCCLHQRLQRHFY----------------- 212
Tspan5   EKWLQ--DNLTIVAGIFIGIALLQIFGICLAQNLVSDIEAVRASW-------------- 268
Tspan17  EKWLQ--DNLIVVAGVFMGIALLQIFGICLAQNLEQ-ME-------------------- 263
Tspan14  ESWLP--RNIYIVAGVFIAISLLQIFGIFLARTLISDIEAVKAGHHF------------ 270
Tspan33  VNWIH--SNLFLLGGVALGLAIPQLVGILLSQILVNQIKDQIKLQLYNQQHRADPWY--- 283
Tspan15  IIWFM--DNYTIMAGILLGILLPQFLGVLLTLLYITRVEDIIMEHSVTDGLLGPGAKPSV 281
ROM1     LEHLQ--DLAGTLGSMLAVTFLLQALVLLGLRYLQTALEGLGGVIDAGGETQGYLFPSGL 315
RDS      LSYYS--SLMNSMGVVTLLIWLFEVTITIGLRYLQTSLDGVSNPEESESESQGWLLERSV 312
Tspan18  LNTFE--TYVYLAGALAIGVLAIELFAMIFAMCLFRGIQ--------------------- 248
Tspan1   LYDIR--TNAVTVGGVAAGIGGLELAAMIVSMYLYCNLQ--------------------- 241
UP1A     GHAID--SYTWGISWFGFAILMWTLPVMLIAMYFYTML---------------------- 258
UP1B     SGPMN--RHAWGVAWFGFAILCWTFWVLLGTMFYWSRIEY------------------- 260
Tspan31  LKHSD--EALKILGGVGLFFSFTEILGVWLAMRFRNQKDPRANPSAFL------------ 210
Tspan13  GEYAG--EVLRFVGGIGLFFSFTEILGVWLTYRYRNQKDPRANPSAFL------------ 204
Tspan3   VKKLQ--EIMMHVIWAALAFAAIQLLGMLCACIVLCRRSRDPAYELLITGGTYA------ 228
Tspan19  SAWYN--VNVLTLIGINFGLLTSEVFQVSLTVCFFKNIKNIIHAEM------------- 248
CD63     GGWLR--KNVLVVAAAALGIAFVEVLGIVFACCLVKSIRSGYEVM--------------- 238
Tspan12  YSFLRGTKQLQVLRFLGISIGVTQILAMILTITLLWALYYDRREPGTDQMLSLKNDTSQH 270
Tspan16  RKNYR-----GYNEPDDYSTQWNLVMEKGLSKYFFSSL---------------------- 160
Tspan10  AHYQDDPDLRFLLDQVQLGLRCCGAASYQDWQQNLYCLSSPCSSQ--------------- 235
Tspan32  GPQGQIHPDPTSMWPPAPGAQPLEMLPGWTHTLSPLRSSCYWSKRMLG------------ 259


Tspan9   ------------------------------------
Tspan4   ------------------------------------
CD53     ------------------------------------
CD9      ------------------------------------
Tspan2   ------------------------------------
CD81     ------------------------------------
Tspan8   ------------------------------------
CD82     ------------------------------------
CD37     ------------------------------------
Tspan7   ------------------------------------
Tspan6   ------------------------------------
CD151    ------------------------------------
Tspan11  ------------------------------------
Tspan5   ------------------------------------
Tspan17  ------------------------------------
Tspan14  ------------------------------------
Tspan33  ------------------------------------
```

```
Tspan15  EAAGTGCCLCYPN---------------------- 294
ROM1     KDMLKTAWLQGGVACRPAPEEAPPGEAPPKEDLSEA 351
RDS      PETWKAFLESVKKLGKGNQVEAEGADAGQAPEAG-- 346
Tspan18  ------------------------------------
Tspan1   ------------------------------------
UP1A     ------------------------------------
UP1B     ------------------------------------
Tspan31  ------------------------------------
Tspan13  ------------------------------------
Tspan3   ------------------------------------
Tspan19  ------------------------------------
CD63     ------------------------------------
Tspan12  LSCHSVELLKPSLSRIFEHTSMANSFNTHFEMEEL- 305
Tspan16  ------------------------------------
Tspan10  ------------------------------------
Tspan32  ------------------------------------
```

Appendix 2 Tspan18 signalling is not affected by DMSO

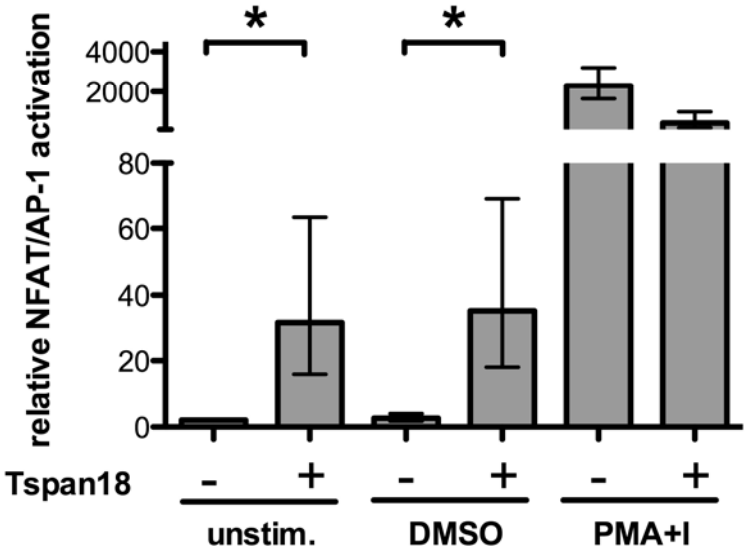


Figure Appendix 2: DT40 cells were transfected with 20 μg of Tspan18 (or control vector), and 20̃ μg of NFAT/AP-1 promoter. Cells were left unstimulated, or were treated with 0.31 μM DMSO or stimulated with PMA and ionomycin. Data were normalized for the relative β-gal value. Error bars represent the standard deviation of three independent experiments. Unstimulated and DMSO samples (both transfected with empty vector or Tspan18) were analysed by ANOVA and Tukey's test, which revealed no significant differences. This experiment was important because certain inhibitors in this thesis were dissolved in DMSO to yield a 0.31 μM final concentration of DMSO.

Appendix 3 siRNA Tspan18 knockdown in HUVEC cells

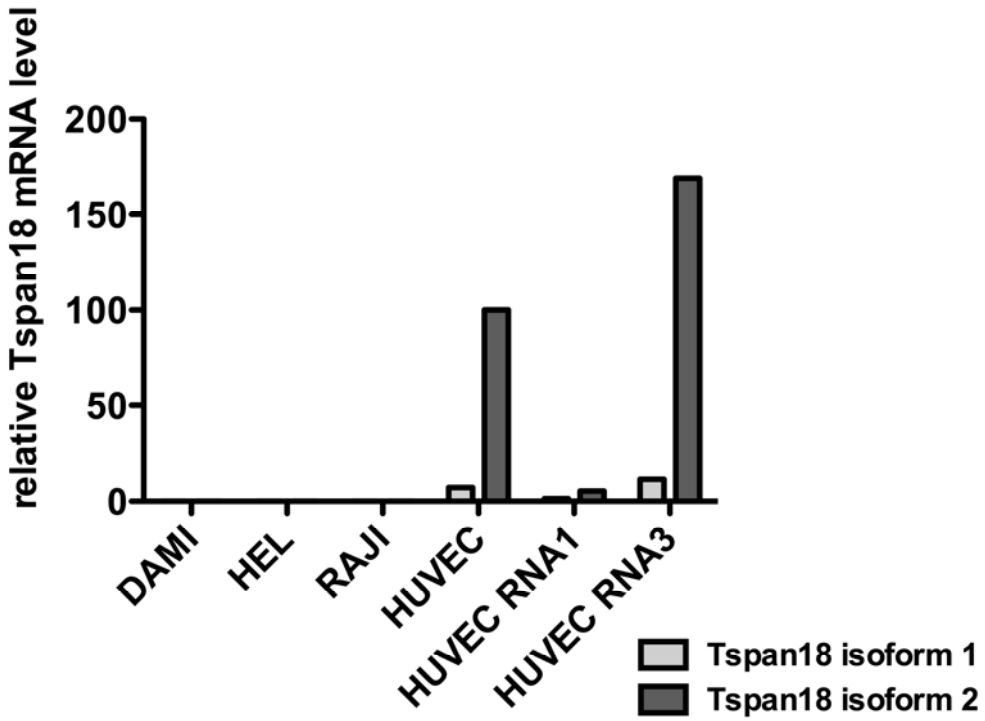


Figure Appendix 3: DAMI, HEL, RAJI and HUVEC cells were tested by qRT-PCR for Tspan18 isoform1 and Tspan18 isoform 2 expression. Three days prior to qRT-PCR analysis, HUVEC cells were transfected with two different Tspan18 siRNA Stealth duplexes from Invitrogen (RNA1 and RNA3), using RNAiMAX transfection reagent (Invitrogen). One tested siRNA duplex successfully inhibited Tspan18 isoform 2 expression by 90% (HUVEC RNA1). This figure is courtesy of Dr Mike Tomlinson, who performed this work.

Appendix 4 Tspan18 isoform 1 is not detected by PCR from HUVEC cDNA

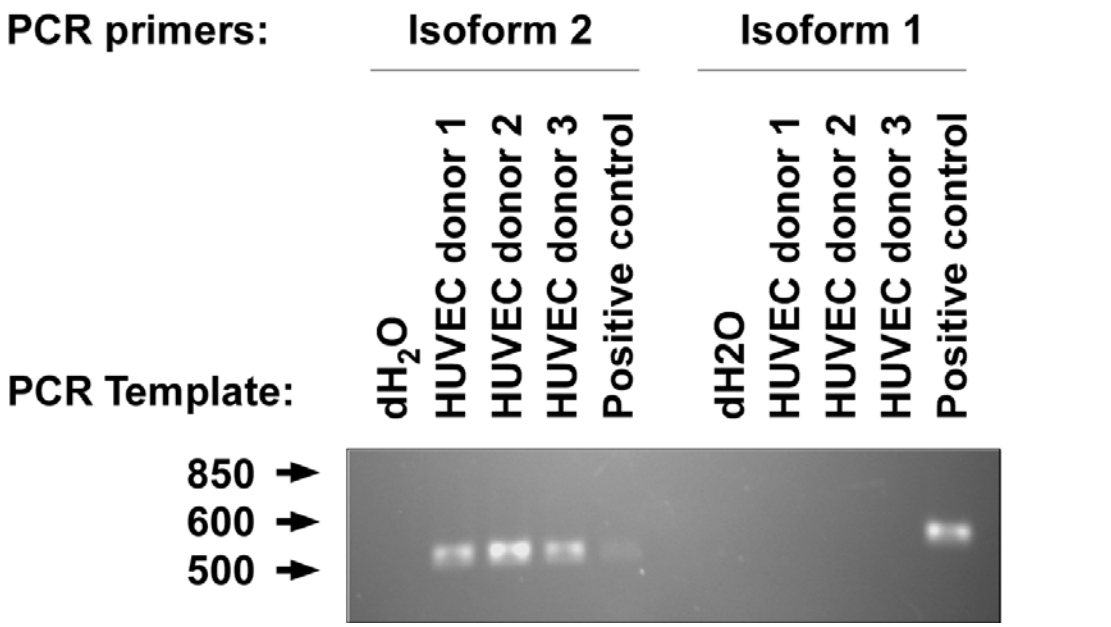


Figure Appendix 4: cDNA from three different donors was tested for the presence of Tspan18 isofom 1 and isoform 2 by PCR. The positive control lanes represent PCR reactions from isoform 1 and 2 cDNA constructs. This experiment is important because it includes a positive control for isoform 1, which was not included in Figure 4.5B. This figure is courtesy of Dr Mike Tomlinson, who performed this work.

.

Appendix 5 Identification of Tspan9 as a novel platelet tetraspanin and the collagen receptor GPVI as a component of tetraspanin microdomains

BJ www.biochemj.org

Biochem. J. (2009) 417, 391–400 (Printed in Great Britain) doi:10.1042/BJ20081126

Identification of Tspan9 as a novel platelet tetraspanin and the collagen receptor GPVI as a component of tetraspanin microdomains

Majd B. PROTTY*, Nicholas A. WATKINS†, Dario COLOMBO*, Steven G. THOMAS*, Victoria L. HEATH*, John M. J. HERBERT*, Roy BICKNELL*, Yotis A. SENIS*, Leonie K. ASHMAN‡, Fedor BERDITCHEVSKI§, Willem H. OUWEHAND†‖, Steve P. WATSON* and Michael G. TOMLINSON*[1]

*Centre for Cardiovascular Sciences, Institute of Biomedical Research, The Medical School, University of Birmingham, Birmingham B15 2TT, U.K., †Department of Haematology, University of Cambridge and National Health Service Blood and Transplant, Cambridge CB2 2PT, U.K., ‡School of Biomedical Sciences, University of Newcastle, Callaghan, NSW 2308, Australia, §Cancer Research U.K. Institute for Cancer Studies, University of Birmingham, Birmingham B15 2TT, U.K., and ‖Wellcome Trust Sanger Institute, Hinxton, Cambridge CB10 1SA, U.K.

Platelets are essential for wound healing and inflammatory processes, but can also play a deleterious role by causing heart attack and stroke. Normal platelet activation is dependent on tetraspanins, a superfamily of glycoproteins that function as 'organisers' of cell membranes by recruiting other receptors and signalling proteins into tetraspanin-enriched microdomains. However, our understanding of how tetraspanin microdomains regulate platelets is hindered by the fact that only four of the 33 mammalian tetraspanins have been identified in platelets. This is because of a lack of antibodies to most tetraspanins and difficulties in measuring mRNA, due to low levels in this anucleate cell. To identify potentially platelet-expressed tetraspanins, mRNA was measured in their nucleated progenitor cell, the megakaryocyte, using serial analysis of gene expression and DNA microarrays. Amongst 19 tetraspanins identified in megakaryocytes, Tspan9, a previously uncharacterized tetraspanin, was relatively specific to these cells. Through generating the first Tspan9 antibodies, Tspan9 expression was found to be tightly regulated in platelets. The relative levels of CD9, CD151, Tspan9 and CD63 were 100, 14, 6 and 2 respectively. Since CD9 was expressed at 49 000 cell surface copies per platelet, this suggested a copy number of 2800 Tspan9 molecules. Finally, Tspan9 was shown to be a component of tetraspanin microdomains that included the collagen receptor GPVI (glycoprotein VI) and integrin $\alpha 6\beta 1$, but not the von Willebrand receptor GPIbα or the integrins αIIb$\beta 3$ or $\alpha 2\beta 1$. These findings suggest a role for Tspan9 in regulating platelet function in concert with other platelet tetraspanins and their associated proteins.

Key words: glycoprotein VI (GPVI), megakaryocyte, membrane microdomain, platelet, tetraspanin, Tspan9.

INTRODUCTION

Platelets play an essential role in preventing excessive blood loss at sites of vascular injury by plugging holes in damaged blood vessels and promoting the clotting cascade. Platelets are also important in inflammation through recruitment of leukocytes, and in blood vessel repair through the release of growth factors. However, platelets also play a deleterious role in causing heart attack and stroke when activated in diseased vessels [1]. Critical to these events are a variety of well-characterized surface receptor proteins which combine to induce powerful platelet activation when they encounter exposed sub-endothelial matrix proteins in the damaged vessel wall [2]. It is becoming increasingly clear that such cell surface proteins and their associated signalling proteins are not randomly distributed in the plasma membrane, but are instead compartmentalized into membrane microdomains [3].

The tetraspanins are a large family of four-transmembrane glycoproteins that are thought to function as 'molecular organizers' by forming a type of membrane microdomain that is distinct from lipid rafts [4–6]. This is achieved through tetraspanin–tetraspanin associations and specific tetraspanin interactions with so-called partner proteins, which include integrins, immunoglobulin superfamily proteins and G-protein-coupled receptors. Such a fundamental organizing role is consistent with tetraspanin expression throughout the fungi, plant and animal kingdoms and their essential roles in processes such as cell adhesion, motility and signal transduction [4].

An essential role for tetraspanins in platelets was demonstrated by mild bleeding phenotypes in mice deficient for either of the tetraspanins CD151 or Tspan32 (previously named TSSC6 or PHEMX) [7,8]. These similar phenotypes appear to be due to impaired signalling by the major platelet integrin αIIb$\beta 3$, but their weak nature suggests functional redundancy between tetraspanins. However, only two other tetraspanins, CD9 and CD63, have been identified on platelets using antibodies [9]. Identification of other platelet tetraspanins is impaired by a lack of antibodies to most of the 33 mammalian tetraspanins. Additionally, the anucleate platelet has minimal mRNA levels [10], which makes RT–PCR (reverse transcription–PCR) challenging due to the issue of contamination with mRNA from other blood cells.

The present study aimed to identify a novel platelet tetraspanin through genomic analyses of megakaryocytes, the platelet progenitors, and subsequent antibody generation to confirm expression on platelets.

MATERIALS AND METHODS

Genomics

The SAGE (serial analysis of gene expression) library from primary mouse megakaryocytes was generated using the I-SAGE

Abbreviations used: DIC, differential interference contrast; ECL, enhanced chemiluminescence; GP, glycoprotein; HEK-293T cells, HEK (human embryonic kidney)-293 cells expressing the large T-antigen of SV40 (simian virus 40); PNGase F, peptide N-glycosidase F; RT–PCR, reverse transcription–PCR; SAGE, serial analysis of gene expression.

[1] To whom correspondence should be addressed (email m.g.tomlinson@bham.ac.uk).

Long Kit (Invitrogen) as described previously [11]. SAGE tags were identified using SAGE2000 4.5 Analysis Software (Invitrogen) and each tetraspanin tag was confirmed manually by referring to GenBank entries in the National Center for Biotechnology Information public database. The HaemAtlas microarray data was generated as described [12] from monocytes ($CD14^+$), B-cells ($CD19^+$), helper T-cells ($CD4^+$), natural killer cells ($CD56^+$), granulocytes ($CD66b^+$) and cytotoxic T cells ($CD8^+$) isolated from seven human volunteers, and erythroblasts and megakaryocytes differentiated from $CD34^-$ haematopoietic stem cells.

Cell culture

The HEK-293T [HEK (human embryonic kidney)-293 cells expressing the large T-antigen of SV40 (simian virus 40)] cell line was cultured in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10% fetal bovine serum, penicillin, streptomycin, and glutamine. The DG75 and Raji human B-cell lines, the HPB-ALL and Jurkat human T-cell lines, the DAMI and MEG-01 human megakaryocyte-like cell lines, and the HEL human erythroleukaemia line, were all cultured in RPMI supplemented with 10% fetal bovine serum, penicillin, streptomycin, and glutamine.

FLAG-tagged tetraspanin constructs

Tetraspanin cDNAs were generated by PCR from the following sources: DAMI human megakaryocyte-like cell line cDNA for CD9 and CD151; specific cDNA for CD63 [13]; and mouse primary megakaryocyte cDNA for Tspan9 [11]. These were cloned into the pEF6/Myc-His vector (Invitrogen) which had been modified to include an in-frame, upstream sequence for the FLAG epitope tag. The endogenous stop codons were retained for each tetraspanin construct to prevent translation of the Myc and His epitope tags.

Antibodies

The mouse negative control monoclonal antibody (MOPC 21) and the rabbit anti-FLAG antibody were from Sigma, while the normal rabbit control IgG was from Upstate. The mouse monoclonal antibodies to human tetraspanins CD9 (C9-BB and 1AA2) [14,15], CD63 (6H1 and 12F12) [16,17] and CD151 (11B1) [15] and to human GPVI (glycoprotein VI) (204-11) [18] and GPIbα (WM23) [19], were as described, whereas those to integrins αIIb (SZ22), α2 (Gi9) and α6 (4F10) were from Beckman Coulter, Immunotech and Serotec respectively.

Generation of Tspan9 polyclonal antibodies

A peptide with the sequence QHIHRTGKKYDA, corresponding to the C-terminus of human and mouse Tspan9, conjugated to keyhole limpet haemocyanin via an additional N-terminal cysteine residue, was used to immunize one rabbit and one chicken by Eurogentec. IgY was purified from chicken yolks using the Pierce Eggcellent Chicken IgY Purification Kit (Perbio). Rabbit and chicken anti-Tspan9 antibodies were then peptide-purified using the Pierce SulfoLink Kit according to the manufacturer's protocol (Perbio).

Confocal microscopy

Fetal liver mouse megakaryocytes were prepared and cultured as described [20], and mature megakaryocytes were collected after five days by passing the culture over a bovine serum albumin step gradient. Megakaryocytes were allowed to spread for two hours on coverslips coated with 100 μg/ml fibrinogen. HEK-293T cells were allowed to adhere to glass coverslips prior to calcium phosphate transfection with FLAG-tagged Tspan9, and incubated for 24 h. Both megakaryocytes and HEK-293T cells were fixed and prepared for fluorescence microscopy as described previously [21]. The rabbit anti-Tspan9 antibody was used at a concentration of 2 μg/ml. Images were acquired using a DMIRE2 Leica confocal microscope (Leica) with a 63× oil immersion 1.40 NA (numerical aperture) plan-apochromat oil immersion lens. Images were captured using Leica Confocal Software and offline analysis was performed with Adobe Photoshop.

Quantification of tetraspanins in human platelets

Tetraspanins were quantified using six human donors: three males of Caucasian, Chinese and Indian origin, and three females of Arabic, Caucasian and Japanese origin. Washed platelets were prepared as previously described [22], but with the modification that platelets were taken from the top third of the platelet-rich plasma to ensure that they were essentially free of other blood cells. Consent was obtained from each donor and platelet preparation was carried out in accordance with the Declaration of Helsinki (2000) of the World Medical Association, and was approved by the University of Birmingham Ethics Committee.

The number of copies of CD9 per platelet cell surface was determined using the anti-CD9 monoclonal antibody 1AA2 [15] and the Platelet Calibrator Kit (Biocytex), according to the manufacturer's instructions. The relative expression levels of Tspan9 compared with CD9, CD63 and CD151 in platelets were determined using a quantitative Western blotting method that we have used previously to quantify protein tyrosine kinases in lymphocytes [23,24]. Reference samples of FLAG-tagged forms of each tetraspanin were first generated by transiently transfecting each construct into HEK-293T cells using the calcium phosphate protocol, lysing the cells two days later with 1% Nonidet P40 and 1% dodecylmaltoside lysis buffer (the latter to fully solubilize lipid rafts) [1% Nonidet P40, 1% dodecylmaltoside, 10 mM Tris/HCl, pH 7.5, 150 mM NaCl, 0.01% sodium azide, 1 mM EDTA, 200 μg/ml 4-(2-amino ethyl)-benzenesulfonyl fluoride hydrochloride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 μg/ml pepstatin A, 10 mM NaF and 2 mM sodium orthovanadate], immunoprecipitating with mouse anti-FLAG (M2) agarose beads (Sigma), washing with stringent RIPA buffer [1% Nonidet P40, 1% deoxycholic acid, 0.1% SDS, 10 mM Tris/HCl, pH 7.5, 150 mM NaCl, 0.01% sodium azide, 1 mM EDTA, 200 μg/ml 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 μg/ml pepstatin A, 10 mM NaF and 2 mM sodium orthovanadate] to remove associated proteins, Western blotting under non-reducing conditions with rabbit anti-FLAG (Sigma), and quantifying the bands using ECL (enhanced chemiluminescence) in combination with the GeneGnome quantitative Western blotting system (Syngene). Specific FLAG-tetraspanin reference samples and human platelet lysates, lysed as for the HEK-293T cells, were then Western blotted under non-reducing conditions using tetraspanin-specific antibodies (C9-BB for CD9, 6H1 for CD63, 11B1 for CD151 and rabbit anti-Tspan9), and the bands were quantified using the GeneGnome. Finally, the relative expression levels of each tetraspanin were calculated by comparing the anti-FLAG and anti-tetraspanin quantification data, with the expression of CD9 arbitrarily set to 100.

Platelet biotinylation and biochemical analyses

Washed human platelets were suspended at a concentration of 5×10^8 per ml in PBS containing 1 mg/ml sulfo-NHS-SS-biotin

(Perbio) and gently rotated for 30 min at room temperature (20 °C). The biotinylation reaction was quenched with 100 mM glycine in PBS, and the platelets pelleted and washed once more with PBS. Platelets (5×10^8) were lysed on ice in 1 ml of either 1 % Brij 97 (containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$) lysis buffer or 1 % Triton X-100 lysis buffer (containing 1 mM EDTA); both lysis buffers otherwise contained 10 mM Tris/HCl, pH 7.5, 150 mM NaCl, 0.01 % sodium azide, 200 μg/ml 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 μg/ml pepstatin A, 10 mM NaF and 2 mM sodium orthovanadate. Lysates were pre-cleared at 4 °C for 30 min each with 10 μl Protein G Sepharose (Sigma) followed by addition of 10 μl Protein G Sepharose pre-coupled with 1 μg of either MOPC mouse or normal rabbit control Ig. Pre-cleared lysates were immunoprecipitated at 4 °C for 90 min with specific antibodies (1AA2 for CD9, 11B1 for CD151, rabbit anti-Tspan9, 4F10 for α6 and Gi9 for α2), pre-coupled to 10 μl Protein G Sepharose, and washed four times with lysis buffer on ice. Non-reducing samples were separated on 4–12 % gradient gels (Invitrogen) for blotting with IRDye 800CW-conjugated streptavidin (LI-COR). The blots were visualized using the Odyssey Infrared Imaging System (LI-COR).

Co-immunoprecipitations using non-biotinylated platelets were performed as described above, with the exception that Western blots were visualized using Western Lightning chemiluminescence reagents (Perkin Elmer) and Hyperfilm (Amersham Biosciences), which was developed using a Compact X4 film processor (Xograph Imaging Systems).

Cleavage of N-linked oligosaccharides from Tspan9 *in vitro* was performed using PNGase F (peptide N-glycosidase F) according to the manufacturer's protocol (New England BioLabs).

RESULTS

Primary mouse megakaryocytes express mRNA for 19 tetraspanins

A total of 33 tetraspanins have been identified in the human and mouse genomes, but only four have been identified as platelet-expressed using specific antibodies, namely CD9, CD63, CD151 and Tspan32 [9]. This is primarily due to a lack of antibodies and to the technical difficulties in performing genomics in the platelet [10]. To identify tetraspanins that are potentially expressed in platelets, we examined mRNA expression in their nucleated precursor cell, the megakaryocyte, through re-analysis of our previously reported SAGE library [11]. SAGE provides a quantitative measure of relative mRNA levels by essentially converting each mRNA into a short piece of DNA, termed a SAGE tag, which can be ligated with other tags and readily sequenced [25]. SAGE identified 16 tetraspanins in primary mouse megakaryocytes (Figure 1). The expression of each was confirmed by RT–PCR, which also identified an additional three tetraspanins (CD82, Tspan5 and Tspan18) that fell below the threshold of detection in our SAGE library (results not shown). The most abundant tetraspanin was CD63, followed by CD9 and CD151, each of which is known to be expressed in platelets. The other platelet tetraspanin, Tspan32, was also relatively highly expressed in megakaryocytes. Of the other 15 tetraspanins identified, most have not been reported as megakaryocyte-expressed and remain unstudied at the protein level.

Tspan9 mRNA is relatively specific to human megakaryocytes versus other haematopoietic cells

Since SAGE is quantitative, different SAGE libraries can be compared with each other to identify cell type-specific genes.

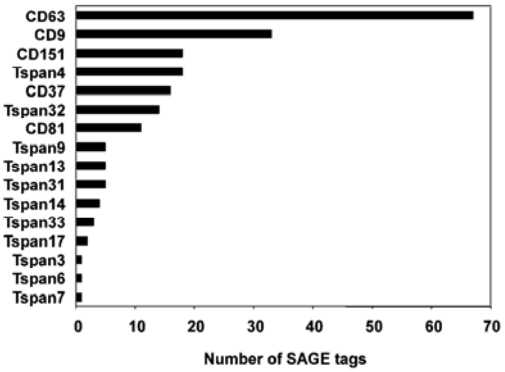


Figure 1 Tetraspanin expression in primary mouse megakaryocytes

A LongSAGE library was generated from mouse megakaryocytes grown *in vitro* from bone marrow precursors [11]. The bar chart shows the number of SAGE tags, for those tetraspanins that were identified, out of 53046 total tags.

We have previously performed such comparisons to show that the most megakaryocyte-specific genes tend to be important for platelet function [11]. Similar analyses of tetraspanins, however, revealed that none were specific to megakaryocytes (results not shown), although the fact that nine of the tetraspanins had only five tags or less made it difficult to determine statistically whether any of these were over-represented in megakaryocytes compared with other cell types. To address this issue, we used our blood cell expression atlas data [12] to compare tetraspanin mRNA expression in megakaryocytes with seven other haematopoietic cell types. The Illumina HumanWG-6 v2 Expression BeadChips, used in this study, is a recently developed bead-based DNA microarray that largely overcomes the problems of intra- and inter-array reproducibility associated with earlier microarray types [26]. Interestingly, the tetraspanins that were more highly expressed in megakaryocytes than other cell types were the known platelet tetraspanins CD9, CD63, CD151 and Tspan32, as well as Tspan4 and Tspan9 (Supplementary Figure 1, at http://www.BiochemJ.org/bj/417/bj4170391add.htm). The most megakaryocyte-specific of these was Tspan9 (Figure 2), a functionally unstudied tetraspanin that was originally named NET-5 [27], and which we and others had identified as a candidate platelet tetraspanin using proteomic analyses of the human platelet surface [11,28] and DNA microarray analyses of human megakaryocytes [29]. Moreover, Tspan9 is remarkably highly conserved during evolution, with amino acid identities of 97 % for human compared with mouse and 91 % for human compared with frog, suggesting that this tetraspanin may be functionally important in megakaryocyte and platelet biology.

Tspan9 protein is expressed in megakaryocytes and platelets

To determine whether Tspan9 is indeed a new platelet tetraspanin, we generated the first antibodies to this protein in the form of rabbit and chicken polyclonal antisera to the C-terminal cytoplasmic tail of Tspan9, a region that is identical in amino acid sequence between human and mouse. The antisera were peptide-purified and their efficacy confirmed by Western blotting lysates of HEK-293T cells transiently transfected with mouse Tspan9 (Figure 3A). The multiple bands detected in the Western blot of transfected Tspan9 are likely to represent immature glycoforms

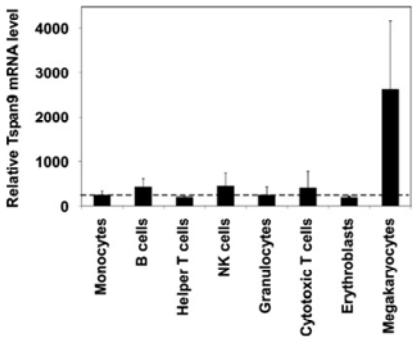


Figure 2 Tspan9 is relatively specific to human megakaryocytes when compared with other haematopoietic cell types

Whole genome expression data was obtained for eight different human blood cells using Illumina HumanWG-6 v2 Expression BeadChips. Mean normalized intensity values are shown, with error bars representing S.E.M from either 4 or 7 replicates. The dashed line represents the cut-off for present/absent.

that may be relatively abundant in an over-expression system. Indeed, Tspan9 has a single predicted N-linked glycosylation site, and treatment of Tspan9 immunoprecipitates with an N-glycosidase reduced the banding pattern to a single species (Figure 3B). Moreover, N-glycosidase treatment of Tspan9 immunoprecipitates from human platelet lysates confirmed that Tspan9 is a glycoprotein that is expressed in this cell type (Figure 3B). Confocal microscopy analysis of Tspan9-transfected cells, in which not all of the cells were positive due to the transient nature of the transfection, suggested a predominantly plasma membrane localization for Tspan9 (Figure 3C).

The Tspan9 antibody was used to Western blot whole cell lysates of human platelets and a panel of haematopoietic cell lines which had been normalized for protein content (Figure 3D). Tspan9 was readily detected in platelets but not in the other cell types, including the megakaryocyte-like cell lines DAMI and MEG-01, suggesting that Tspan9 is predominantly restricted to primary cells of this lineage (Figure 3D). Tspan9 was similarly detected in mouse platelet lysates, as well as in lung, with relatively weak expression in brain, kidney, liver and spleen (Figure 3E). Bone marrow, a major site for megakaryocytopoiesis, was positive upon longer exposure (results not shown), but its weak signal was probably due to the relatively low numbers of megakaryocytes in this tissue. Indeed, expression of Tspan9 protein in primary mouse megakaryocytes (Figure 3F), and the proplatelet structures that they form (Figure 3G), was confirmed by fluorescence microscopy, a technique that was used because of difficulties in obtaining enough mature megakaryocytes for Western blotting. Tspan9 staining throughout the megakaryocyte suggests localization to the demarcation membrane system, which is an extensive network of membrane channels that is connected to, and is thought to originate from, the plasma membrane [30].

Quantification of tetraspanins in human platelets

Quantification of Tspan9 expression levels on platelets may provide clues as to potential binding partners, which may be expressed at similar levels. The human platelet cell surface is relatively well characterized with respect to copy numbers of the major glycoproteins, but amongst tetraspanins only CD9 has been quantified. Three different groups, using iodinated CD9

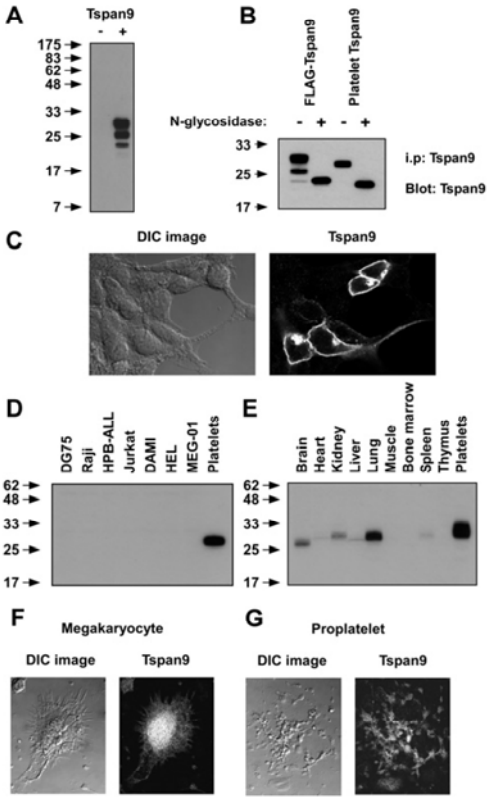


Figure 3 Generation of a new antibody identifies the Tspan9 glycoprotein as a novel megakaryocyte/platelet tetraspanin

(**A**) HEK-293T cells were transiently transfected with empty vector (−) or FLAG-tagged mouse Tspan9 (+) and whole cell lysates were Western blotted with the peptide-purified rabbit anti-Tspan9 antibody. Essentially identical data were generated with the chicken anti-Tspan9 antibody (results not shown). (**B**) FLAG-tagged mouse Tspan9, transiently transfected into HEK-293T cells, and endogenous Tspan9 from human platelets, were immunoprecipitated with rabbit anti-Tspan9 antibody and subjected to control (−) or N-glycosidase (+) treatment with the enzyme PNGase F. Samples were analysed by Western blotting with chicken anti-Tspan9 antibody. (**C**) HEK-293T cells were transiently transfected with FLAG-tagged mouse Tspan9, fixed, permeabilized and analysed by fluorescence confocal microscopy with the rabbit anti-Tspan9 antibody. The left panel identifies the cells by DIC (differential interference contrast) microscopy and the right panel is a mid-plane confocal section showing Tspan9 expression in a subpopulation of the cells. (**D**) Whole cell lysates of the human cell lines DG75 and Raji (B-cells), HPB-ALL and Jurkat (T-cells), DAMI (megakaryocyte-like), HEL (erythroleukaemia) and MEG-01 (megakaryocyte-like), and human washed platelets were normalized for protein content and then Western blotted with the rabbit anti-Tspan9 antibody. (**E**) Whole cell lysates of various mouse tissues and washed mouse platelets were normalized for protein content and then Western blotted with the rabbit anti-Tspan9 antibody. In this panel and the previous panel, essentially identical data were generated with the chicken anti-Tspan9 antibody (results not shown). (**F**) Mouse megakaryocytes and (**G**) proplatelets were generated from cultured fetal liver cells, allowed to spread on fibrinogen, fixed, permeabilized and analysed by fluorescence confocal microscopy with the rabbit anti-Tspan9 antibody. The left panel identifies the cells by DIC and the right panel shows Tspan9 staining. Control staining with normal rabbit immunoglobulin did not yield a substantial signal (results not shown).

antibodies, reported 65000 [31], 46000 [32] and 38000 [33] copies per platelet. To determine whether these discrepancies were due to donor variation, we quantified CD9 levels on the

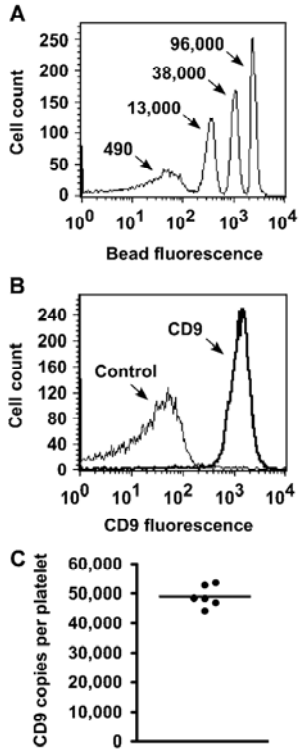


Figure 4 CD9 is expressed at 49 000 surface copies per cell on human platelets

The number of surface copies of CD9 per platelet was determined using the mouse IgG1 anti-CD9 monoclonal antibody 1AA2 and the Platelet Calibrator Kit from Biocytex. (**A**) The calibrator beads, coated with the indicated numbers of mouse IgG1 antibody molecules, were stained with FITC-conjugated anti-mouse antibody and analysed by flow cytometry. (**B**) Washed human platelets from a representative donor were stained with an IgG1 control or the CD9 monoclonal antibody, followed by FITC-conjugated anti-mouse antibody and analysed by flow cytometry. (**C**) The geometric mean fluorescence intensities from (**A**) and (**B**) were compared, to quantify CD9 on human platelets. Data are shown for six donors of diverse ethnic backgrounds.

platelets of six donors, of diverse ethnic backgrounds, using a flow cytometry-based method that we have previously used to quantify other platelet glycoproteins [34]. CD9 levels were similar between individuals, with a mean of 49 000 copies per platelet, with a S.D. of 3560 (Figure 4). This suggests that the 65 000 to 38 000 range in previously reported copy numbers [31–33] reflects differences in antibodies and methodology. Nevertheless, a copy number of approx. 50 000 ranks CD9 as a major component of the platelet cell surface, compared to the two other major glycoproteins, integrin αIIbβ3 (80 000 copies) [35] and GPIb-IX-V (25 000) [36].

Quantification of Tspan9 by the flow cytometry-based method is not readily applicable to intracellular epitopes, such as the C-terminal cytoplasmic tail of Tspan9 to which our antibody was raised. Therefore we quantified Tspan9 in human platelets relative to CD9, and other tetraspanins, using a Western blotting protocol with epitope-tagged standards, an approach we used previously to quantify protein tyrosine kinases in lymphocytes [23,24]. The platelet lysates were made in a lysis buffer that included dodecylmaltoside to fully solubilize lipid rafts, although Tspan9 and other platelet tetraspanins appear to be largely excluded from lipid rafts (results not shown and [37]). Using the same six donors as for the previous CD9 quantification (Figure 4), we found Tspan9 to be expressed at 6 % of the level of CD9, with similar expression between donors (Figure 5). In comparison, CD151 was expressed at a 2.3-fold greater level than Tspan9, whereas CD63 was 3-fold lower than Tspan9 (Figure 5). These results suggest that the Tspan9 expression level, similar to that of other tetraspanins, is tightly regulated in human platelets, and can be estimated at approximately 2800 surface copies, with a S.D. of 600, assuming the same cell surface to intracellular localization ratio for CD9 and Tspan9.

Tspan9 is a component of tetraspanin microdomains on human platelets

To determine whether Tspan9 is a component of tetraspanin microdomains, Tspan9-associated cell surface proteins were analysed following Tspan9 immunoprecipitation in two different detergents, Brij 97 and Triton X-100. In the presence of calcium and magnesium ions, 1 % Brij 97 is a relatively weak detergent that is known to largely maintain tetraspanin–tetraspanin interactions. In contrast, the more stringent detergent 1 % Triton X-100 disrupts tetraspanin–tetraspanin interactions but maintains certain tetraspanin–partner protein interactions [5,38].

As shown in Figure 6(A) (left panel), Tspan9 immunoprecipitation from Brij 97 lysates of surface biotinylated platelets contained several proteins. Similar patterns of immunoprecipitated proteins were observed in immunoprecipitations for CD9, CD151 and the tetraspanin-associated laminin-binding integrin $\alpha6\beta1$ [4] (Figure 6A, left panel). Consistent with its relatively high expression level, CD9 immunoprecipitation yielded more intense bands. As a control, the collagen-binding $\alpha2\beta1$ integrin, which is not thought to be tetraspanin-associated [4], was predominantly immunoprecipitated as a doublet corresponding to the molecular weights of the integrin $\alpha2$ and $\beta1$ chains (Figure 6A, left panel). For each immunoprecipitation in Triton X-100 (Figure 6A, upper right panel), the major bands were the immunoprecipitated proteins themselves, with some weaker bands, presumably representing relatively tightly associated proteins, detected on a longer exposure (Figure 6A, lower right panel). For Tspan9, the most prominent of these was a 43 kDa protein (p43) that was also present in CD151, but not CD9, immunoprecipitates (Figure 6A, lower right panel). A 60 kDa protein (p60) was also identified in Tspan9 and $\alpha6$ immunoprecipitates (Figure 6A, lower right panel), which corresponded to a major component of tetraspanin microdomains observed in Brij 97 immunoprecipitates (Figure 6A, left panel). These two proteins may represent novel binding partners for Tspan9.

Since Tspan9 and other tetraspanins co-immunoprecipitate a similar pattern of surface biotinylated proteins in Brij 97, this suggests that Tspan9 is a component of tetraspanin microdomains. To provide further supporting evidence for this idea, immunoprecipitations from non-biotinylated platelets were blotted for Tspan9 (Figure 6B, upper panels). In Brij 97, Tspan9 was detected in CD9, CD151 and $\alpha6$ immunoprecipitates, but not in those for control antibody or $\alpha2$, suggesting that Tspan9 is tetraspanin-associated. These interactions were not observed in more stringent Triton X-100 lysis conditions that are thought to disrupt tetraspanin–tetraspanin interactions [5,38]. Interestingly, Tspan9 was immunoprecipitated more efficiently in Triton X-100 than in Brij 97, despite similar solubilities in the two detergents

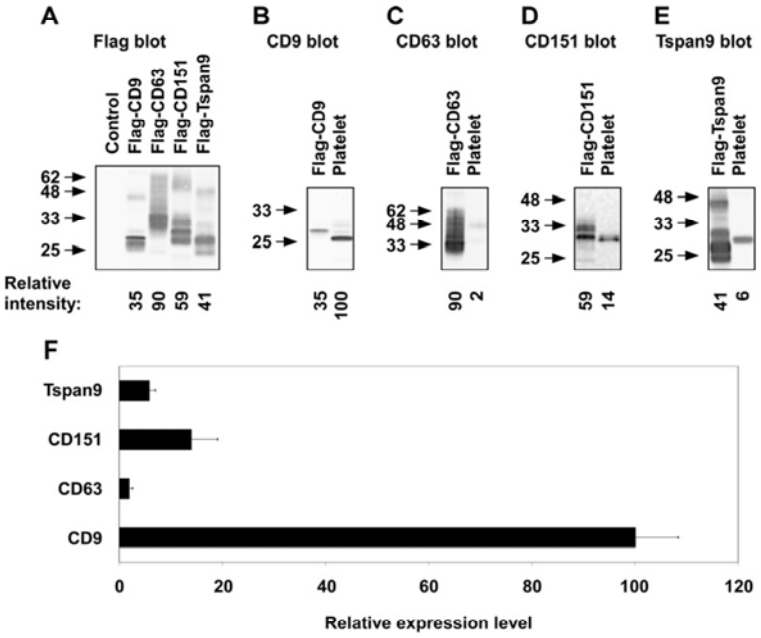


Figure 5 Quantification of Tspan9, CD63 and CD151, *relative to CD9, in human platelets*

(**A**) HEK-293T cells were transiently transfected with empty vector control, FLAG-tagged human CD9, CD63, CD151 or mouse Tspan9 (note that the Tspan9 antibody epitope is identical in protein sequence between human and mouse). Mouse anti-FLAG immunoprecipitates were Western blotted with a rabbit anti-FLAG antibody and each tetraspanin quantified, following ECL chemiluminescence, using the GeneGnome quantitative Western blotting system from Syngene. These FLAG–tetraspanin samples were then compared to whole cell lysates of washed human platelets by Western blotting for (**B**) CD9, (**C**) CD63, (**D**) CD151 and (**E**) Tspan9 and the relative intensities quantified as in (**A**). (**F**) The data in (**A**–**E**), for platelets from six human donors of diverse ethnic backgrounds, were used to quantify the relative expression levels of each tetraspanin in human platelets. The data are presented as mean relative expression levels ± S.D.

as shown by Western blotting of whole cell lysates (Figure 6B, upper panels). This result is consistent with Figure 6(A), in which a biotinylated 27 kDa protein band corresponding to Tspan9 was immunoprecipitated more efficiently in Triton X-100. These findings suggest that the epitope for the Tspan9 antibody, which is 12 amino acids long, C-terminal and membrane-proximal, is less accessible to an immunoprecipitating antibody in the context of intact tetraspanin microdomains in Brij 97, perhaps due to steric hindrance by other tetraspanins or associated proteins.

Tetraspanin microdomains on human platelets contain GPVI but not GPIbα or αIIbβ3

The identities of most of the tetraspanin-associated platelet proteins, observed in tetraspanin immunoprecipitations from Brij 97 lysates (Figure 6A, left panel), are not known, but their molecular weights suggest as candidates the major platelet glycoproteins GPIbα (the von Willebrand receptor), αIIbβ3 (the major platelet integrin) and GPVI (the main signalling receptor for collagen). However, GPIbα and αIIb were not detected in Western blots of tetraspanin immunoprecipitates (Figure 6B, middle and lower panels), suggesting that these proteins are not tetraspanin-associated. A similar experiment proved difficult for GPVI because in Western blots this protein was obscured by the immunoglobulin heavy chain from the immunoprecipitating antibodies (results not shown). To overcome this problem, GPVI immunoprecipitates were analysed for tetraspanin-associated proteins (Figure 6C) and tetraspanins themselves (Figure 6D). GPVI immunoprecipitation from Brij 97 lysates of biotinylated platelets yielded a strikingly similar pattern of biotinylated proteins to CD151 immunoprecipitation (Figure 6C). In Triton X-100, most of these interactions were lost, leaving a major 62 kDa band in GPVI immunoprecipitates and a major 27 kDa band in CD151 immunoprecipitates (Figure 6C), corresponding to the molecular weights of GPVI and CD151, respectively. To confirm that GPVI was associated with tetraspanins in these immunoprecipitation experiments, specific CD9 and CD151 blots were performed (Figure 6D). In Brij 97 lysates, both tetraspanins were detected in GPVI immunoprecipitates, but not in those for control or α2 antibodies. These interactions were lost in Triton X-100 lysates. The GPVI–tetraspanin interaction appeared to be less robust than the positive control α6β1–tetraspanin interaction, the latter of which was even detected in more stringent Triton X-100 lysates (Figure 6D), consistent with the direct and relatively strong nature of the CD151 interaction with this integrin [4]. Together these data suggest that GPVI is a novel tetraspanin-associated protein.

DISCUSSION

In the present study, we found 19 tetraspanins to be expressed in megakaryocytes and raised the first antibodies to the previously

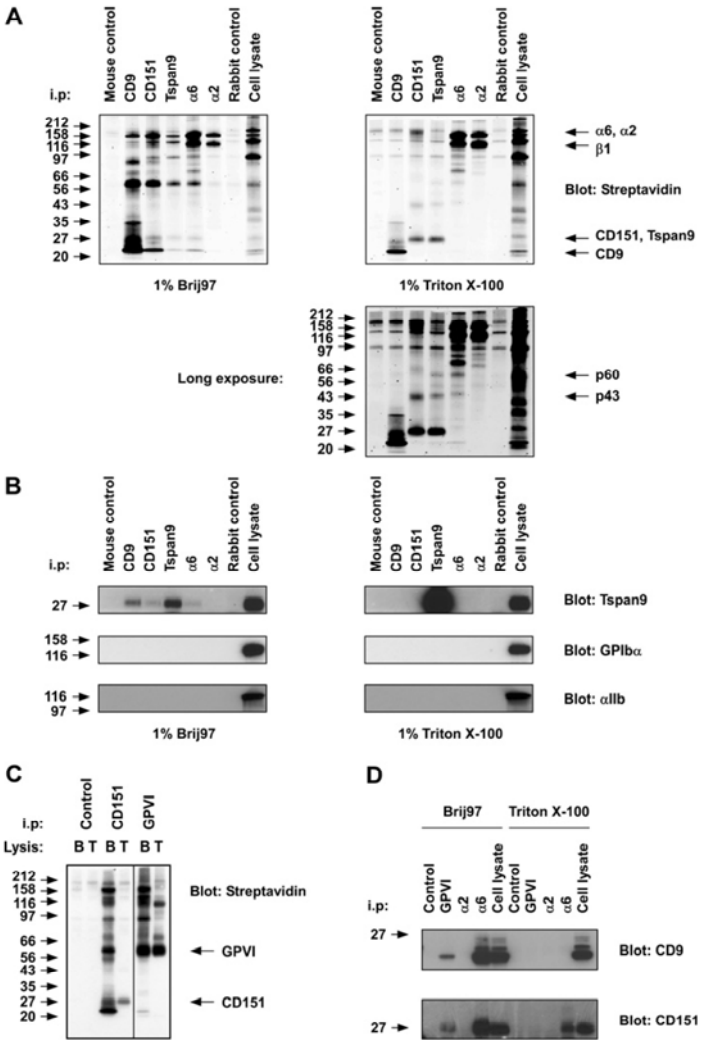


Figure 6 Tspan9 is a component of platelet tetraspanin microdomains that include GPVI and $\alpha6\beta1$, but not $\alpha IIb\beta3$, $\alpha2\beta1$ or GPIbα

(**A**) Human washed platelets were surface biotinylated, lysed in 1% Brij 97 (left panel) or more stringent 1% Triton X-100 (right panels, including a longer exposure), immunoprecipitated with the indicated antibodies and the biotinylated proteins were detected by Western blotting with streptavidin using the Odyssey Infrared Imaging System from LI-COR. The position of certain proteins is indicated by arrows. These results are representative of four experiments using different donors. (**B**) Immunoprecipitations were performed as described in (**A**), with the exception that non-biotinylated platelets were used, that Western blotting was performed with Tspan9, GPIbα and $\alpha IIb\beta$ antibodies, and that blots were visualized using ECL and film. (**C**) CD151 and GPVI immunoprecipitations were performed from 1% Brij 97 (B) or 1% Triton X-100 (T) lysates of biotinylated platelets. Biotinylated proteins were detected by Western blotting with streptavidin and visualized using chemiluminescence and film. The dividing line indicates that images were grouped from two parts of the same gel. (**D**) GPVI, $\alpha2$ and $\alpha6$ immunoprecipitations were Western blotted with CD9 and CD151 antibodies as described in (**B**).

uncharacterized tetraspanin Tspan9, which was originally named NET-5 [27]. Tspan9 protein expression was relatively specific to platelets when compared to other blood cell types. Moreover, biochemical analyses demonstrated that Tspan9 was a novel component of tetraspanin microdomains on the platelet surface.

This is the first characterization of the tetraspanin Tspan9 at the protein level through the generation of antibodies. Of the 33 tetraspanins in the human genome, antibodies have been reported for less than half. As such, we do not know the full complement of tetraspanins that are expressed in a given cell type and which

other proteins are recruited to tetraspanin microdomains. The probable reasons for the lack of antibody reagents are firstly the relatively small size of tetraspanins and their association with larger proteins in tetraspanin microdomains [39]. This may render them virtually inaccessible to antibody recognition in the context of intact cells, which are the predominant immunogens used to date for the generation of tetraspanin monoclonal antibodies. Secondly, tetraspanin protein sequences are highly conserved during evolution [40], so the number of tetraspanin non-self epitopes is very low. However, we managed to overcome these problems by raising antibodies in rabbit and chicken to the intracellular C-terminal tail of human Tspan9. Although this approach was successful for Tspan9, it is not applicable to all tetraspanins, since some have cytoplasmic tails that are predicted to contain as few as four amino acids and are therefore too short to be used as immunogens. Moreover, we have failed to raise good antibodies to Tspan3, Tspan15, Tspan17, Tspan32 and Tspan33 using this method, although an antibody that recognized both Tspan13 and Tspan31 was successfully generated (M.B.P. and M.G.T., unpublished work).

In order to identify platelet tetraspanins in the absence of antibodies, we measured mRNA levels in megakaryocytes, the platelet progenitor cells, since platelets have extremely low levels of mRNA [10]. To do this, we took advantage of a mouse megakaryocyte SAGE library that we had generated previously [11], and more recent DNA microarray analyses of human megakaryocytes and seven other haematopoietic cell types [12]. SAGE, which detects essentially all expressed genes in a quantitative manner, identified the four platelet tetraspanins, CD63, CD9, CD151 and Tspan32, amongst the six most highly expressed mouse megakaryocyte tetraspanins. We identified an additional 15 tetraspanins, at generally lower expression levels, most of which have not been reported on megakaryocytes or platelets [9], but which nevertheless may have important roles in, for example, the regulation of G protein-coupled receptors that are expressed at relatively low levels. A total of 19 tetraspanins in megakaryocytes may be typical for other cell types. For example, a figure of 20 tetraspanins has been estimated for leukocytes [41], and we have found 22 for endothelial cells upon analyses of 11 publicly-available SAGE libraries (J.M.J.H., R.B. and M.G.T., unpublished data). Unlike SAGE, the microarray data is not a reliable indicator of relative tetraspanin expression in megakaryocytes, due to the potential for differential hybridization efficiencies for different genes. However, the microarrays do enable a comparison of the expression levels of individual tetraspanins between different cell types. Those tetraspanins that have been reported to be expressed in human platelets using antibodies (CD9, CD63, CD151 and Tspan32) [9], or by mass spectrometry peptide sequencing (Tspan9, Tspan15 and Tspan33) [11,28], were relatively highly represented in human megakaryocytes. In contrast, a number of tetraspanins were not detected by either SAGE or DNA microarray, for example the CD9-related Tspan2 and the CD151-related Tspan11, suggesting that they are not expressed by the megakaryocyte/platelet lineage and thus do not compensate for CD9 and CD151 when these genes are knocked out in platelets. Finally, CD37, CD81 and Tspan4 were identified at the mRNA level in megakaryocytes but are absent at the protein level from human platelets (results not shown). It is possible that these tetraspanins are also not expressed at the protein level in megakaryocytes. Alternatively they may be megakaryocyte-expressed but actively excluded from platelets during platelet formation.

Genomic approaches, such as SAGE and DNA microarrays, do not prove expression at the protein level. However, using our new antibodies to Tspan9, we confirmed its expression on megakaryocytes and platelets by immunofluorescence microscopy, Western blotting and immunoprecipitation from surface-biotinylated cells. Consistent with its relative mRNA specificity for megakaryocytes within the haematopoietic lineage, Tspan9 was not readily detected in human B- and T-cell lines, or mouse thymus, and only weakly in spleen. Tspan9 was only detected in primary megakaryocytes and proplatelets, and not human megakaryocyte-like cell lines, highlighting that these cells are not good models for primary cells. Tspan9 is not entirely restricted to megakaryocytes and platelets, though, since expression was also detected in mouse lung, brain and kidney. In human platelets, we found that Tspan9 expression was tightly regulated between donors at a level of 6 % of CD9, 43 % of CD151 and three times the level of CD63 (this relatively low protein expression level for the latter in platelets contrasts with its high mRNA expression in megakaryocytes, for reasons that are currently unknown). Since we also showed that CD9 is the major platelet tetraspanin at 49000 cell surface copies per cell, we can estimate a Tspan9 copy number of approximately 2800 on the platelet surface, assuming that the plasma membrane to intracellular ratio is the same for each protein. Therefore it seems unlikely that the major platelet glycoproteins, such as integrin αIIbβ3 (80000 copies) [35] or GPIb-IX-V (25000 copies) [36], would be direct binding partners for Tspan9. We did detect 43 and 60 kDa proteins in relatively stringent co-immunoprecipitations with Tspan9, and we are working to identify these proteins using proteomics.

Immunoprecipitation of tetraspanins CD9, CD151 and Tspan9, and the CD151-associated integrin, $\alpha 6\beta 1$, from surface biotinylated platelets revealed a distinct pattern of proteins in the relatively weak detergent Brij 97, in which tetraspanin microdomains are thought to remain largely intact [5,38]. These proteins are likely to represent the major components of tetraspanin microdomains on the platelet cell surface. Using specific antibodies, we found that these tetraspanin-associated proteins do not include three of the major platelet glycoproteins, namely GPIb-IX-V, $\alpha 2\beta 1$ or αIIbβ3. The latter result was somewhat surprising, since αIIbβ3 was reported to co-immunoprecipitate with CD151 in HEL cells lysed in the mild detergent CHAPS [42], and with CD151 and Tspan32 in mouse platelets lysed in Triton X-100 [7,8]. However, we did identify GPVI as a novel component of tetraspanin microdomains using co-immunoprecipitation experiments with CD9 and CD151 in Brij 97. GPVI interaction with CD9 and CD151 was lost in more stringent Triton X-100 detergent, which does not preserve tetraspanin–tetraspanin interactions and hence microdomain integrity [5,38]. This suggests that either the GPVI-tetraspanin interaction is relatively weak or that GPVI interacts more strongly with an unidentified tetraspanin. If the latter is true, Tspan9 is unlikely to be the direct binding partner for GPVI because we could not detect Tspan9 in GPVI immunoprecipitates (results not shown). The functional consequences of GPVI-tetraspanin association are currently unknown. Interestingly, the antigen receptors on B- and T-lymphocytes, which signal via an ITAM (immunoreceptor tyrosine-based activation motif)-based mechanism in common with the GPVI/FcRγ complex [43], are dependent on tetraspanins for their normal function [44–48]. Particularly well characterized is the role of the tetraspanin CD81 on B-cells, where it interacts with the CD19 glycoprotein. CD19 is a signalling molecule which forms a complex with the complement receptor CD21, and is recruited to the B-cell receptor complex upon its engagement by complement-opsonized antigen. In the absence of CD81, the activated B-cell receptor fails to localize correctly to lipid raft microdomains and fails to induce sustained signalling [44,45]. Moreover, CD19 biosynthetic maturation and trafficking to the plasma membrane are impaired [49]. Our future studies will aim

to determine whether a specific platelet tetraspanin can similarly regulate GPVI maturation, trafficking and/or signal transduction.

In summary, we have shown that Tspan9 is relatively highly expressed in the megakaryocyte/platelet lineage and is a component of tetraspanin microdomains on the platelet surface. Tspan9 is remarkably highly conserved during evolution, even relative to most other tetraspanins. A feature of conserved amino acids is their involvement in intra-molecular or inter-molecular interactions [50]. This raises the possibility that almost the entire surface of Tspan9 is engaged in protein–protein interactions. Indeed, we hypothesize that Tspan9 has multiple simultaneous interacting partners and functions as an interaction 'hub' within tetraspanin microdomains. Tspan9 may therefore be essential for microdomain organization and for the function of microdomain-associated proteins on the platelet surface. The generation of Tspan9-deficient mice is in progress to test this hypothesis.

ACKNOWLEDGEMENTS

We are grateful to members of the Watson Lab for their advice and comments. We thank Michael Berndt (University College Cork, Republic of Ireland) and Masaaki Moroi (Kumme University, Japan) for providing the anti-GPIbα and GPVI mAbs respectively, Jon Frampton, John Gordon, Martin Rowe (University of Birmingham, U.K.) and Arthur Weiss (University of California at San Francsisco, U.S.A.) for gifts of cell lines, Kate Fitzpatrick-Ellis for generating the Tspan9 construct, and Alex Mazharian and Sonia Severin for helping to establish the methodology for megakaryocyte culture from fetal liver.

FUNDING

M. G. T. is supported by a MRC (Medical Research Council) New Investigator Award and a Wellcome Trust Value in People Award; D. C. holds a BHF (British Heart Foundation) Studentship; S. P. W. holds a BHF Chair; N. A. W. and W. H. O. are funded by grants from the 6th Framework Program of the European Union [grant number LSHM-CT-2004-503485] and from the National Institute for Health Research (NIHR) to NHS (National Health Service) Blood and Transplant.

REFERENCES

1 Langer, H. F. and Gawaz, M. (2008) Platelet-vessel wall interactions in atherosclerotic disease. Thromb. Haemost. **99**, 480–486

2 Varga-Szabo, D., Pleines, I. and Nieswandt, B. (2008) Cell adhesion mechanisms in platelets. Arterioscler. Thromb. Vasc. Biol. **28**, 403–412

3 Jacobson, K., Mouritsen, O. G. and Anderson, R. G. (2007) Lipid rafts: at a crossroad between cell biology and physics. Nat. Cell Biol. **9**, 7–14

4 Hemler, M. E. (2005) Tetraspanin functions and associated microdomains. Nat. Rev. Mol. Cell Biol. **6**, 801–811

5 Le Naour, F., Andre, M., Boucheix, C. and Rubinstein, E. (2006) Membrane microdomains and proteomics: lessons from tetraspanin microdomains and comparison with lipid rafts. Proteomics **6**, 6447–6454

6 Levy, S. and Shoham, T. (2005) The tetraspanin web modulates immune-signalling complexes. Nat. Rev. Immunol. **5**, 136–148

7 Lau, L. M., Wee, J. L., Wright, M. D., Moseley, G. W., Hogarth, P. M., Ashman, L. K. and Jackson, D. E. (2004) The tetraspanin superfamily member CD151 regulates outside-in integrin αIIbβ3 signaling and platelet function. Blood **104**, 2368–2375

8 Goschnick, M. W., Lau, L. M., Wee, J. L., Liu, Y. S., Hogarth, P. M., Robb, L. M., Hickey, M. J., Wright, M. D. and Jackson, D. E. (2006) Impaired "outside-in" integrin αIIbβ3 signaling and thrombus stability in TSSC6-deficient mice. Blood **108**, 1911–1918

9 Goschnick, M. W. and Jackson, D. E. (2007) Tetraspanins-structural and signalling scaffolds that regulate platelet function. Mini Rev. Med. Chem. **7**, 1248–1254

10 Gnatenko, D. V., Dunn, J. J., McCorkle, S. R., Weissmann, D., Perrotta, P. L. and Bahou, W. F. (2003) Transcript profiling of human platelets using microarray and serial analysis of gene expression. Blood **101**, 2285–2293

11 Senis, Y. A., Tomlinson, M. G., Garcia, A., Dumon, S., Heath, V. L., Herbert, J., Cobbold, S. P., Spalton, J. C., Ayman, S., Antrobus, R. et al. (2007) A comprehensive proteomics and genomics analysis reveals novel transmembrane proteins in human platelets and mouse megakaryocytes including G6b-B, a novel immunoreceptor tyrosine-based inhibitory motif protein. Mol. Cell. Proteomics **6**, 548–564

12 Watkins, N. A., Gusnanto, A., de Bono, B., De, S., Miranda-Saavedra, D., Angenent, W. D. J., Attwood, A. P., Ellis, P. D., Erber, W., Foad, N. S. et al. (in preparation) The HaemAtlas: characterising gene expression in differentiated human blood cells.

13 Berditchevski, F., Chang, S., Bodorova, J. and Hemler, M. E. (1997) Generation of monoclonal antibodies to integrin-associated proteins. Evidence that α3β1 complexes with EMMPRIN/basigin/OX47/M6. J. Biol. Chem. **272**, 29174–29180

14 Berditchevski, F., Zutter, M. M. and Hemler, M. E. (1996) Characterization of novel complexes on the cell surface between integrins and proteins with 4 transmembrane domains (TM4 proteins). Mol. Biol. Cell **7**, 193–207

15 Sincock, P. M., Mayrhofer, G. and Ashman, L. K. (1997) Localization of the transmembrane 4 superfamily (TM4SF) member PETA-3 (CD151) in normal human tissues: comparison with CD9, CD63, and α5β1 integrin. J. Histochem. Cytochem. **45**, 515–525

16 Berditchevski, F., Bazzoni, G. and Hemler, M. E. (1995) Specific association of CD63 with the VLA-3 and VLA-6 integrins. J. Biol. Chem. **270**, 17784–17790

17 Zannettino, A. C., Rayner, J. R., Ashman, L. K., Gonda, T. J. and Simmons, P. J. (1996) A powerful new technique for isolating genes encoding cell surface antigens using retroviral expression cloning. J. Immunol. **156**, 611–620

18 Moroi, M., Mizuguchi, J., Kawashima, S., Nagamatsu, M., Miura, Y., Nakagaki, T., Ito, K. and Jung, S. M. (2003) A new monoclonal antibody, mAb 204-11, that influences the binding of platelet GPVI to fibrous collagen. Thromb. Haemost. **89**, 996–1003

19 Berndt, M. C., Gregory, C., Kabral, A., Zola, H., Fournier, D. and Castaldi, P. A. (1985) Purification and preliminary characterization of the glycoprotein Ib complex in the human platelet membrane. Eur. J. Biochem. **151**, 637–649

20 Lecine, P., Blank, V. and Shivdasani, R. (1998) Characterization of the hematopoietic transcription factor NF-E2 in primary murine megakaryocytes. J. Biol. Chem. **273**, 7572–7578

21 Thomas, S. G., Calaminus, S. D., Auger, J. M., Watson, S. P. and Machesky, L. M. (2007) Studies on the actin-binding protein HS1 in platelets. BMC Cell Biol. **8**, 46

22 McCarty, O. J., Calaminus, S. D., Berndt, M. C., Machesky, L. M. and Watson, S. P. (2006) von Willebrand factor mediates platelet spreading through glycoprotein Ib and α(IIb)β3 in the presence of botrocetin and ristocetin, respectively. J. Thromb. Haemost. **4**, 1367–1378

23 Tomlinson, M. G., Kurosaki, T., Berson, A. E., Fujii, G. H., Johnston, J. A. and Bolen, J. B. (1999) Reconstitution of Btk signaling by the atypical Tec family tyrosine kinases Bmx and Txk. J. Biol. Chem. **274**, 13577–13585

24 Tomlinson, M. G., Kane, L. P., Su, J., Kadlecek, T. A., Mollenauer, M. N. and Weiss, A. (2004) Expression and function of Tec, Itk, and Btk in lymphocytes: evidence for a unique role for Tec. Mol. Cell. Biol. **24**, 2455–2466

25 Saha, S., Sparks, A. B., Rago, C., Akmaev, V., Wang, C. J., Vogelstein, B., Kinzler, K. W. and Velculescu, V. E. (2002) Using the transcriptome to annotate the genome. Nat. Biotechnol. **20**, 508–512

26 Kuhn, K., Baker, S. C., Chudin, E., Lieu, M. H., Oeser, S., Bennett, H., Rigault, P., Barker, D., McDaniel, T. K. and Chee, M. S. (2004) A novel, high-performance random array platform for quantitative gene expression profiling. Genome Res. **14**, 2347–2356

27 Serru, V., Dessen, P., Boucheix, C. and Rubinstein, E. (2000) Sequence and expression of seven new tetraspans. Biochim. Biophys. Acta **1478**, 159–163

28 Moebius, J., Zahedi, R. P., Lewandrowski, U., Berger, C., Walter, U. and Sickmann, A. (2005) The human platelet membrane proteome reveals several new potential membrane proteins. Mol. Cell. Proteomics **4**, 1754–1761

29 Macaulay, I. C., Tijssen, M. R., Thijssen-Timmer, D. C., Gusnanto, A., Steward, M., Burns, P., Langford, C. F., Ellis, P. D., Dudbridge, F., Zwaginga, J. J. et al. (2007) Comparative gene expression profiling of *in vitro* differentiated megakaryocytes and erythroblasts identifies novel activatory and inhibitory platelet membrane proteins. Blood **109**, 3260–3269

30 Battinelli, E. M., Hartwig, J. H. and Italiano, Jr, J. E. (2007) Delivering new insight into the biology of megakaryopoiesis and thrombopoiesis. Curr. Opin. Hematol. **14**, 419–426

31 Miller, J. L., Kupinski, J. M. and Hustad, K. O. (1986) Characterization of a platelet membrane protein of low molecular weight associated with platelet activation following binding by monoclonal antibody AG-1. Blood **68**, 743–751

32 Hato, T., Ikeda, K., Yasukawa, M., Watanabe, A. and Kobayashi, Y. (1988) Exposure of platelet fibrinogen receptors by a monoclonal antibody to CD9 antigen. Blood **72**, 224–229

33 Higashihara, M., Takahata, K., Yatomi, Y., Nakahara, K. and Kurokawa, K. (1990) Purification and partial characterization of CD9 antigen of human platelets. FEBS Lett. **264**, 270–274

34 Best, D., Senis, Y. A., Jarvis, G. E., Eagleton, H. J., Roberts, D. J., Saito, T., Jung, S. M., Moroi, M., Harrison, P., Green, F. R. and Watson, S. P. (2003) GPVI levels in platelets: relationship to platelet function at high shear. Blood **102**, 2811–2818

35 Wagner, C. L., Mascelli, M. A., Neblock, D. S., Weisman, H. F., Coller, B. S. and Jordan, R. E. (1996) Analysis of GPIIb/IIIa receptor number by quantification of 7E3 binding to human platelets. Blood **88**, 907–914
36 Du, X., Beutler, L., Ruan, C., Castaldi, P. A. and Berndt, M. C. (1987) Glycoprotein Ib and glycoprotein IX are fully complexed in the intact platelet membrane. Blood **69**, 1524–1527
37 Israels, S. J. and McMillan-Ward, E. M. (2007) Platelet tetraspanin complexes and their association with lipid rafts. Thromb. Haemost. **98**, 1081–1087
38 Hemler, M. E. (2003) Tetraspanin proteins mediate cellular penetration, invasion, and fusion events and define a novel type of membrane microdomain. Annu. Rev. Cell Dev. Biol. **19**, 397–422
39 Min, G., Wang, H., Sun, T. T. and Kong, X. P. (2006) Structural basis for tetraspanin functions as revealed by the cryo-EM structure of uroplakin complexes at 6-A resolution. J. Cell Biol. **173**, 975–983
40 Garcia-Espana, A., Chung, P. J., Sarkar, I. N., Stiner, E., Sun, T. T. and Desalle, R. (2008) Appearance of new tetraspanin genes during vertebrate evolution. Genomics **91**, 326–334
41 Tarrant, J. M., Robb, L., van Spriel, A. B. and Wright, M. D. (2003) Tetraspanins: molecular organisers of the leukocyte surface. Trends Immunol. **24**, 610–617
42 Fitter, S., Sincock, P. M., Jolliffe, C. N. and Ashman, L. K. (1999) Transmembrane 4 superfamily protein CD151 (PETA-3) associates with β 1 and α IIb β 3 integrins in haemopoietic cell lines and modulates cell-cell adhesion. Biochem. J. **338**, 61–70
43 Watson, S. P., Auger, J. M., McCarty, O. J. and Pearce, A. C. (2005) GPVI and integrin αIIb β3 signaling in platelets. J. Thromb. Haemost. **3**, 1752–1762
44 Cherukuri, A., Carter, R. H., Brooks, S., Bornmann, W., Finn, R., Dowd, C. S. and Pierce, S. K. (2004) B cell signaling is regulated by induced palmitoylation of CD81. J. Biol. Chem. **279**, 31973–31982
45 Cherukuri, A., Shoham, T., Sohn, H. W., Levy, S., Brooks, S., Carter, R. and Pierce, S. K. (2004) The tetraspanin CD81 is necessary for partitioning of coligated CD19/CD21-B cell antigen receptor complexes into signaling-active lipid rafts. J. Immunol. **172**, 370–380
46 Tarrant, J. M., Groom, J., Metcalf, D., Li, R., Borobokas, B., Wright, M. D., Tarlinton, D. and Robb, L. (2002) The absence of Tssc6, a member of the tetraspanin superfamily, does not affect lymphoid development but enhances *in vitro* T-cell proliferative responses. Mol. Cell. Biol. **22**, 5006–5018
47 van Spriel, A. B., Puls, K. L., Sofi, M., Pouniotis, D., Hochrein, H., Orinska, Z., Knobeloch, K. P., Plebanski, M. and Wright, M. D. (2004) A regulatory role for CD37 in T cell proliferation. J. Immunol. **172**, 2953–2961
48 Wright, M. D., Geary, S. M., Fitter, S., Moseley, G. W., Lau, L. M., Sheng, K. C., Apostolopoulos, V., Stanley, E. G., Jackson, D. E. and Ashman, L. K. (2004) Characterization of mice lacking the tetraspanin superfamily member CD151. Mol. Cell. Biol. **24**, 5978–5988
49 Shoham, T., Rajapaksa, R., Kuo, C. C., Haimovich, J. and Levy, S. (2006) Building of the tetraspanin web: distinct structural domains of CD81 function in different cellular compartments. Mol. Cell. Biol. **26**, 1373–1385
50 Kim, P. M., Lu, L. J., Xia, Y. and Gerstein, M. B. (2006) Relating three-dimensional structures to protein networks provides evolutionary insights. Science **314**, 1938–1941

Received 4 June 2008/8 September 2008; accepted 16 September 2008
Published as BJ Immediate Publication 16 September 2008, doi:10.1042/BJ20081126

Biochem. J. (2009) **417**, 391–400 (Printed in Great Britain) doi:10.1042/BJ20081126

online data

SUPPLEMENTARY ONLINE DATA

Identification of Tspan9 as a novel platelet tetraspanin and the collagen receptor GPVI as a component of tetraspanin microdomains

Majd B. PROTTY*, Nicholas A. WATKINS†, Dario COLOMBO*, Steven G. THOMAS*, Victoria L. HEATH*, John M. J. HERBERT*, Roy BICKNELL*, Yotis A. SENIS*, Leonie K. ASHMAN‡, Fedor BERDITCHEVSKI§, Willem H. OUWEHAND†‖, Steve P. WATSON* and Michael G. TOMLINSON*[1]

*Centre for Cardiovascular Sciences, Institute of Biomedical Research, The Medical School, University of Birmingham, Birmingham B15 2TT, U.K., †Department of Haematology, University of Cambridge and National Health Service Blood and Transplant, Cambridge CB2 2PT, U.K., ‡School of Biomedical Sciences, University of Newcastle, Callaghan, NSW 2308, Australia, §Cancer Research U.K. Institute for Cancer Studies, University of Birmingham, Birmingham B15 2TT, U.K., and ‖Wellcome Trust Sanger Institute, Hinxton, Cambridge CB10 1SA, U.K.

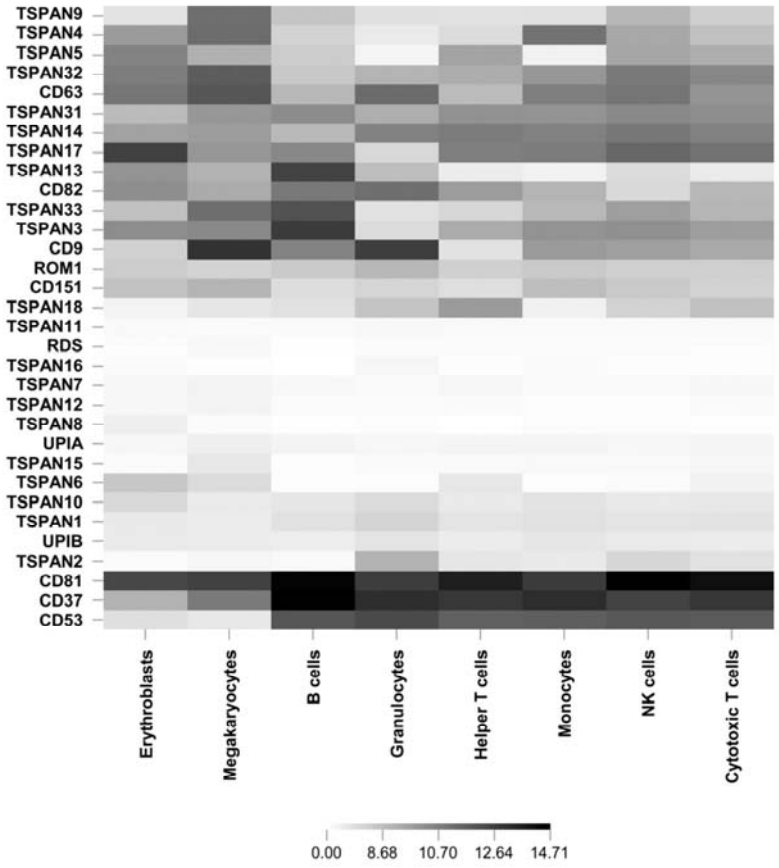


Figure S1 DNA microarray analyses of the 33 human tetraspanins in primary haematopoietic cells

Experiments using Illumina Human WG-6 v2 Expression BeadChips were performed using mRNA from eight different human cell types from four to seven donors. Normalized intensity values (Log2) for each tetraspanin are presented as a heat map, with white panels representing no expression and black panels representing the highest expression level.

Received 4 June 2008/8 September 2008; accepted 16 September 2008
Published as BJ Immediate Publication 16 September 2008, doi:10.1042/BJ20081126

[1] To whom correspondence should be addressed (email m.g.tomlinson@bham.ac.uk).

REFERENCES

Abdullaev, I. F., Bisaillon, J. M., Potier, M., Gonzalez, J. C., Motiani, R. K. and Trebak, M. (2008) Stim1 and Orai1 mediate CRAC currents and store-operated calcium entry important for endothelial cell proliferation. Circ Res 103, 1289-99.

Ades, E. W., Candal, F. J., Swerlick, R. A., George, V. G., Summers, S., Bosse, D. C. and Lawley, T. J. (1992) HMEC-1: establishment of an immortalized human microvascular endothelial cell line. J Invest Dermatol 99, 683-90.

Arduise, C., Abache, T., Li, L., Billard, M., Chabanon, A., Ludwig, A., Mauduit, P., Boucheix, C., Rubinstein, E. and Le Naour, F. (2008) Tetraspanins regulate ADAM10-mediated cleavage of TNF-alpha and epidermal growth factor. J Immunol 181, 7002-13.

Ashman, L. K., Aylett, G. W., Cambareri, A. C. and Cole, S. R. (1991) Different epitopes of the CD31 antigen identified by monoclonal antibodies: cell type-specific patterns of expression. Tissue Antigens 38, 199-207.

Baldwin, G., Novitskaya, V., Sadej, R., Pochec, E., Litynska, A., Hartmann, C., Williams, J., Ashman, L., Eble, J. A. and Berditchevski, F. (2008) Tetraspanin cd151 regulates glycosylation of alpha3beta1 integrin. J Biol Chem.

Barreiro, O., Zamai, M., Yanez-Mo, M., Tejera, E., Lopez-Romero, P., Monk, P. N., Gratton, E., Caiolfa, V. R. and Sanchez-Madrid, F. (2008) Endothelial adhesion receptors are recruited to adherent leukocytes by inclusion in preformed tetraspanin nanoplatforms. J Cell Biol 183, 527-42.

Bartosch, B., Vitelli, A., Granier, C., Goujon, C., Dubuisson, J., Pascale, S., Scarselli, E., Cortese, R., Nicosia, A. and Cosset, F. L. (2003) Cell entry of hepatitis C virus requires a set of co-receptors that include the CD81 tetraspanin and the SR-B1 scavenger receptor. J Biol Chem 278, 41624-30.

Berditchevski, F. and Odintsova, E. (2007) Tetraspanins as regulators of protein trafficking. Traffic 8, 89-96.

Berditchevski, F., Odintsova, E., Sawada, S. and Gilbert, E. (2002) Expression of the palmitoylation-deficient CD151 weakens the association of alpha 3 beta 1 integrin with the tetraspanin-enriched microdomains and affects integrin-dependent signaling. J Biol Chem 277, 36991-7000.

Berridge, M. J. (1993) Inositol trisphosphate and calcium signalling. Nature 361, 315-25.

Berridge, M. J., Bootman, M. D. and Roderick, H. L. (2003) Calcium signalling: dynamics, homeostasis and remodelling. Nat Rev Mol Cell Biol 4, 517-29.

Birling, M. C., Tait, S., Hardy, R. J. and Brophy, P. J. (1999) A novel rat tetraspan protein in cells of the oligodendrocyte lineage. J Neurochem 73, 2600-8.

Bodin, S., Tronchere, H. and Payrastre, B. (2003) Lipid rafts are critical membrane domains in blood platelet activation processes. Biochim Biophys Acta 1610, 247-57.

Boesze-Battaglia, K., Clayton, S. T. and Schimmel, R. J. (1996) Cholesterol redistribution within human platelet plasma membrane: evidence for a stimulus-dependent event. Biochemistry 35, 6664-73.

Boesze-Battaglia, K., Stefano, F. P., Fitzgerald, C. and Muller-Weeks, S. (2007) ROM-1 potentiates photoreceptor specific membrane fusion processes. Exp Eye Res 84, 22-31.

Boucheix, C., Soria, C., Mirshahi, M., Soria, J., Perrot, J. Y., Fournier, N., Billard, M. and Rosenfeld, C. (1983) Characteristics of platelet aggregation induced by the monoclonal antibody ALB6 (acute lymphoblastic leukemia antigen p 24). Inhibition of aggregation by ALB6Fab. FEBS Lett 161, 289-95.

Broad, L. M., Braun, F. J., Lievremont, J. P., Bird, G. S., Kurosaki, T. and Putney, J. W., Jr. (2001) Role of the phospholipase C-inositol 1,4,5-trisphosphate pathway in calcium release-activated calcium current and capacitative calcium entry. J Biol Chem 276, 15945-52.

Buday, L. and Downward, J. (2008) Many faces of Ras activation. Biochim Biophys Acta 1786, 178-87.

Burack, W. R. and Shaw, A. S. (2000) Signal transduction: hanging on a scaffold. Curr Opin Cell Biol 12, 211-6.

Callard, R. E., Rigley, K. P., Smith, S. H., Thurstan, S. and Shields, J. G. (1992) CD19 regulation of human B cell responses. B cell proliferation and antibody secretion are inhibited or enhanced by ligation of the CD19 surface glycoprotein depending on the stimulating signal used. J Immunol 148, 2983-7.

Canobbio, I., Trionfini, P., Guidetti, G. F., Balduini, C. and Torti, M. (2008) Targeting of the small GTPase Rap2b, but not Rap1b, to lipid rafts is promoted by palmitoylation at Cys176 and Cys177 and is required for efficient protein activation in human platelets. Cell Signal 20, 1662-70.

Carter, R. H. and Fearon, D. T. (1992) CD19: lowering the threshold for antigen receptor stimulation of B lymphocytes. Science 256, 105-7.

Carter, R. H., Tuveson, D. A., Park, D. J., Rhee, S. G. and Fearon, D. T. (1991) The CD19 complex of B lymphocytes. Activation of phospholipase C by a protein tyrosine kinase-dependent pathway that can be enhanced by the membrane IgM complex. J Immunol 147, 3663-71.

Charrin, S., Le Naour, F., Labas, V., Billard, M., Le Caer, J. P., Emile, J. F., Petit, M. A., Boucheix, C. and Rubinstein, E. (2003) EWI-2 is a new component of the tetraspanin web in hepatocytes and lymphoid cells. Biochem J 373, 409-21.

Charrin, S., Le Naour, F., Oualid, M., Billard, M., Faure, G., Hanash, S. M., Boucheix, C. and Rubinstein, E. (2001) The major CD9 and CD81 molecular partner. Identification and characterization of the complexes. J Biol Chem 276, 14329-37.

Charrin, S., le Naour, F., Silvie, O., Milhiet, P. E., Boucheix, C. and Rubinstein, E. (2009) Lateral organization of membrane proteins: tetraspanins spin their web. Biochem J 420, 133-54.

Charrin, S., Manie, S., Oualid, M., Billard, M., Boucheix, C. and Rubinstein, E. (2002) Differential stability of tetraspanin/tetraspanin interactions: role of palmitoylation. FEBS Lett 516, 139-44.

Chen, C. Y., Gherzi, R., Andersen, J. S., Gaietta, G., Jurchott, K., Royer, H. D., Mann, M. and Karin, M. (2000) Nucleolin and YB-1 are required for JNK-mediated interleukin-2 mRNA stabilization during T-cell activation. Genes Dev 14, 1236-48.

Cheng, P. C., Dykstra, M. L., Mitchell, R. N. and Pierce, S. K. (1999) A role for lipid rafts in B cell antigen receptor signaling and antigen targeting. J Exp Med 190, 1549-60.

Cherukuri, A., Cheng, P. C., Sohn, H. W. and Pierce, S. K. (2001) The CD19/CD21 complex functions to prolong B cell antigen receptor signaling from lipid rafts. Immunity 14, 169-79.

Cherukuri, A., Shoham, T., Sohn, H. W., Levy, S., Brooks, S., Carter, R. and Pierce, S. K. (2004) The tetraspanin CD81 is necessary for partitioning of coligated CD19/CD21-B cell antigen receptor complexes into signaling-active lipid rafts. J Immunol 172, 370-80.

Chow, C. W., Dong, C., Flavell, R. A. and Davis, R. J. (2000) c-Jun NH(2)-terminal kinase inhibits targeting of the protein phosphatase calcineurin to NFATc1. Mol Cell Biol 20, 5227-34.

Churchill, G. C., Okada, Y., Thomas, J. M., Genazzani, A. A., Patel, S. and Galione, A. (2002) NAADP mobilizes Ca(2+) from reserve granules, lysosome-related organelles, in sea urchin eggs. Cell 111, 703-8.

Clapham, D. E. (2007) Calcium signaling. Cell 131, 1047-58.

Clapham, D. E. (2009) A STIMulus Package puts orai calcium channels to work. Cell 136, 814-6.

Clark, M. R., Campbell, K. S., Kazlauskas, A., Johnson, S. A., Hertz, M., Potter, T. A., Pleiman, C. and Cambier, J. C. (1992) The B cell antigen receptor complex: association of Ig-alpha and Ig-beta with distinct cytoplasmic effectors. Science 258, 123-6.

Clark, M. R., Johnson, S. A. and Cambier, J. C. (1994) Analysis of Ig-alpha-tyrosine kinase interaction reveals two levels of binding specificity and tyrosine phosphorylated Ig-alpha stimulation of Fyn activity. Embo J 13, 1911-9.

Clemetson, K. J. and Clemetson, J. M. (2001) Platelet collagen receptors. Thromb Haemost 86, 189-97.

Cnops, G., Neyt, P., Raes, J., Petrarulo, M., Nelissen, H., Malenica, N., Luschnig, C., Tietz, O., Ditengou, F., Palme, K., Azmi, A., Prinsen, E. and Van Lijsebettens, M. (2006)

The TORNADO1 and TORNADO2 genes function in several patterning processes during early leaf development in Arabidopsis thaliana. Plant Cell 18, 852-66.

Cnops, G., Wang, X., Linstead, P., Van Montagu, M., Van Lijsebettens, M. and Dolan, L. (2000) Tornado1 and tornado2 are required for the specification of radial and circumferential pattern in the Arabidopsis root. Development 127, 3385-94.

Cocquerel, L., Voisset, C. and Dubuisson, J. (2006) Hepatitis C virus entry: potential receptors and their biological functions. J Gen Virol 87, 1075-84.

Cormier, E. G., Tsamis, F., Kajumo, F., Durso, R. J., Gardner, J. P. and Dragic, T. (2004) CD81 is an entry coreceptor for hepatitis C virus. Proc Natl Acad Sci U S A 101, 7270-4.

Cserzo, M., Eisenhaber, F., Eisenhaber, B. and Simon, I. (2002) On filtering false positive transmembrane protein predictions. Protein Eng 15, 745-52

Cserzo, M., Wallin, E., Simon, I., von Heijne, G. and Elofsson, A. (1997) Prediction of transmembrane alpha-helices in prokaryotic membrane proteins: the dense alignment surface method. Protein Eng 10, 673-6

Dale, G. L., Remenyi, G. and Friese, P. (2009) Tetraspanin CD9 is required for microparticle release from coated-platelets. Platelets, 1-6.

Deneka, M., Pelchen-Matthews, A., Byland, R., Ruiz-Mateos, E. and Marsh, M. (2007) In macrophages, HIV-1 assembles into an intracellular plasma membrane domain containing the tetraspanins CD81, CD9, and CD53. J Cell Biol 177, 329-41.

Dorahy, D. J., Lincz, L. F., Meldrum, C. J. and Burns, G. F. (1996) Biochemical isolation of a membrane microdomain from resting platelets highly enriched in the plasma membrane glycoprotein CD36. Biochem J 319 (Pt 1), 67-72.

Dubuisson, J., Helle, F. and Cocquerel, L. (2008) Early steps of the hepatitis C virus life cycle. Cell Microbiol 10, 821-7.

Espenel, C., Margeat, E., Dosset, P., Arduise, C., Le Grimellec, C., Royer, C. A., Boucheix, C., Rubinstein, E. and Milhiet, P. E. (2008) Single-molecule analysis of CD9 dynamics and partitioning reveals multiple modes of interaction in the tetraspanin web. J Cell Biol 182, 765-76.

Feng, J., Garrity, D., Call, M. E., Moffett, H. and Wucherpfennig, K. W. (2005) Convergence on a distinctive assembly mechanism by unrelated families of activating immune receptors. Immunity 22, 427-38.

Fitter, S., Sincock, P. M., Jolliffe, C. N. and Ashman, L. K. (1999) Transmembrane 4 superfamily protein CD151 (PETA-3) associates with beta 1 and alpha IIb beta 3 integrins in haemopoietic cell lines and modulates cell-cell adhesion. Biochem J 338 (Pt 1), 61-70.

Fradkin, L. G., Kamphorst, J. T., DiAntonio, A., Goodman, C. S. and Noordermeer, J. N. (2002) Genomewide analysis of the Drosophila tetraspanins reveals a subset with

similar function in the formation of the embryonic synapse. Proc Natl Acad Sci U S A 99, 13663-8.

Fu, C. and Chan, A. C. (1997) Identification of two tyrosine phosphoproteins, pp70 and pp68, which interact with phospholipase Cgamma, Grb2, and Vav after B cell antigen receptor activation. J Biol Chem 272, 27362-8.

Fukudome, K., Furuse, M., Imai, T., Nishimura, M., Takagi, S., Hinuma, Y. and Yoshie, O. (1992) Identification of membrane antigen C33 recognized by monoclonal antibodies inhibitory to human T-cell leukemia virus type 1 (HTLV-1)-induced syncytium formation: altered glycosylation of C33 antigen in HTLV-1-positive T cells. J Virol 66, 1394-401.

Garcia, E., Pion, M., Pelchen-Matthews, A., Collinson, L., Arrighi, J. F., Blot, G., Leuba, F., Escola, J. M., Demaurex, N., Marsh, M. and Piguet, V. (2005) HIV-1 trafficking to the dendritic cell-T-cell infectious synapse uses a pathway of tetraspanin sorting to the immunological synapse. Traffic 6, 488-501.

Gardiner, E. E., Karunakaran, D., Shen, Y., Arthur, J. F., Andrews, R. K. and Berndt, M. C. (2007) Controlled shedding of platelet glycoprotein (GP)VI and GPIb-IX-V by ADAM family metalloproteinases. J Thromb Haemost 5, 1530-7.

Gomez del Arco, P., Martinez-Martinez, S., Maldonado, J. L., Ortega-Perez, I. and Redondo, J. M. (2000) A role for the p38 MAP kinase pathway in the nuclear shuttling of NFATp. J Biol Chem 275, 13872-8.

Gordon-Alonso, M., Yanez-Mo, M., Barreiro, O., Alvarez, S., Munoz-Fernandez, M. A., Valenzuela-Fernandez, A. and Sanchez-Madrid, F. (2006) Tetraspanins CD9 and CD81 modulate HIV-1-induced membrane fusion. J Immunol 177, 5129-37.

Goschnick, M. W., Lau, L. M., Wee, J. L., Liu, Y. S., Hogarth, P. M., Robb, L. M., Hickey, M. J., Wright, M. D. and Jackson, D. E. (2006) Impaired "outside-in" integrin alphaIIbbeta3 signaling and thrombus stability in TSSC6-deficient mice. Blood 108, 1911-8.

Graham, F. L., Smiley, J., Russell, W. C. and Nairn, R. (1977) Characteristics of a human cell line transformed by DNA from human adenovirus type 5. J Gen Virol 36, 59-74.

Grande, S. M., Bannish, G., Fuentes-Panana, E. M., Katz, E. and Monroe, J. G. (2007) Tonic B-cell and viral ITAM signaling: context is everything. Immunol Rev 218, 214-34.

Greenberg, S. M., Rosenthal, D. S., Greeley, T. A., Tantravahi, R. and Handin, R. I. (1988) Characterization of a new megakaryocytic cell line: the Dami cell. Blood 72, 1968-77.

Grigorov, B., Attuil-Audenis, V., Perugi, F., Nedelec, M., Watson, S., Pique, C., Darlix, J. L., Conjeaud, H. and Muriaux, D. (2009) A role for CD81 on the late steps of HIV-1 replication in a chronically infected T cell line. Retrovirology 6, 28.

Guibert, C., Ducret, T. and Savineau, J. P. (2008) Voltage-independent calcium influx in smooth muscle. Prog Biophys Mol Biol 98, 10-23.

Hashimoto, S., Iwamatsu, A., Ishiai, M., Okawa, K., Yamadori, T., Matsushita, M., Baba, Y., Kishimoto, T., Kurosaki, T. and Tsukada, S. (1999) Identification of the SH2 domain binding protein of Bruton's tyrosine kinase as BLNK--functional significance of Btk-SH2 domain in B-cell antigen receptor-coupled calcium signaling. Blood 94, 2357-64.

Hato, T., Ikeda, K., Yasukawa, M., Watanabe, A. and Kobayashi, Y. (1988) Exposure of platelet fibrinogen receptors by a monoclonal antibody to CD9 antigen. Blood 72, 224-9.

Heikens, M. J., Cao, T. M., Morita, C., Dehart, S. L. and Tsai, S. (2007) Penumbra encodes a novel tetraspanin that is highly expressed in erythroid progenitors and promotes effective erythropoiesis. Blood 109, 3244-52.

Hemler, M. E. (2005) Tetraspanin functions and associated microdomains. Nat Rev Mol Cell Biol 6, 801-11.

Hemler, M. E. (2008) Targeting of tetraspanin proteins - potential benefits and strategies. Nat Rev Drug Discov 7, 747-58.

Hempel, W. M. and DeFranco, A. L. (1991) Expression of phospholipase C isozymes by murine B lymphocytes. J Immunol 146, 3713-20.

Hewavitharana, T., Deng, X., Soboloff, J. and Gill, D. L. (2007) Role of STIM and Orai proteins in the store-operated calcium signaling pathway. Cell Calcium 42, 173-82.

Higashihara, M., Takahata, K., Yatomi, Y., Nakahara, K. and Kurokawa, K. (1990) Purification and partial characterization of CD9 antigen of human platelets. FEBS Lett 264, 270-4.

Higashiyama, S., Iwamoto, R., Goishi, K., Raab, G., Taniguchi, N., Klagsbrun, M. and Mekada, E. (1995) The membrane protein CD9/DRAP 27 potentiates the juxtacrine growth factor activity of the membrane-anchored heparin-binding EGF-like growth factor. J Cell Biol 128, 929-38.

Ho, S. H., Martin, F., Higginbottom, A., Partridge, L. J., Parthasarathy, V., Moseley, G. W., Lopez, P., Cheng-Mayer, C. and Monk, P. N. (2006) Recombinant extracellular domains of tetraspanin proteins are potent inhibitors of the infection of macrophages by human immunodeficiency virus type 1. J Virol 80, 6487-96.

Hogan, P. G. and Rao, A. (2007) Dissecting ICRAC, a store-operated calcium current. Trends Biochem Sci 32, 235-45.

Horii, K., Kahn, M. L. and Herr, A. B. (2006) Structural basis for platelet collagen responses by the immune-type receptor glycoprotein VI. Blood 108, 936-42.

Horvath, G., Serru, V., Clay, D., Billard, M., Boucheix, C. and Rubinstein, E. (1998) CD19 is linked to the integrin-associated tetraspans CD9, CD81, and CD82. J Biol Chem 273, 30537-43.

Hu, C. C., Liang, F. X., Zhou, G., Tu, L., Tang, C. H., Zhou, J., Kreibich, G. and Sun, T. T. (2005) Assembly of urothelial plaques: tetraspanin function in membrane protein trafficking. Mol Biol Cell 16, 3937-50.

Huang, H., Sossey-Alaoui, K., Beachy, S. H. and Geradts, J. (2007) The tetraspanin superfamily member NET-6 is a new tumor suppressor gene. J Cancer Res Clin Oncol 133, 761-9.

Inada, H., Iida, T. and Tominaga, M. (2006) Different expression patterns of TRP genes in murine B and T lymphocytes. Biochem Biophys Res Commun 350, 762-7.

Ishiai, M., Sugawara, H., Kurosaki, M. and Kurosaki, T. (1999) Cutting edge: association of phospholipase C-gamma 2 Src homology 2 domains with BLNK is critical for B cell antigen receptor signaling. J Immunol 163, 1746-9.

Israels, S. J. and McMillan-Ward, E. M. (2005) CD63 modulates spreading and tyrosine phosphorylation of platelets on immobilized fibrinogen. Thromb Haemost 93, 311-8.

Israels, S. J. and McMillan-Ward, E. M. (2007) Platelet tetraspanin complexes and their association with lipid rafts. Thromb Haemost 98, 1081-7.

Israels, S. J., McMillan-Ward, E. M., Easton, J., Robertson, C. and McNicol, A. (2001) CD63 associates with the alphaIIb beta3 integrin-CD9 complex on the surface of activated platelets. Thromb Haemost 85, 134-41.

Iwamoto, R., Higashiyama, S., Mitamura, T., Taniguchi, N., Klagsbrun, M. and Mekada, E. (1994) Heparin-binding EGF-like growth factor, which acts as the diphtheria toxin receptor, forms a complex with membrane protein DRAP27/CD9, which up-regulates functional receptors and diphtheria toxin sensitivity. Embo J 13, 2322-30.

Jacobson, K. and Dietrich, C. (1999) Looking at lipid rafts? Trends Cell Biol 9, 87-91.

Jamshad, M., Rajesh, S., Stamataki, Z., McKeating, J. A., Dafforn, T., Overduin, M. and Bill, R. M. (2008) Structural characterization of recombinant human CD81 produced in Pichia pastoris. Protein Expr Purif 57, 206-16.

Jin, L. and Lloyd, R. V. (1997) In situ hybridization: methods and applications. J Clin Lab Anal 11, 2-9.

Jolly, C. and Sattentau, Q. J. (2007) Human immunodeficiency virus type 1 assembly, budding, and cell-cell spread in T cells take place in tetraspanin-enriched plasma membrane domains. J Virol 81, 7873-84.

Jones, D. T. (2007) Improving the accuracy of transmembrane protein topology prediction using evolutionary information. Bioinformatics 23, 538-44.

Jones, D. T., Taylor, W. R. and Thornton, J. M. (1994) A model recognition approach to the prediction of all-helical membrane protein structure and topology. Biochemistry 33, 3038-49.

Joyoti, B. (2004) Protein palmytoylation and dynamic modulation of protein function. Current science 87, 212-218.

Kim, Y. H., Yang, I., Bae, Y. S. and Park, S. R. (2008) Performance evaluation of thermal cyclers for PCR in a rapid cycling condition. Biotechniques 44, 495-6, 498, 500 passim.

Kiselyov, K., Shin, D. M., Shcheynikov, N., Kurosaki, T. and Muallem, S. (2001) Regulation of Ca2+-release-activated Ca2+ current (Icrac) by ryanodine receptors in inositol 1,4,5-trisphosphate-receptor-deficient DT40 cells. Biochem J 360, 17-22.

Kitadokoro, K., Bordo, D., Galli, G., Petracca, R., Falugi, F., Abrignani, S., Grandi, G. and Bolognesi, M. (2001) CD81 extracellular domain 3D structure: insight into the tetraspanin superfamily structural motifs. Embo J 20, 12-8.

Knobeloch, K. P., Wright, M. D., Ochsenbein, A. F., Liesenfeld, O., Lohler, J., Zinkernagel, R. M., Horak, I. and Orinska, Z. (2000) Targeted inactivation of the tetraspanin CD37 impairs T-cell-dependent B-cell response under suboptimal costimulatory conditions. Mol Cell Biol 20, 5363-9.

Koretzky, G. A., Abtahian, F. and Silverman, M. A. (2006) SLP76 and SLP65: complex regulation of signalling in lymphocytes and beyond. Nat Rev Immunol 6, 67-78.

Kovalenko, O. V., Yang, X., Kolesnikova, T. V. and Hemler, M. E. (2004) Evidence for specific tetraspanin homodimers: inhibition of palmitoylation makes cysteine residues available for cross-linking. Biochem J 377, 407-17.

Kovalenko, O. V., Yang, X. H. and Hemler, M. E. (2007) A novel cysteine cross-linking method reveals a direct association between claudin-1 and tetraspanin CD9. Mol Cell Proteomics 6, 1855-67.

Krementsov, D. N., Weng, J., Lambele, M., Roy, N. H. and Thali, M. (2009) Tetraspanins regulate cell-to-cell transmission of HIV-1. Retrovirology 6, 64.

Ku, G. and McManus, M. T. (2008) Behind the scenes of a small RNA gene-silencing pathway. Hum Gene Ther 19, 17-26.

Kurosaki, T. (1997) Molecular mechanisms in B cell antigen receptor signaling. Curr Opin Immunol 9, 309-18.

Kurosaki, T., Maeda, A., Ishiai, M., Hashimoto, A., Inabe, K. and Takata, M. (2000) Regulation of the phospholipase C-gamma2 pathway in B cells. Immunol Rev 176, 19-29.

Kushnir, A., Betzenhauser, M. J. and Marks, A. R. Ryanodine receptor studies using genetically engineered mice. FEBS Lett 584, 1956-65.

Lambou, K., Tharreau, D., Kohler, A., Sirven, C., Marguerettaz, M., Barbisan, C., Sexton, A. C., Kellner, E. M., Martin, F., Howlett, B. J., Orbach, M. J. and Lebrun, M. H. (2008) Fungi have three tetraspanin families with distinct functions. BMC Genomics 9, 63.

Lammerding, J., Kazarov, A. R., Huang, H., Lee, R. T. and Hemler, M. E. (2003) Tetraspanin CD151 regulates alpha6beta1 integrin adhesion strengthening. Proc Natl Acad Sci U S A 100, 7616-21.

Langer, H. F. and Gawaz, M. (2008) Platelet-vessel wall interactions in atherosclerotic disease. Thromb Haemost 99, 480-6.

Latysheva, N., Muratov, G., Rajesh, S., Padgett, M., Hotchin, N. A., Overduin, M. and Berditchevski, F. (2006) Syntenin-1 is a new component of tetraspanin-enriched microdomains: mechanisms and consequences of the interaction of syntenin-1 with CD63. Mol Cell Biol 26, 7707-18.

Lau, L. M., Wee, J. L., Wright, M. D., Moseley, G. W., Hogarth, P. M., Ashman, L. K. and Jackson, D. E. (2004) The tetraspanin superfamily member CD151 regulates outside-in integrin alphaIIbbeta3 signaling and platelet function. Blood 104, 2368-75.

Le Naour, F., Andre, M., Greco, C., Billard, M., Sordat, B., Emile, J. F., Lanza, F., Boucheix, C. and Rubinstein, E. (2006) Profiling of the tetraspanin web of human colon cancer cells. Mol Cell Proteomics 5, 845-57.

Le Naour, F., Rubinstein, E., Jasmin, C., Prenant, M. and Boucheix, C. (2000) Severely reduced female fertility in CD9-deficient mice. Science 287, 319-21.

Levy, S. and Shoham, T. (2005) The tetraspanin web modulates immune-signalling complexes. Nat Rev Immunol 5, 136-48.

Lewandrowski, U., Wortelkamp, S., Lohrig, K., Zahedi, R. P., Wolters, D. A., Walter, U. and Sickmann, A. (2009) Platelet membrane proteomics: a novel repository for functional research. Blood 114, e10-9.

Liang, F. X., Riedel, I., Deng, F. M., Zhou, G., Xu, C., Wu, X. R., Kong, X. P., Moll, R. and Sun, T. T. (2001) Organization of uroplakin subunits: transmembrane topology, pair formation and plaque composition. Biochem J 355, 13-8.

Lin, J., Weiss, A. and Finco, T. S. (1999) Localization of LAT in glycolipid-enriched microdomains is required for T cell activation. J Biol Chem 274, 28861-4.

Liou, J., Kim, M. L., Heo, W. D., Jones, J. T., Myers, J. W., Ferrell, J. E., Jr. and Meyer, T. (2005) STIM is a Ca2+ sensor essential for Ca2+-store-depletion-triggered Ca2+ influx. Curr Biol 15, 1235-41.

Liu, J., Masuda, E. S., Tsuruta, L., Arai, N. and Arai, K. (1999) Two independent calcineurin-binding regions in the N-terminal domain of murine NF-ATx1 recruit calcineurin to murine NF-ATx1. J Immunol 162, 4755-61.

Liu, J. O. (2009) Calmodulin-dependent phosphatase, kinases, and transcriptional corepressors involved in T-cell activation. Immunol Rev 228, 184-98.

Locke, D., Chen, H., Liu, Y., Liu, C. and Kahn, M. L. (2002) Lipid rafts orchestrate signaling by the platelet receptor glycoprotein VI. J Biol Chem 277, 18801-9.

Long, S. B., Campbell, E. B. and Mackinnon, R. (2005) Voltage sensor of Kv1.2: structural basis of electromechanical coupling. Science 309, 903-8.

Lopez-Rodriguez, C., Aramburu, J., Rakeman, A. S., Copeland, N. G., Gilbert, D. J., Thomas, S., Disteche, C., Jenkins, N. A. and Rao, A. (1999) NF-AT5: the NF-AT family of transcription factors expands in a new direction. Cold Spring Harb Symp Quant Biol 64, 517-26.

Lozzio, C. B. and Lozzio, B. B. (1975) Human chronic myelogenous leukemia cell-line with positive Philadelphia chromosome. Blood 45, 321-34.

Luik, R. M., Wang, B., Prakriya, M., Wu, M. M. and Lewis, R. S. (2008) Oligomerization of STIM1 couples ER calcium depletion to CRAC channel activation. Nature 454, 538-42.

Macian, F. (2005) NFAT proteins: key regulators of T-cell development and function. Nat Rev Immunol 5, 472-84.

Macian, F., Lopez-Rodriguez, C. and Rao, A. (2001) Partners in transcription: NFAT and AP-1. Oncogene 20, 2476-89.

Maecker, H. T., Todd, S. C. and Levy, S. (1997) The tetraspanin superfamily: molecular facilitators. FASEB J 11, 428-42.

Mangin, P. H., Kleitz, L., Boucheix, C., Gachet, C. and Lanza, F. (2009) CD9 negatively regulates integrin alphaIIbbeta3 activation and could thus prevent excessive platelet recruitment at sites of vascular injury. J Thromb Haemost 7, 900-2.

Martin, F., Roth, D. M., Jans, D. A., Pouton, C. W., Partridge, L. J., Monk, P. N. and Moseley, G. W. (2005) Tetraspanins in viral infections: a fundamental role in viral biology? J Virol 79, 10839-51.

Meyer-Wentrup, F., Figdor, C. G., Ansems, M., Brossart, P., Wright, M. D., Adema, G. J. and van Spriel, A. B. (2007) Dectin-1 interaction with tetraspanin CD37 inhibits IL-6 production. J Immunol 178, 154-62.

Miller, J. L., Kupinski, J. M. and Hustad, K. O. (1986) Characterization of a platelet membrane protein of low molecular weight associated with platelet activation following binding by monoclonal antibody AG-1. Blood 68, 743-51.

Min, G., Wang, H., Sun, T. T. and Kong, X. P. (2006) Structural basis for tetraspanin functions as revealed by the cryo-EM structure of uroplakin complexes at 6-A resolution. J Cell Biol 173, 975-83.

Mori, J., Pearce, A. C., Spalton, J. C., Grygielska, B., Eble, J. A., Tomlinson, M. G., Senis, Y. A. and Watson, S. P. (2008) G6b-B inhibits constitutive and agonist-induced signaling by glycoprotein VI and CLEC-2. J Biol Chem 283, 35419-27.

Moribe, H., Yochem, J., Yamada, H., Tabuse, Y., Fujimoto, T. and Mekada, E. (2004) Tetraspanin protein (TSP-15) is required for epidermal integrity in Caenorhabditis elegans. J Cell Sci 117, 5209-20.

Myung, P. S., Boerthe, N. J. and Koretzky, G. A. (2000) Adapter proteins in lymphocyte antigen-receptor signaling. Curr Opin Immunol 12, 256-66.

Ng, S. W., Nelson, C. and Parekh, A. B. (2009) Coupling of Ca(2+) microdomains to spatially and temporally distinct cellular responses by the tyrosine kinase Syk. J Biol Chem 284, 24767-72.

Nieswandt, B. and Watson, S. P. (2003) Platelet-collagen interaction: is GPVI the central receptor? Blood 102, 449-61.

Nieuwenhuis, H. K., van Oosterhout, J. J., Rozemuller, E., van Iwaarden, F. and Sixma, J. J. (1987) Studies with a monoclonal antibody against activated platelets: evidence that a secreted 53,000-molecular weight lysosome-like granule protein is exposed on the surface of activated platelets in the circulation. Blood 70, 838-45.

Nydegger, S., Khurana, S., Krementsov, D. N., Foti, M. and Thali, M. (2006) Mapping of tetraspanin-enriched microdomains that can function as gateways for HIV-1. J Cell Biol 173, 795-807.

Oh-hora, M. and Rao, A. (2008) Calcium signaling in lymphocytes. Curr Opin Immunol 20, 250-8.

Okamura, H., Aramburu, J., Garcia-Rodriguez, C., Viola, J. P., Raghavan, A., Tahiliani, M., Zhang, X., Qin, J., Hogan, P. G. and Rao, A. (2000) Concerted dephosphorylation of the transcription factor NFAT1 induces a conformational switch that regulates transcriptional activity. Mol Cell 6, 539-50.

Ottinger, E. A., Botfield, M. C. and Shoelson, S. E. (1998) Tandem SH2 domains confer high specificity in tyrosine kinase signaling. J Biol Chem 273, 729-35.

Ovalle, S., Gutierrez-Lopez, M. D., Olmo, N., Turnay, J., Lizarbe, M. A., Majano, P., Molina-Jimenez, F., Lopez-Cabrera, M., Yanez-Mo, M., Sanchez-Madrid, F. and Cabanas, C. (2007) The tetraspanin CD9 inhibits the proliferation and tumorigenicity of human colon carcinoma cells. Int J Cancer 121, 2140-52.

Parekh, A. B. and Putney, J. W., Jr. (2005) Store-operated calcium channels. Physiol Rev 85, 757-810.

Park, C. Y., Hoover, P. J., Mullins, F. M., Bachhawat, P., Covington, E. D., Raunser, S., Walz, T., Garcia, K. C., Dolmetsch, R. E. and Lewis, R. S. (2009) STIM1 clusters and activates CRAC channels via direct binding of a cytosolic domain to Orai1. Cell 136, 876-90.

Parthasarathy, V., Martin, F., Higginbottom, A., Murray, H., Moseley, G. W., Read, R. C., Mal, G., Hulme, R., Monk, P. N. and Partridge, L. J. (2009) Distinct roles for tetraspanins CD9, CD63 and CD81 in the formation of multinucleated giant cells. Immunology 127, 237-48.

Penna, A., Demuro, A., Yeromin, A. V., Zhang, S. L., Safrina, O., Parker, I. and Cahalan, M. D. (2008) The CRAC channel consists of a tetramer formed by Stim-induced dimerization of Orai dimers. Nature 456, 116-20.

Perchonock, C. E., Fernando, M. C., Quinn, W. J., 3rd, Nguyen, C. T., Sun, J., Shapiro, M. J. and Shapiro, V. S. (2006) Negative regulation of interleukin-2 and p38 mitogen-activated protein kinase during T-cell activation by the adaptor ALX. Mol Cell Biol 26, 6005-15.

Perron, J. C. and Bixby, J. L. (1999) Tetraspanins expressed in the embryonic chick nervous system. FEBS Lett 461, 86-90.

Philipp, S., Strauss, B., Hirnet, D., Wissenbach, U., Mery, L., Flockerzi, V. and Hoth, M. (2003) TRPC3 mediates T-cell receptor-dependent calcium entry in human T-lymphocytes. J Biol Chem 278, 26629-38.

Pietschmann, T. (2009) Virology: Final entry key for hepatitis C. Nature 457, 797-8.

Pike, L. J. (2009) The challenge of lipid rafts. J Lipid Res 50 Suppl, S323-8.

Pileri, P., Uematsu, Y., Campagnoli, S., Galli, G., Falugi, F., Petracca, R., Weiner, A. J., Houghton, M., Rosa, D., Grandi, G. and Abrignani, S. (1998) Binding of hepatitis C virus to CD81. Science 282, 938-41.

Pique, C., Lagaudriere-Gesbert, C., Delamarre, L., Rosenberg, A. R., Conjeaud, H. and Dokhelar, M. C. (2000) Interaction of CD82 tetraspanin proteins with HTLV-1 envelope glycoproteins inhibits cell-to-cell fusion and virus transmission. Virology 276, 455-65.

Ploss, A., Evans, M. J., Gaysinskaya, V. A., Panis, M., You, H., de Jong, Y. P. and Rice, C. M. (2009) Human occludin is a hepatitis C virus entry factor required for infection of mouse cells. Nature 457, 882-6.

Porter, C. M., Havens, M. A. and Clipstone, N. A. (2000) Identification of amino acid residues and protein kinases involved in the regulation of NFATc subcellular localization. J Biol Chem 275, 3543-51.

Prakriya, M., Feske, S., Gwack, Y., Srikanth, S., Rao, A. and Hogan, P. G. (2006) Orai1 is an essential pore subunit of the CRAC channel. Nature 443, 230-3.

Prakriya, M. and Lewis, R. S. (2001) Potentiation and inhibition of Ca(2+) release-activated Ca(2+) channels by 2-aminoethyldiphenyl borate (2-APB) occurs independently of IP(3) receptors. J Physiol 536, 3-19.

Protty, M. B., Watkins, N. A., Colombo, D., Thomas, S. G., Heath, V. L., Herbert, J. M., Bicknell, R., Senis, Y. A., Ashman, L. K., Berditchevski, F., Ouwehand, W. H., Watson, S. P. and Tomlinson, M. G. (2009) Identification of Tspan9 as a novel platelet tetraspanin and the collagen receptor GPVI as a component of tetraspanin microdomains. Biochem J 417, 391-400.

Putney, J. W., Jr. (2005) Capacitative calcium entry: sensing the calcium stores. J Cell Biol 169, 381-2.

Qin, L., Zhao, D., Liu, X., Nagy, J. A., Hoang, M. V., Brown, L. F., Dvorak, H. F. and Zeng, H. (2006) Down syndrome candidate region 1 isoform 1 mediates angiogenesis through the calcineurin-NFAT pathway. Mol Cancer Res 4, 811-20.

Rao, A., Luo, C. and Hogan, P. G. (1997) Transcription factors of the NFAT family: regulation and function. Annu Rev Immunol 15, 707-47.

Reth, M. (1989) Antigen receptor tail clue. Nature 338, 383-4.

Rhee, S. G., Suh, P. G., Ryu, S. H. and Lee, S. Y. (1989) Studies of inositol phospholipid-specific phospholipase C. Science 244, 546-50.

Robb, L., Tarrant, J., Groom, J., Ibrahim, M., Li, R., Borobakas, B. and Wright, M. D. (2001) Molecular characterisation of mouse and human TSSC6: evidence that TSSC6 is a genuine member of the tetraspanin superfamily and is expressed specifically in haematopoietic organs. Biochim Biophys Acta 1522, 31-41.

Roos, J., DiGregorio, P. J., Yeromin, A. V., Ohlsen, K., Lioudyno, M., Zhang, S., Safrina, O., Kozak, J. A., Wagner, S. L., Cahalan, M. D., Velicelebi, G. and Stauderman, K. A. (2005) STIM1, an essential and conserved component of store-operated Ca2+ channel function. J Cell Biol 169, 435-45.

Rosker, C., Meur, G., Taylor, E. J. and Taylor, C. W. (2009) Functional ryanodine receptors in the plasma membrane of RINm5F pancreatic beta-cells. J Biol Chem 284, 5186-94.

Rost, B., Fariselli, P. and Casadio, R. (1996) Topology prediction for helical transmembrane proteins at 86% accuracy. Protein Sci 5, 1704-18.

Rubinstein, E., Le Naour, F., Lagaudriere-Gesbert, C., Billard, M., Conjeaud, H. and Boucheix, C. (1996) CD9, CD63, CD81, and CD82 are components of a surface tetraspan network connected to HLA-DR and VLA integrins. Eur J Immunol 26, 2657-65.

Rubinstein, E., Ziyyat, A., Prenant, M., Wrobel, E., Wolf, J. P., Levy, S., Le Naour, F. and Boucheix, C. (2006) Reduced fertility of female mice lacking CD81. Dev Biol 290, 351-8.

Rudolph, V. and Freeman, B. A. (2009) Cardiovascular consequences when nitric oxide and lipid signaling converge. Circ Res 105, 511-22.

Ruiz-Mateos, E., Pelchen-Matthews, A., Deneka, M. and Marsh, M. (2008) CD63 is not required for production of infectious human immunodeficiency virus type 1 in human macrophages. J Virol 82, 4751-61.

Runge, K. E., Evans, J. E., He, Z. Y., Gupta, S., McDonald, K. L., Stahlberg, H., Primakoff, P. and Myles, D. G. (2007) Oocyte CD9 is enriched on the microvillar membrane and required for normal microvillar shape and distribution. Dev Biol 304, 317-25.

Samelson, L. E. (2002) Signal transduction mediated by the T cell antigen receptor: the role of adapter proteins. Annu Rev Immunol 20, 371-94.

Sanyal, M., Fernandez, R. and Levy, S. (2009) Enhanced B cell activation in the absence of CD81. Int Immunol 21, 1225-37.

Sato, K., Aoki, J., Misawa, N., Daikoku, E., Sano, K., Tanaka, Y. and Koyanagi, Y. (2008) Modulation of human immunodeficiency virus type 1 infectivity through incorporation of tetraspanin proteins. J Virol 82, 1021-33.

Schabbauer, G., Schweighofer, B., Mechtcheriakova, D., Lucerna, M., Binder, B. R. and Hofer, E. (2007) Nuclear factor of activated T cells and early growth response-1

cooperate to mediate tissue factor gene induction by vascular endothelial growth factor in endothelial cells. Thromb Haemost 97, 988-97.

Schamel, W. W. and Reth, M. (2000) Monomeric and oligomeric complexes of the B cell antigen receptor. Immunity 13, 5-14.

Schneider, U., Schwenk, H. U. and Bornkamm, G. (1977) Characterization of EBV-genome negative "null" and "T" cell lines derived from children with acute lymphoblastic leukemia and leukemic transformed non-Hodgkin lymphoma. Int J Cancer 19, 621-6.

Schroder, J., Lullmann-Rauch, R., Himmerkus, N., Pleines, I., Nieswandt, B., Orinska, Z., Koch-Nolte, F., Schroder, B., Bleich, M. and Saftig, P. (2009) Deficiency of the tetraspanin CD63 associated with kidney pathology but normal lysosomal function. Mol Cell Biol 29, 1083-94.

Seigneuret, M. (2006) Complete predicted three-dimensional structure of the facilitator transmembrane protein and hepatitis C virus receptor CD81: conserved and variable structural domains in the tetraspanin superfamily. Biophys J 90, 212-27.

Senis, Y. A., Tomlinson, M. G., Garcia, A., Dumon, S., Heath, V. L., Herbert, J., Cobbold, S. P., Spalton, J. C., Ayman, S., Antrobus, R., Zitzmann, N., Bicknell, R., Frampton, J., Authi, K. S., Martin, A., Wakelam, M. J. and Watson, S. P. (2007) A comprehensive proteomics and genomics analysis reveals novel transmembrane proteins in human platelets and mouse megakaryocytes including G6b-B, a novel immunoreceptor tyrosine-based inhibitory motif protein. Mol Cell Proteomics 6, 548-64.

Serru, V., Le Naour, F., Billard, M., Azorsa, D. O., Lanza, F., Boucheix, C. and Rubinstein, E. (1999) Selective tetraspan-integrin complexes (CD81/alpha4beta1, CD151/alpha3beta1, CD151/alpha6beta1) under conditions disrupting tetraspan interactions. Biochem J 340 (Pt 1), 103-11.

Shoham, T., Rajapaksa, R., Boucheix, C., Rubinstein, E., Poe, J. C., Tedder, T. F. and Levy, S. (2003) The tetraspanin CD81 regulates the expression of CD19 during B cell development in a postendoplasmic reticulum compartment. J Immunol 171, 4062-72.

Shoham, T., Rajapaksa, R., Kuo, C. C., Haimovich, J. and Levy, S. (2006) Building of the tetraspanin web: distinct structural domains of CD81 function in different cellular compartments. Mol Cell Biol 26, 1373-85.

Simons, K. and Ikonen, E. (1997) Functional rafts in cell membranes. Nature 387, 569-72.

Sincock, P. M., Mayrhofer, G. and Ashman, L. K. (1997) Localization of the transmembrane 4 superfamily (TM4SF) member PETA-3 (CD151) in normal human tissues: comparison with CD9, CD63, and alpha5beta1 integrin. J Histochem Cytochem 45, 515-25.

Slupsky, J. R., Kamiguti, A. S., Rhodes, N. P., Cawley, J. C., Shaw, A. R. and Zuzel, M. (1997) The platelet antigens CD9, CD42 and integrin alpha IIb beta IIIa can be topographically associated and transduce functionally similar signals. Eur J Biochem 244, 168-75.

Soboloff, J., Spassova, M., Hewavitharana, T., He, L. P., Luncsford, P., Xu, W., Venkatachalam, K., van Rossum, D., Patterson, R. L. and Gill, D. L. (2007) TRPC channels: integrators of multiple cellular signals. Handb Exp Pharmacol, 575-91.

Sonnhammer, E. L., von Heijne, G. and Krogh, A. (1998) A hidden Markov model for predicting transmembrane helices in protein sequences. Proc Int Conf Intell Syst Mol Biol 6, 175-82.

Stathopulos, P. B., Zheng, L., Li, G. Y., Plevin, M. J. and Ikura, M. (2008) Structural and mechanistic insights into STIM1-mediated initiation of store-operated calcium entry. Cell 135, 110-22

Sterk, L. M., Geuijen, C. A., van den Berg, J. G., Claessen, N., Weening, J. J. and Sonnenberg, A. (2002) Association of the tetraspanin CD151 with the laminin-binding integrins alpha3beta1, alpha6beta1, alpha6beta4 and alpha7beta1 in cells in culture and in vivo. J Cell Sci 115, 1161-73.

Stipp, C. S., Kolesnikova, T. V. and Hemler, M. E. (2001) EWI-2 is a major CD9 and CD81 partner and member of a novel Ig protein subfamily. J Biol Chem 276, 40545-54.

Stipp, C. S., Kolesnikova, T. V. and Hemler, M. E. (2003) EWI-2 regulates alpha3beta1 integrin-dependent cell functions on laminin-5. J Cell Biol 163, 1167-77.

Sugawara, H., Kurosaki, M., Takata, M. and Kurosaki, T. (1997) Genetic evidence for involvement of type 1, type 2 and type 3 inositol 1,4,5-trisphosphate receptors in signal transduction through the B-cell antigen receptor. Embo J 16, 3078-88.

Suh, P. G., Park, J. I., Manzoli, L., Cocco, L., Peak, J. C., Katan, M., Fukami, K., Kataoka, T., Yun, S. and Ryu, S. H. (2008) Multiple roles of phosphoinositide-specific phospholipase C isozymes. BMB Rep 41, 415-34.

Takahashi, M., Sugiura, T., Abe, M., Ishii, K. and Shirasuna, K. (2007) Regulation of c-Met signaling by the tetraspanin KAI-1/CD82 affects cancer cell migration. Int J Cancer 121, 1919-29.

Takata, M., Homma, Y. and Kurosaki, T. (1995) Requirement of phospholipase C-gamma 2 activation in surface immunoglobulin M-induced B cell apoptosis. J Exp Med 182, 907-14.

Takeda, Y., Kazarov, A. R., Butterfield, C. E., Hopkins, B. D., Benjamin, L. E., Kaipainen, A. and Hemler, M. E. (2007) Deletion of tetraspanin Cd151 results in decreased pathologic angiogenesis in vivo and in vitro. Blood 109, 1524-32.

Takeda, Y., Tachibana, I., Miyado, K., Kobayashi, M., Miyazaki, T., Funakoshi, T., Kimura, H., Yamane, H., Saito, Y., Goto, H., Yoneda, T., Yoshida, M., Kumagai, T., Osaki, T., Hayashi, S., Kawase, I. and Mekada, E. (2003) Tetraspanins CD9 and CD81 function to prevent the fusion of mononuclear phagocytes. J Cell Biol 161, 945-56.

Tardif, M. R. and Tremblay, M. J. (2005) Tetraspanin CD81 provides a costimulatory signal resulting in increased human immunodeficiency virus type 1 gene expression in

primary CD4+ T lymphocytes through NF-kappaB, NFAT, and AP-1 transduction pathways. J Virol 79, 4316-28.

Tarrant, J. M., Groom, J., Metcalf, D., Li, R., Borobokas, B., Wright, M. D., Tarlinton, D. and Robb, L. (2002) The absence of Tssc6, a member of the tetraspanin superfamily, does not affect lymphoid development but enhances in vitro T-cell proliferative responses. Mol Cell Biol 22, 5006-18.

Tarrant, J. M., Robb, L., van Spriel, A. B. and Wright, M. D. (2003) Tetraspanins: molecular organisers of the leukocyte surface. Trends Immunol 24, 610-7.

Taylor, C. W., Prole, D. L. and Rahman, T. (2009) Ca(2+) channels on the move. Biochemistry 48, 12062-80.

Taylor, C. W., Rahman, T., Tovey, S. C., Dedos, S. G., Taylor, E. J. and Velamakanni, S. (2009) IP_3 receptors: some lessons from DT40 cells. Immunol Rev 231, 23-44.

Taylor, M. E. and Drickamer, K. (2006) Introduction to glycobiology, pp. xix, 255 p., Oxford University Press, Oxford.

Tedder, T. F., Inaoki, M. and Sato, S. (1997) The CD19-CD21 complex regulates signal transduction thresholds governing humoral immunity and autoimmunity. Immunity 6, 107-18.

Todaro, G. J. and Green, H. (1963) Quantitative studies of the growth of mouse embryo cells in culture and their development into established lines. J Cell Biol 17, 299-313.

Todd, S. C., Lipps, S. G., Crisa, L., Salomon, D. R. and Tsoukas, C. D. (1996) CD81 expressed on human thymocytes mediates integrin activation and interleukin 2-dependent proliferation. J Exp Med 184, 2055-60.

Todres, E., Nardi, J. B. and Robertson, H. M. (2000) The tetraspanin superfamily in insects. Insect Mol Biol 9, 581-90.

Tomlinson, M. G. (2009) Platelet tetraspanins: small but interesting. J Thromb Haemost.

Tomlinson, M. G., Calaminus, S. D., Berlanga, O., Auger, J. M., Bori-Sanz, T., Meyaard, L. and Watson, S. P. (2007) Collagen promotes sustained glycoprotein VI signaling in platelets and cell lines. J Thromb Haemost 5, 2274-83.

Tomlinson, M. G., Kane, L. P., Su, J., Kadlecek, T. A., Mollenauer, M. N. and Weiss, A. (2004) Expression and function of Tec, Itk, and Btk in lymphocytes: evidence for a unique role for Tec. Mol Cell Biol 24, 2455-66.

Treisman, R. (1996) Regulation of transcription by MAP kinase cascades. Curr Opin Cell Biol 8, 205-15.

Tuveson, D. A., Carter, R. H., Soltoff, S. P. and Fearon, D. T. (1993) CD19 of B cells as a surrogate kinase insert region to bind phosphatidylinositol 3-kinase. Science 260, 986-9.

van Spriel, A. B., Puls, K. L., Sofi, M., Pouniotis, D., Hochrein, H., Orinska, Z., Knobeloch, K. P., Plebanski, M. and Wright, M. D. (2004) A regulatory role for CD37 in T cell proliferation. J Immunol 172, 2953-61.

VanGuilder, H. D., Vrana, K. E. and Freeman, W. M. (2008) Twenty-five years of quantitative PCR for gene expression analysis. Biotechniques 44, 619-26.

Varga-Szabo, D., Braun, A., Kleinschnitz, C., Bender, M., Pleines, I., Pham, M., Renne, T., Stoll, G. and Nieswandt, B. (2008) The calcium sensor STIM1 is an essential mediator of arterial thrombosis and ischemic brain infarction. J Exp Med 205, 1583-91.

Venkataraman, L., Francis, D. A., Wang, Z., Liu, J., Rothstein, T. L. and Sen, R. (1994) Cyclosporin-A sensitive induction of NF-AT in murine B cells. Immunity 1, 189-96.

Vig, M., Peinelt, C., Beck, A., Koomoa, D. L., Rabah, D., Koblan-Huberson, M., Kraft, S., Turner, H., Fleig, A., Penner, R. and Kinet, J. P. (2006) CRACM1 is a plasma membrane protein essential for store-operated Ca2+ entry. Science 312, 1220-3.

Wang, Y., Deng, X., Zhou, Y., Hendron, E., Mancarella, S., Ritchie, M. F., Tang, X. D., Baba, Y., Kurosaki, T., Mori, Y., Soboloff, J. and Gill, D. L. (2009) STIM protein coupling in the activation of Orai channels. Proc Natl Acad Sci U S A 106, 7391-6.

Watson, S. P., Auger, J. M., McCarty, O. J. and Pearce, A. C. (2005) GPVI and integrin alphaIIb beta3 signaling in platelets. J Thromb Haemost 3, 1752-62.

Wen, W., Yan, J. and Zhang, M. (2006) Structural characterization of the split pleckstrin homology domain in phospholipase C-gamma1 and its interaction with TRPC3. J Biol Chem 281, 12060-8.

Wonerow, P. and Watson, S. P. (2001) The transmembrane adapter LAT plays a central role in immune receptor signalling. Oncogene 20, 6273-83.

Wright, M. D., Moseley, G. W. and van Spriel, A. B. (2004) Tetraspanin microdomains in immune cell signalling and malignant disease. Tissue Antigens 64, 533-42.

Wu, H., Li, J., Peng, L., Liu, H., Wu, W., Zhou, Y., Hou, Q. and Ke, D. (2000) Anti-human platelet tetraspanin (CD9) monoclonal antibodies induce platelet integrin alphaIIbbeta3 activation in a Fc receptor-independent fashion. Chin Med Sci J 15, 145-9.

Yang, T. T., Xiong, Q., Enslen, H., Davis, R. J. and Chow, C. W. (2002) Phosphorylation of NFATc4 by p38 mitogen-activated protein kinases. Mol Cell Biol 22, 3892-904.

Yang, X., Claas, C., Kraeft, S. K., Chen, L. B., Wang, Z., Kreidberg, J. A. and Hemler, M. E. (2002) Palmitoylation of tetraspanin proteins: modulation of CD151 lateral interactions, subcellular distribution, and integrin-dependent cell morphology. Mol Biol Cell 13, 767-81.

Yang, X., Kovalenko, O. V., Tang, W., Claas, C., Stipp, C. S. and Hemler, M. E. (2004) Palmitoylation supports assembly and function of integrin-tetraspanin complexes. J Cell Biol 167, 1231-40.

Yauch, R. L., Kazarov, A. R., Desai, B., Lee, R. T. and Hemler, M. E. (2000) Direct extracellular contact between integrin alpha(3)beta(1) and TM4SF protein CD151. J Biol Chem 275, 9230-8.

Yokozeki, T., Adler, K., Lankar, D. and Bonnerot, C. (2003) B cell receptor-mediated Syk-independent activation of phosphatidylinositol 3-kinase, Ras, and mitogen-activated protein kinase pathways. J Immunol 171, 1328-35.

Yoshida, T., Kawano, Y., Sato, K., Ando, Y., Aoki, J., Miura, Y., Komano, J., Tanaka, Y. and Koyanagi, Y. (2008) A CD63 mutant inhibits T-cell tropic human immunodeficiency virus type 1 entry by disrupting CXCR4 trafficking to the plasma membrane. Traffic 9, 540-58.

Young, R. K., Cailleau, R. M., Mackay, B. and Reeves, W. J. (1974) Establishment of epithelial cell line MDA-MB-157 from metastatic pleural effusion of human breast carcinoma. In Vitro 9, 239-45.

Zhang, S. L., Yeromin, A. V., Zhang, X. H., Yu, Y., Safrina, O., Penna, A., Roos, J., Stauderman, K. A. and Cahalan, M. D. (2006) Genome-wide RNAi screen of Ca(2+) influx identifies genes that regulate Ca(2+) release-activated Ca(2+) channel activity. Proc Natl Acad Sci U S A 103, 9357-62.

Zijlstra, A., Lewis, J., Degryse, B., Stuhlmann, H. and Quigley, J. P. (2008) The inhibition of tumor cell intravasation and subsequent metastasis via regulation of in vivo tumor cell motility by the tetraspanin CD151. Cancer Cell 13, 221-34.

Zoller, M. (2006) Gastrointestinal tumors: metastasis and tetraspanins. Z Gastroenterol 44, 573-86.

Zoller, M. (2009) Tetraspanins: push and pull in suppressing and promoting metastasis. Nat Rev Cancer 9, 40-55.