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

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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 and integrin  $\alpha 6\beta 1$ , but not the von Willebrand receptor GPIb $\alpha$  or the integrins  $\alpha IIb\beta 3$  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:** 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  $\alpha IIb\beta 3$ , 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 Long Kit (Invitrogen) as described previously [11]. SAGE

Abbreviations used: DIC, differential interference contrast; ECL, enhanced chemiluminescence; 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.

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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<sup>+</sup>), B-cells (CD19<sup>+</sup>), helper T-cells (CD4<sup>+</sup>), natural killer cells (CD56<sup>+</sup>), granulocytes (CD66b<sup>+</sup>) and cytotoxic T cells (CD8<sup>+</sup>) isolated from seven human volunteers, and erythroblasts and megakaryocytes differentiated from CD34<sup>+</sup> 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 (204-11) [18] and GPIIb $\alpha$  (WM23) [19], were as described, whereas those to integrins  $\alpha$ IIb (SZ22),  $\alpha$ 2 (Gi9) and  $\alpha$ 6 (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  $\mu$ g/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  $\mu$ g/ml. Images were acquired using a DMIRE2 Leica confocal microscope (Leica) with a 63 $\times$  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. Q1

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), immunoprecipitating with mouse anti-FLAG (M2) agarose beads (Sigma), washing with stringent RIPA buffer 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. Q2  
Q3

### Platelet biotinylation and biochemical analyses

Washed human platelets were suspended at a concentration of  $5 \times 10^8$  per ml in PBS containing 1 mg/ml sulfo-NHS-SS-biotin (Perbio) and gently rotated for 30 min at room temperature (?? $^{\circ}$ C). The biotinylation reaction was quenched with 100 mM glycine in PBS, and the platelets pelleted and washed once more with PBS. Platelets ( $5 \times 10^8$ ) were lysed on ice in 1 ml of either 1% Brij 97 (containing 1 mM CaCl<sub>2</sub> and 1 mM MgCl<sub>2</sub>) 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  $\mu$ g/ml 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride, 10  $\mu$ g/ml aprotinin, 10  $\mu$ g/ml leupeptin, 1  $\mu$ g/ml pepstatin A, 10  $\mu$ M NaF and 2 mM sodium orthovanadate. Lysates were pre-cleared at Q4

4°C for 30 min each with 10  $\mu$ l Protein G sepharose (Sigma) followed by addition of 10  $\mu$ l Protein G sepharose pre-coupled with 1  $\mu$ g of either MOPC mouse or normal rabbit control Ig. Pre-cleared lysates were immunoprecipitated at 4°C for 90 minutes with specific antibodies (1AA2 for CD9, 11B1 for CD151, rabbit anti-Tspan9, 4F10 for  $\alpha$ 6 and Gi9 for  $\alpha$ 2), pre-coupled to 10  $\mu$ l 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).

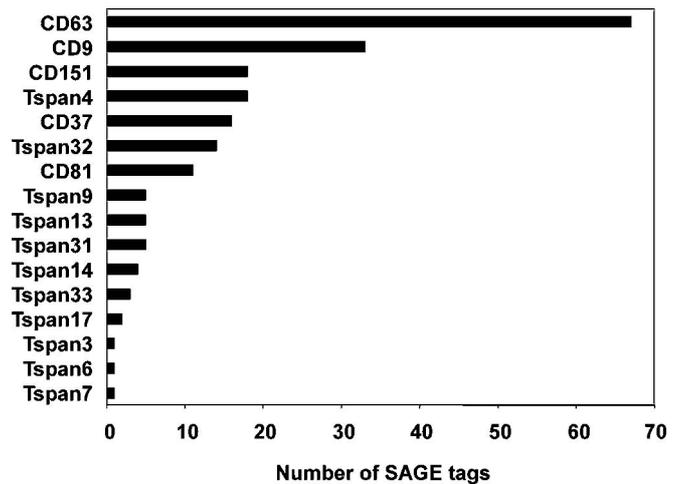
## RESULTS

### 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.

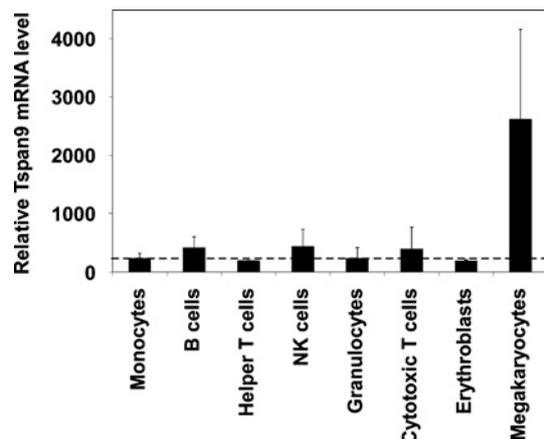
### Tspan9 mRNA is relatively specific to human megakaryocytes versus other haematopoietic cells

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



**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.



**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.

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/416/bj416ppppadd.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.

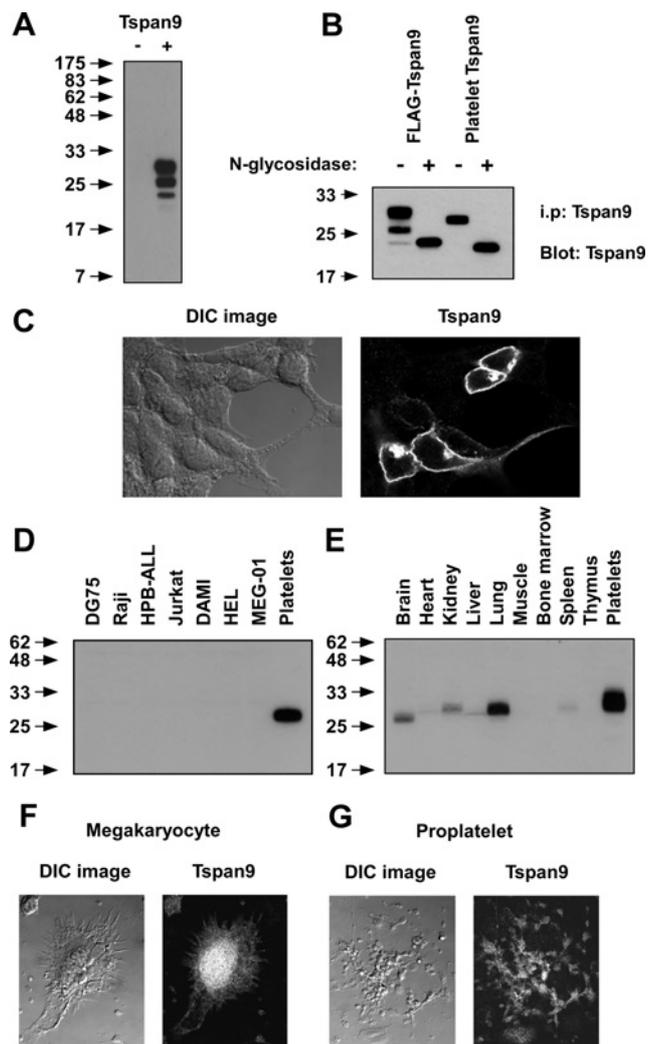
### Tspan9 protein is expressed in megakaryocytes and platelets

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 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

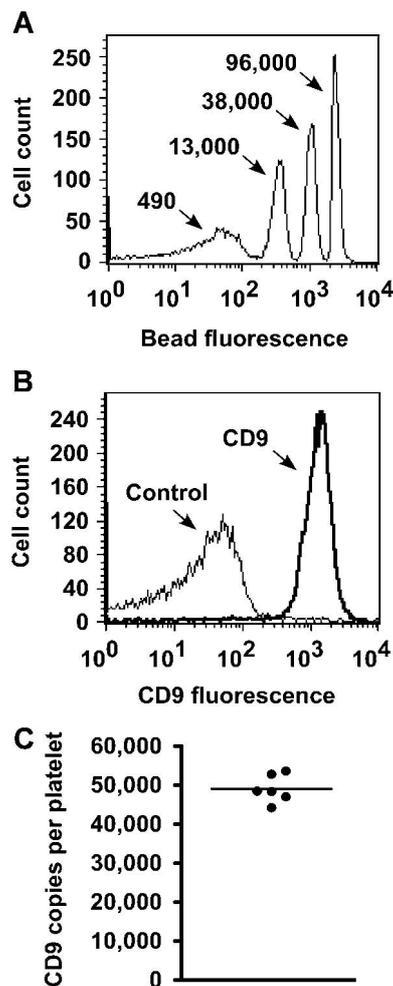
Quantification of Tspan9 expression levels on platelets may provide clues as to potential binding partners, which may be expressed at similar levels. The human platelet cell surface is relatively well characterized with respect to copy numbers of the major glycoproteins, but amongst tetraspanins only CD9 has been quantified. Three different groups, using iodinated CD9 antibodies, reported 65 000 [31], 46 000 [32] and 38 000 [33] copies per platelet. To determine whether these discrepancies were due to donor variation, we quantified CD9 levels on the 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



**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).

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



**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 Biotec. (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.

the platelet cell surface, compared to the two other major glycoproteins, integrin  $\alpha$ IIb $\beta$ 3 (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 simi-

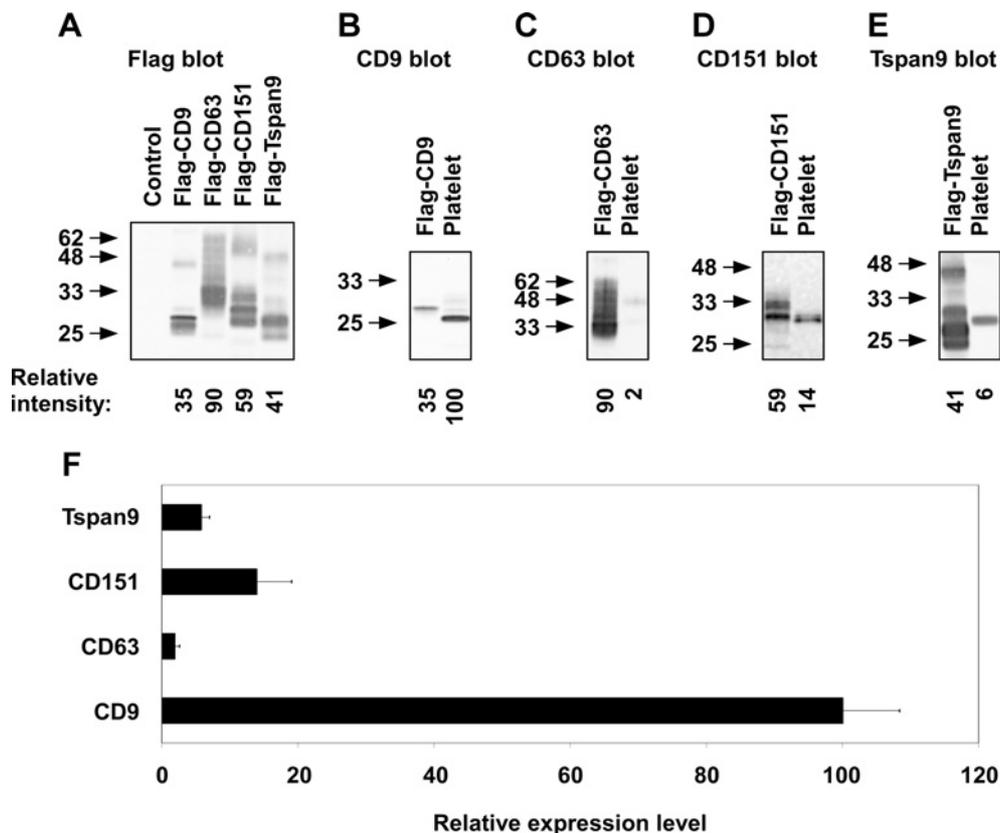
lar expression between donors (Figure 5). In comparison, CD151 was expressed at a 2.3-fold greater level than Tspan9, whereas CD63 was 3-fold lower than Tspan9 (Figure 5). These results suggest that the Tspan9 expression level, similar that of other tetraspanins, is tightly regulated in human platelets, 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  $\alpha$ 6 $\beta$ 1 [4] (Figure 6A, left panel). Consistent with its relatively high expression level, CD9 immunoprecipitation yielded more intense bands. As a control, the collagen-binding  $\alpha$ 2 $\beta$ 1 integrin, which is not thought to be tetraspanin-associated [4], was predominantly immunoprecipitated as a doublet corresponding to the molecular weights of the integrin  $\alpha$ 2 and  $\beta$ 1 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  $\alpha$ 6 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  $\alpha$ 6 immunoprecipitates, but not in those for control antibody or  $\alpha$ 2, 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 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



**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  $\pm$  S.D.

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 GPIb $\alpha$ or $\alpha$ IIB $\beta$ 3

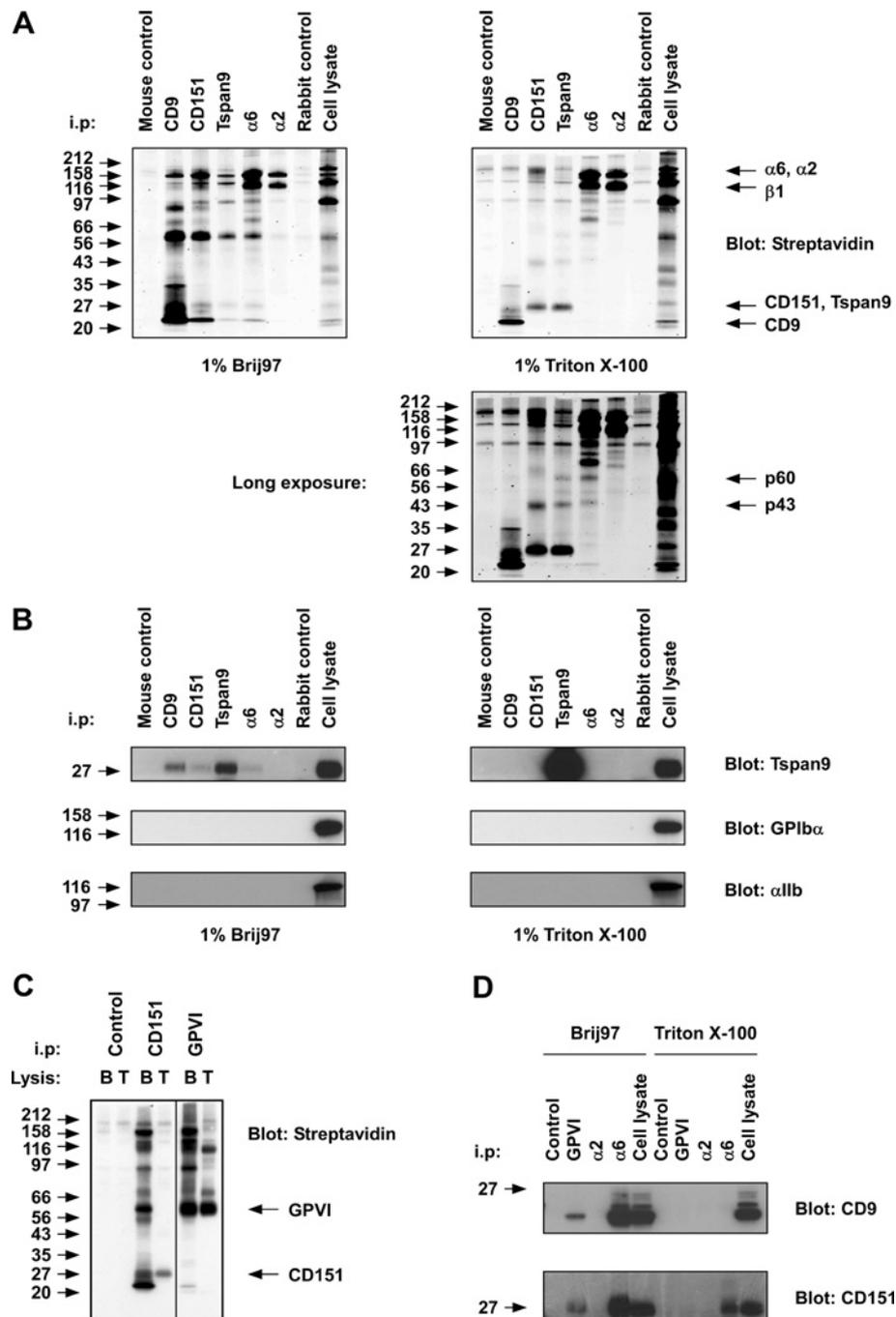
The identities of most of the tetraspanin-associated platelet proteins, observed in tetraspanin immunoprecipitations from Brij 97 lysates (Figure 6A, left panel), are not known, but their molecular weights suggest as candidates the major platelet glycoproteins GPIb $\alpha$  (the von Willebrand receptor),  $\alpha$ IIB $\beta$ 3 (the major platelet integrin) and GPVI (the main signalling receptor for collagen). However, GPIb $\alpha$  and  $\alpha$ IIB $\beta$ 3 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 kD band in GPVI immunoprecipitates and a major 27 kD 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  $\alpha$ 2 antibodies. These interactions were lost in Triton X-100 lysates. The GPVI–tetraspanin interaction appeared to be less robust than the positive control  $\alpha$ 6 $\beta$ 1–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 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



**Figure 6** Tspan9 is a component of platelet tetraspanin microdomains that include GPVI and  $\alpha 6\beta 1$ , but not  $\alpha IIb\beta 3$ ,  $\alpha 2\beta 1$  or GPIb $\alpha$

(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, GPIb $\alpha$  and  $\alpha IIb$  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,  $\alpha 2$  and  $\alpha 6$  immunoprecipitations were Western blotted with CD9 and CD151 antibodies as described in (B).

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.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  $\alpha$ IIb $\beta$ 3 (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  $\alpha$ IIb $\beta$ 3. The latter result was somewhat surprising, since  $\alpha$ IIb $\beta$ 3 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/Fc $\gamma$  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.

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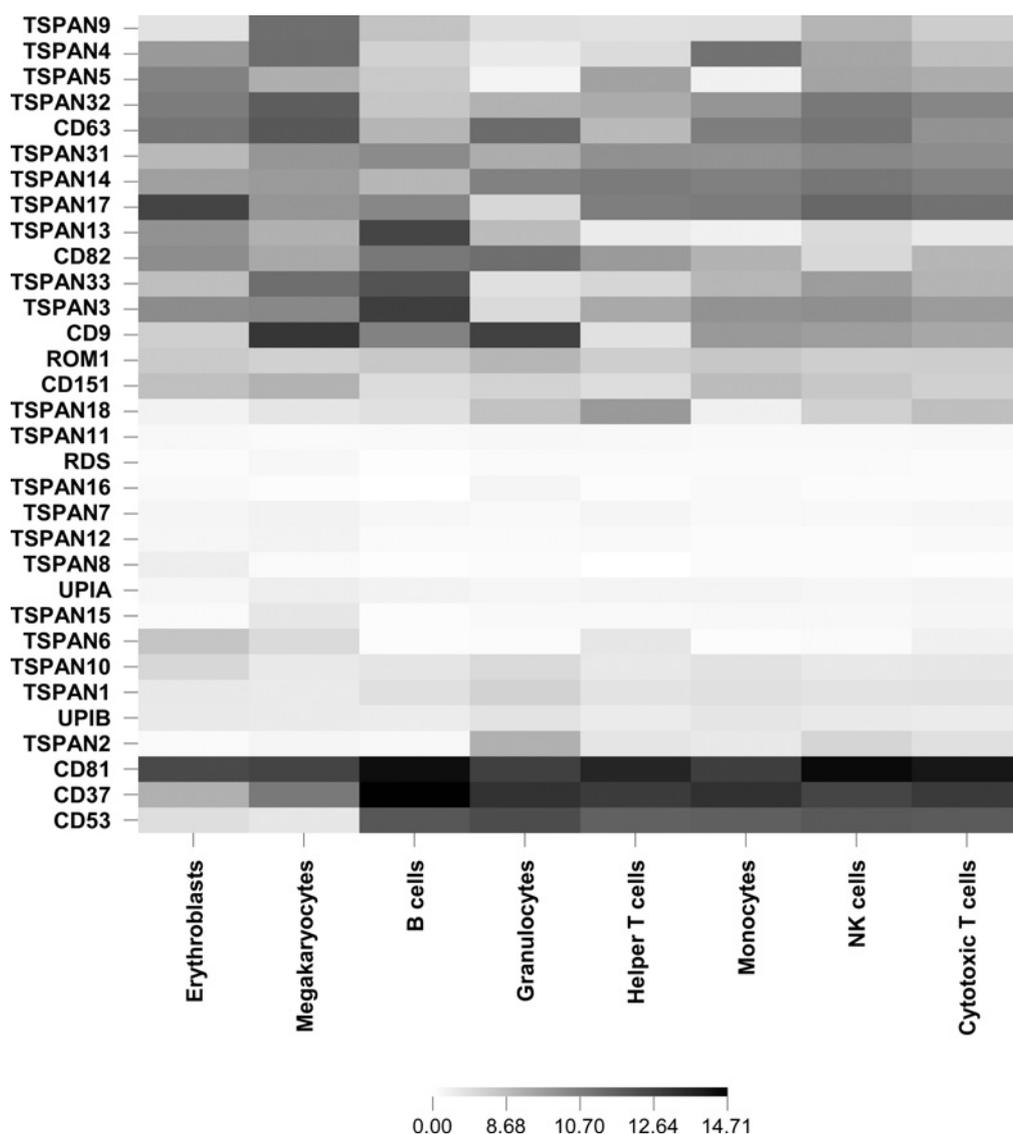
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## SUPPLEMENTARY ONLINE DATA

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

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**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 (Log<sub>2</sub>) for each tetraspanin are presented as a heat map, with white panels representing no expression and black panels representing the highest expression level.

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