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Vav family proteins are required for optimal regulation of PLCγ2 by integrin αIIbβ3

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Abstract

Vav proteins belong to the family of guanine nucleotide exchange factors for the Rho/Rac family of small G proteins. In addition, they serve as important adapter proteins for the activation of PLCγ isoforms by ITAM receptors, including the platelet collagen receptor GPVI. Vav proteins are also regulated downstream of integrins, including the major platelet integrin αIIbβ3, which has recently been shown to regulate PLCγ2. In the present study we have investigated the role of Vav family proteins in filopodia and lamellipodia formation on fibrinogen using platelets deficient in Vav1 and Vav3. Wild type mouse platelets undergo a limited degree of spreading on fibrinogen, characterised by formation of numerous filopodia and limited lamellipodia structures. Platelets deficient in Vav1 and Vav3 exhibit reduced filopodia and lamellipodia formation during spreading on fibrinogen. This is accompanied by reduced αIIbβ3-mediated PLCγ2 tyrosine phosphorylation and reduced Ca^{2+} mobilisation. In contrast, the G protein agonist thrombin stimulates full spreading of control and Vav1/3-deficient platelets. Consistent with this, stimulation of F-actin formation and Rac activation by thrombin is not altered in Vav deficient cells. These results demonstrate that Vav1 and Vav3 are required for optimal spreading and regulation of PLCγ2 by integrin αIIbβ3, but that their requirement is by-passed upon G protein receptor activation.

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Introduction

Platelets are small anucleate cells that circulate in a quiescent state in the vasculature. Following vascular damage, platelets are recruited to the site of injury and undergo explosive activation to stem the loss of blood from the wound. Following tethering, platelets are activated by prolonged close proximity of the platelet immunoglobulin receptor GPVI with subendothelial collagen (reviewed in[1]). GPVI stimulates an increase in the affinity of the major platelet integrin αIIbβ3 for its ligands, von Willebrand factor (VWF) and fibrinogen. Outside-in signalling by the integrin contributes to spreading over the subendothelial matrix and recruitment of additional platelets which become crosslinked by fibrinogen, thereby generating a vascular plug [2]. Finally, the clot is retracted and stabilised by an active process dependent on αIIbβ3 and the cytoskeleton [3].

The Vav family of GTP exchange factors (GEFs) consists of three members [4-7] which share a common structural arrangement. The amino terminus contains a calponin homology domain and an acidic region, which contains regulatory tyrosine phosphorylation sites. This is followed by Dbl homology, pleckstrin homology (PH) and zinc finger domains, which form the GDP/GTP exchange factor region of Vav family proteins. The C terminal portion contains a short proline rich region and an SH3-SH2-SH3 region. Vav2 and Vav3 are widely expressed, whereas Vav1 is specifically expressed in haematopoietic cells [5-7]. The guanine nucleotide exchange activity of Vav proteins is specific for the Rho family of small G proteins. The individual Vav proteins have specificity towards different Rho family G proteins. For example, Vav1 selectively activates Rac1, Rac2 and RhoG, whereas Vav2 and Vav3 show less activity towards Rac1 [6, 8]. The GTP/GDP exchange activity of all three Vav proteins is modulated through tyrosine phosphorylation of regulatory tyrosine residues by Src and Syk family kinases [9].

Vav proteins are prominent tyrosine kinase substrates downstream of ITAM receptors, including the platelet collagen receptor GPVI, and the T- and B-cell antigen receptors [6, 9-12]. The importance of Vav in signalling by ITAM receptors has been demonstrated by studying mice that lack individual or combinations of Vav family proteins [13-21]. In all cases, Vav proteins have been shown to play a critical role in the regulation of PLCγ by ITAM receptors. Vav1 deficient T cells have significantly reduced TCR signalling with a more pronounced phenotype observed in Vav1/3$^{-/-}$ and

Vav1/2/3$^{-/-}$ cells. B cells deficient in Vav1 have a mild reduction in BCR signalling, while Vav1/2$^{-/-}$ cells have a severe phenotype that is even more pronounced in Vav1/2/3-deficient cells [17, 18, 20]. These observations therefore demonstrate a limited redundancy between the three members of the Vav family.

Vav1 and Vav3 are expressed at significant levels in platelets, whereas there is only residual expression of Vav2 [10, 11, 22]. In striking contrast to B- and T-cells, however, Vav1 and Vav3 are completely redundant in platelets[11]. Platelets deficient in Vav1 or Vav3 respond normally in response to GPVI stimulation, whereas cells deficient in both Vav isoforms display a severe block in activation through the collagen receptor[10, 11]. Vav2 is not phosphorylated in platelets and cells deficient in Vav1, 2 and 3 exhibit a similar defect in response to GPVI agonists as platelets deficient in Vav1 and 3[10, 11].

Importantly, Vav family proteins have been demonstrated to play a role in ITAM signalling independent of GEF activity and Rac activation, most notably through regulation of PLCγ isoforms downstream of the antigen receptors [12, 21, 23-26]. This action has been attributed to the adapter function of Vav isoforms in the formation and stabilisation of signalling complexes [21, 23, 27-29]. There appear to be at least two pathways through which Vav regulates PLCγ downstream of ITAM receptors, namely via regulation of Tec family kinases and through assembly of a LAT-Gads-SLP-76-PLCγ signalosome [12, 21, 23-26, 30]. The regulation of Tec family kinases is dependent on PI 3-kinase and has been proposed to be regulated through Rac1 [30-32]. On the other hand, the role of Vav in the assembly of the LAT-Gads-SLP-76-PLCγ signalosome is independent of PI 3-kinase and, presumably, Rac activation. In addition, Rac has been shown to directly activate PLCγ isoforms *in vitro* independently of PI3 kinase [33]. Therefore Vav is able to control PLCγ activity via at least 2 pathways that differ in their dependence on PI 3-kinase and Rac, although the relative contribution of these pathways is unclear.

Vav family proteins have been shown to be tyrosine phosphorylated downstream of β1, β2 and β3 integrins in a number of haematopoietic cells, including platelets and in CHO cells transfected with αIIbβ3 [22, 34-38]. Furthermore, Vav proteins have been shown to be critical for outside-in signalling by β2 integrins in neutrophils and to contribute to their stable adhesion and spreading [39]. Vav proteins

have also been shown to bind to tyrosine phosphorylated β3 in a K562 cell line model of αvβ3 function [40].

In platelets the integrin αIIbβ3 activates a signalling pathway that uses many of the same proteins as ITAM receptors (reviewed in Watson *et. al.*[41]) including Src kinases [22, 42], Syk [22, 43], SLP-76 [44] and PLCγ2[45-47]. Platelets deficient in these proteins do not spread on fibrinogen, demonstrating the critical importance of outside-in signalling by the integrin for the spreading response. Vav1 and Vav3 undergo tyrosine phosphorylation downstream of αIIbβ3 when platelets spread on fibrinogen [22] and when platelets undergo aggregation in suspension [37]. In addition, Vav proteins interact with several of the membrane proximal signalling proteins in the αIIbβ3-signalling cascade, including Syk[48] and SLP-76[49-51].

These observations raise the possibility that Vav family proteins may play a role in the regulation of PLCγ2 by integrin αIIbβ3 in platelets. This hypothesis has been investigated in the present study using platelets from mice deficient in Vav1 and Vav3 to assess the role of Vav family proteins in αIIbβ3 outside-in signalling. We show that Vav1/3-deficient platelets have reduced spreading on fibrinogen compared to wild type platelets, which is associated with defective PLCγ2 phosphorylation and Ca^{2+} mobilisation. Pre-treatment of platelets with thrombin overcomes the spreading defect in Vav1/3 deficient platelets. These results demonstrate that Vav family proteins are required for normal regulation of PLCγ2 by αIIbβ3 and for optimal platelet spreading on fibrinogen.

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Materials and Methods

Antibodies and reagents: Anti-phosphotyrosine monoclonal antibody (mAb) 4G10 and anti-Rac mAb were purchased from Upstate Biotechnology (TCS Biologicals Ltd., Bucks, UK). The anti-PLCγ2 and anti-Syk pAbs were kindly supplied by Dr. Mike Tomlinson (DNAX, Palo Alto, CA., USA). The FITC-conjugated rat anti-mouse αIIb (MwReg30), β3 (Luc.A5) and αIIbβ3 (Leo.D2) antibodies and rat IgG were purchased from Emfret Analytics (Wurzburg, Germany). The cDNA for GST-PAK Crib domain was a kind gift from Dr Doreen Cantrell (ICRF, London, UK). FITC-phalloidin and Oregon Green Bapta1-AM were from Molecular Probes (Leiden, The Netherlands). All other reagents were purchased from Sigma (Poole, UK) or obtained from previously described sources[43, 52].

Animals: The generation of mice disrupted in the *vav1* gene ($Vav1^{-/-}$) is described in Turner *et al.* [53]. The generation of mice disrupted in the *vav2* gene ($Vav2^{-/-}$) is described in Doody *et. al.* [17].The generation of mice disrupted in the *vav3* gene ($Vav3^{-/-}$) is described in Fujikawa *et al.* [20]. Compound knockout mice were generated by appropriate crossing of the individual knockout genotypes. Mutant and control mice were age and background matched. All animals were maintained using housing and husbandry in accordance with local and national legal regulations.

Preparation of mouse platelets: Blood was taken from a terminally CO_2-narcosed mouse by cardiac puncture on the day of the experiment into 1:10 (v:v) ACD (85 mM sodium citrate, 110 mM glucose, 71 mM citric acid). Blood was diluted 1:6 (v:v) in Tyrodes-HEPES buffer (134 mM NaCl, 0.34 mM Na_2HPO_4, 2.9 mM KCl, 12 mM $NaHCO_3$, 20 mM HEPES, 5 mM glucose, 1 mM $MgCl_2$, pH 7.3) and centrifuged at 200g to obtain platelet rich plasma (PRP). PRP was centrifuged in the presence of 0.1μg/ml prostacyclin at 1000g and the platelet pellet was resuspended in Tyrodes-HEPES.

Flow cytometry staining: Washed platelets ($1x10^7$/ml) were stained with the indicated FITC-conjugated antibodies for 15 minutes at room temperature. Staining

was quenched with 400µl tyrodes-HEPES buffer and samples were analysed using a FACScalibur flow cytometer and CellQuest software (Becton Dickinson, UK).

Static adhesion spreading assay: Glass coverslips were coated with 100 µg/ml fibrinogen solution overnight at 4^{o}C followed by washing with PBS. Slides were then blocked using 0.5% heat denatured BSA in PBS for 1 hour at room temperature, followed by washing in PBS. Mouse platelets, resuspended at a concentration of 3 x 10^7 platelets/ml in Tyrodes-HEPES containing 2U/ml apyrase and 10µM indomethacin, were transferred to the coverslips and incubated at 37^{o}C for 45 min in a humid atmosphere. Excess platelets were removed and the adherent platelets were fixed with 3.7% paraformaldehyde for 10 min at room temperature. The coverslips were washed in PBS, mounted using HydroMount Mounting Medium (National Diagnostics, Hull, UK) and viewed under differential interference contrast microscopy (DIC) under a 63x oil immersion lens and Slidebook software (Intelligent Imaging Innovations, Colorado, USA). Surface areas were calculated using a Java plug in for ImageJ software.

Immunoprecipitation and immunoblotting: Platelets at 2x10^8/ml were incubated over fibrinogen-coated dishes for 45 minutes at 37°C in the presence of 2U/ml apyrase and 10µM indomethacin. Non-adherent basal platelets were removed and lysed in an equal volume of 2x lysis buffer (2% NP-40, 300 mM NaCl, 20 mM Tris, 2 mM EDTA, 2mM EGTA, 2 mM Na_3VO_4, 200µg/ml AEBSF hydrochloride, 10 µg/ml leupeptin, 10 µg/ml aprotinin, and 1 µg/ml pepstatin A, pH 7.4). Adherent platelets were lysed in a final volume of 1ml 1x lysis buffer. Insoluble cell debris was removed by centrifugation for 5 min at 13,000g, 4°C and cell lysates were precleared using protein A-Sepharose. Platelet lysates were incubated with the indicated primary antibodies and resulting protein complexes and immunoprecipitates were resolved by SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes. Immunoblotting was performed as described previously[11] with detection by enhanced chemiluminescence (ECL, Amersham Biosciences, Bucks, UK).

Rac activation assay: Rac activity was measured as described previously[10]. The CRIB domain of PAK1 (amino acids 67-150) was expressed as a GST fusion protein and bound to glutathione sepharose beads. Platelet stimulations were stopped with an equal volume of 2x Rac assay lysis buffer (2% (v:v) NP-40, 1% (w:v) N-octyl glucoside, 300 mM NaCl, 20 mM Tris, 2 mM EDTA, 2mM EGTA, 20 mM $MgCl_2$, 2 mM Na_3VO_4, 200μg/ml AEBSF hydrochloride, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 μg/ml pepstatin A, pH 7.4). Insoluble material was removed by centrifugation (5 minutes, 13000 rpm, 4°C) and freshly prepared GST-PAK1 was added to the samples and incubated for 1 hour at 4°C. The beads were then washed with 1x Rac assay lysis buffer and the bound protein taken up into Laemmli buffer. The resulting samples were separated by 12% SDS-PAGE and transferred to PVDF membranes for immunoblotting as described above.

F-actin assay: Filamentous actin was measured using a modification of the method of Machesky and Hall as previously described[54]. Briefly, platelets at a concentration of $2x10^8$/ml were fixed with an equal volume of 3.7% formaldehyde containing FITC-phalloidin (20 mM KH_2PO_4, 10 mM Pipes, 5 mM EGTA, 2 mM $MgCl_2$, 0.1% Triton X-100, 3.7% formaldehyde, 2μM FITC-phalloidin) and rotated for 1 hour at room temperature. Platelets were then pelleted for 2 minutes in a microcentrifuge and washed in 0.1% saponin, 20 mM KH_2PO_4, 10 mM Pipes, 5 mM EGTA, 2 mM $MgCl_2$. Platelets were rotated in methanol for 1 hour to extract the FITC-phalloidin. FITC-phalloidin binding was measured by measuring the emission at 520 nm using an excitation wavelength of 488 nm on a spectrofluorimeter.

Ca^{2+} mobilisation assay: Platelets were incubated with 15μM Oregon Green BAPTA 1-AM in DMSO containing 1.2mg/ml pluronic acid for 90 minutes in the dark at room temperature. Excess dye was removed by washing the platelets in Tyrodes-HEPES buffer in the presence of 0.1μg/ml prostacyclin. Platelets were allowed to settle onto fibrinogen-coated coverslips in the presence of 2U/ml apyrase and 10μM indomethacin and imaged in real time using the 63x oil immersion lens on a Zeiss Axiovert 200M microscope using Slidebook software (Intelligent Imaging Innovations, Colorado, USA).

Analysis of data: Experiments were performed on at least 3 occasions and are shown as representative data. Where appropriate data is shown +/- Standard Error. Where statistical significance is indicated data has been subjected to student t test. For comparison of surface areas of platelets in table 1A where multiple comparisons are required, Tukey test has been used. $p<0.01$ was selected to represent statistical significance.

Results

Vav1/3$^{-/-}$ platelets exhibit reduced spreading on fibrinogen

To investigate whether Vav family proteins are involved in outside-in signalling by αIIbβ3, platelets from wild type and Vav1/3$^{-/-}$ mice were incubated on fibrinogen-coated coverslips for 45 minutes and imaged by DIC microscopy. These experiments were carried out in the presence of apyrase and indomethacin to block the effects of the secreted G protein-coupled secondary agonists, ADP and thromboxane A_2. Under these conditions, wild type mouse platelets undergo partial spreading with formation of filopodia and limited lamellipodia-like structures (Figure 1A). This limited spreading response has previously been shown to be independent of activation of Rac, as shown using Rac1/Rac2-deficient platelets [55], but dependent on PLCγ2 [45]. Vav1/3-deficient platelets adhere with the same efficiency as wild type platelets (Figure 1B). However, in contrast to wild type platelets, Vav1/3$^{-/-}$ platelets do not form lamellipodia and have less filopodia than wild type cells (Figure 1A). The number of filopodia formed by wild type and Vav1/3-deficient platelets were counted and plotted as a bar chart. Vav1/3-deficient platelets exhibit significantly less filopodia than wild type cells (Figure 1C). The surface area of each set of platelets was measured and plotted as a frequency distribution. The distribution of surface area of Vav1/3$^{-/-}$ platelets is shifted slightly to the left relative to wild type platelets (Figure 1D), thereby demonstrating a small, but significant reduction in surface area as shown in Table 1. Platelets deficient in Vav1 or Vav3 alone exhibited an intermediate phenotype with a slightly lower average surface area than wild type platelets (Table 1A). The surface area of Vav1/3$^{-/-}$ platelets was significantly reduced relative to the single knockouts demonstrating a degree of redundancy in the role of these proteins in platelet spreading on fibrinogen (Table 1A). Consistent with a residual expression of Vav2 in platelets, the defect in spreading in Vav1/2/3-deficient platelets was not significantly different to that in Vav1/3-deficient platelets (Table 1A).

A potential explanation for the decreased spreading of Vav1/3$^{-/-}$ platelets on fibrinogen could be due to reduced surface expression of the fibrinogen receptor αIIbβ3 in these cells. To investigate this, we have compared the levels of the integrin on wild type and Vav1/3-deficient platelets by staining with antibodies to each of the subunits of the integrin and to the integrin complex. Expression of αIIb, β3 and

αIIbβ3 is identical between wild type and Vav1/3-deficient platelets (Figure 1E). These results demonstrate that the defective spreading of Vav1/3-deficient platelets on fibrinogen is not due to a lower expression level of the receptor.

Activation of PLCγ2 by αIIbβ3 is impaired in Vav1/3$^{-/-}$ platelets

Platelet spreading on fibrinogen has previously been shown to be critically dependent on Ca^{2+}, PKC and PLCγ2 [45, 56]. Since Vav family proteins have been shown to be critical for regulation of PLCγ2 by ITAM receptors in platelets and in other cells, we investigated whether a defect in PLCγ2 regulation by the integrin could underlie the reduced spreading on fibrinogen that is observed in the combined absence of Vav1 and Vav3. Wild type and Vav1/3$^{-/-}$ platelets were incubated over a fibrinogen-coated surface for 45 min, before removal and lysis of basal (non-adherent) and stimulated (adherent) platelets. Following lysis and immunoprecipitation, PLCγ2 phosphorylation was measured by western blotting using the antiphosphotyrosine antibody, 4G10. Wild type platelets exhibit a robust increase in tyrosine phosphorylation of PLCγ2 when spread on fibrinogen, which is reduced by approximately 50% in Vav1/3$^{-/-}$ platelets (Figure 2A). In contrast, tyrosine phosphorylation of the upstream kinase Syk is not significantly different in wild type and Vav1/3$^{-/-}$ platelets (Figure 2A). These results suggest that the same amount of signal is transmitted by the receptor into the cell but that the efficiency of transmitting this signal to PLCγ2 is reduced in the absence of Vav1/3.

In order to investigate if the defect in platelet spreading is due to the defect in signalling of αIIbβ3 to PLCγ2, we assessed the ability of Vav1/3$^{-/-}$ platelets to mobilise Ca^{2+} during spreading on fibrinogen. Platelets from wild type and Vav1/3-deficient mice were labelled with the Ca^{2+} reporter dye Oregon Green BAPTA 1-AM. This dye is a highly sensitive Ca^{2+} reporter dye that can be used to detect fluctuations in intracellular Ca^{2+} levels without chelating sufficient Ca^{2+} to block Ca^{2+}-dependent functional responses (data not shown and [57, 58]). Labelled platelets were incubated over fibrinogen-coated coverslips and spreading was monitored by fluorescence microscopy. Wild type platelets exhibit a series of oscillations in fluorescence, indicative of transient elevation of cytosolic Ca^{2+} as shown in Figure 2B and the supplementary video Figure S1A. In contrast, Vav1/3$^{-/-}$ platelets exhibit significantly less Ca^{2+} transients over a seven minute recording period as shown by the single cell

records (Figure 2B and supplementary video Figure S1B) and pooled data (Figure 2C). These results demonstrate that Vav1 and Vav3 are required for αIIbβ3-mediated Ca^{2+} mobilisation, regulated downstream of PLCγ2.

Vav1 and Vav3 do not regulate Rac in fibrinogen or thrombin stimulated platelets

In addition to their role in regulating PLCγ2 downstream of ITAM receptors, Vav family proteins also function as GTP exchange factors for Rho family G proteins. The defect in spreading that is observed in the absence of Vav1 and Vav3 is unlikely to be due to blockade of Rho activity as platelets are not thought to form stress fibres under these conditions. Furthermore, we have been unable to detect activation of Rho in platelets that have adhered to fibrinogen. However, we have also been unable to detect activation of Cdc42 during adhesion of human or murine platelets to fibrinogen (data not shown). This may reflect that the small G protein is not activated by the integrin or that it is activated at a level below that required for detection. We have previously reported that spreading of mouse platelets on fibrinogen is independent of activation of Rac as shown using Rac1/2-deficient mice [55]. Furthermore, we were unable to detect activation of Rac1/2 in human and mouse platelets using GST-PAK, which selectively binds to the GTP-bound form of the protein, to precipitate active Rac [55]. In the present study we have been able to detect weak activation of Rac in murine platelets that have spread on fibrinogen (Figure 3A). In order to investigate if Rac activation by the integrin is regulated by Vav1/3, Rac activation in Vav1/3-deficient platelets was assayed. αIIbβ3 induced Rac activation is normal in Vav1/3 deficient platelets (Figure 3A). These results demonstrate that Vav1/3 does not lie upstream of Rac in αIIbβ3 signalling.

In contrast to signalling by the integrin, thrombin induces robust activation of Rac. In order to investigate whether Vav1/3 lies upstream of Rac in thrombin-stimulated platelets, active Rac was precipitated from thrombin stimulated platelets as above. Activation of Rac by thrombin is not altered in the absence of Vav1 and Vav3 (Figure 3B). Furthermore, thrombin induced F-actin polymerisation is normal in Vav1/3 deficient platelets (Figure 3C). These results demonstrate that Vav1/3 does not lie upstream of Rac or actin assembly in thrombin stimulated platelets. Importantly this is consistent with the observation that thrombin induces full

spreading and formation of extensive lamellipodia in both wild type and Vav1/Vav3-deficient platelets (Figure 3D) as demonstrated by the indistinguishable frequency distribution curves of platelet surface area (Figure 3E and Table 1).

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Discussion

In the present study we have investigated the role of Vav family proteins in αIIbβ3 mediated platelet activation and spreading on fibrinogen. Platelets deficient in the major Vav family proteins, Vav1 and Vav3, have reduced spreading on fibrinogen, in association with reduced tyrosine phosphorylation of PLCγ2 and reduced elevation of intracellular Ca^{2+}. These results therefore demonstrate a critical role for Vav1 and Vav3 in the regulation of PLCγ2 by αIIbβ3, as has previously been shown to be the case for regulation of PLCγ2 by the ITAM-coupled collagen receptor, GPVI [11]. The defect in spreading in the Vav1/3$^{-/-}$ platelets is by-passed in the presence of the G protein receptor agonist, thrombin, which stimulates powerful activation of PLCβ isoforms in platelets. Thus, the role of Vav1 and Vav3 in mediating spreading on fibrinogen is specifically linked to αIIbβ3-mediated outside-in signalling.

Vav family proteins have been implicated in integrin-dependent responses in a variety of cells [34, 39, 40]. Vav-deficient neutrophils display defective β2-integrin mediated spreading and phagocytosis [39], a K562 model of αvβ3-mediated cell adhesion demonstrates recruitment of Vav1 to phosphorylated β3-subunit in fibronectin-adherent cells [40] and CHO cells over-expressing αIIbβ3, Syk, SLP-76 and Vav1 form extensive lamellipodia on fibrinogen [38]. In the present study, we demonstrate that Vav1 and Vav3 are required for normal platelet spreading on fibrinogen, as a consequence of a reduction in αIIbβ3-mediated activation of PLCγ2 and Ca^{2+} mobilisation. Since β2 integrins in neutrophils also exhibit PLCγ2 dependent Ca^{2+} mobilisation [59], we therefore suspect that Vav proteins are likely to be involved in PLCγ2 regulation by β2-integrins in neutrophils and that this contributes to the defective functional responses in these cells [39]. Interestingly, platelet responses to the G protein coupled agonist thrombin are not affected by deficiency of Vav1 and Vav3. Similarly, neutrophil responses to G protein coupled chemoattractants are normal in Vav deficient cells suggesting that Vav is also dispensable for GPCR signalling in these cells[39].

It is now recognised that integrins use many of the same signalling proteins as ITAM receptors[41]. In platelets, for example, critical roles for Src[22, 42] and Syk[22] tyrosine kinases, the adapter protein SLP-76[44] and PLCγ2[45-47] have been reported downstream of both GPVI and αIIbβ3. Based on the data presented here, a further similarity in the signalling pathways activated by these two classes of

receptors is the requirement of Vav proteins for efficient regulation of PLCγ2[10, 11]. It is noteworthy that Vav proteins appear to be essential for optimal regulation of PLCγ isoforms by all ITAMs. It will therefore be of interest to know whether Vav proteins are required downstream of all integrins for the regulation of PLCγ. Interestingly, we have also recently demonstrated a role for Vav1 and Vav3 in the activation of PLCγ2 by the C-type lectin family receptor, CLEC-2, in platelets which has a single rather than dual YXXL motif in its cytosolic tail[60]. There are however significant differences in signalling by these three classes of receptor in platelets. For example, GPVI signalling takes place in lipid rafts and is critically dependent on the transmembrane adaptor protein, LAT [61, 62], whereas signalling by αIIbβ3 is independent of these specialised membrane domains and LAT [45, 62]. In comparison, signalling by CLEC-2 is partially dependent on the adapters LAT and SLP-76, whereas the latter is essential for responses to GPVI.

Cell spreading involves extensive cytoskeletal re-modifications, which can be regulated by Rho family small G proteins. The small G proteins Cdc42, Rac and Rho are implicated in the formation of filopodia, lamellipodia and stress fibres, respectively [63-65]. In the present study we have assayed spreading directly mediated by the integrin, by performing all studies in the presence of apyrase and indomethacin. Under these conditions, mouse platelets undergo a limited spreading response that is dependent on PLCγ2 [45] but independent of Rac [55]. Consistent with this, we have been able to detect only weak activation of Rac by αIIbβ3 in platelets that have undergone spreading on fibrinogen and this activation is not reduced in Vav1/3-deficient platelets. These results demonstrate that the defective regulation of PLCγ2 by αIIbβ3 in platelets is not due to defective regulation of Rac. Interestingly, we have been unable to detect activation of Cdc42 (data not shown) during spreading on fibrinogen despite robust formation of filopodia. This may represent a low level of activation of the small G protein that is below the level of detection or may represent Cdc42 independent formation of filopodia, as has been reported in other cells [66, 67]. We can therefore not rule out the possibility that the defect in spreading on fibrinogen in the absence of Vav1 and Vav3 is, at least partially, due to a direct inhibition of Cdc42 activation or a related G protein, although it should be noted that a role of Vav family GTP exchange factors in the regulation of Cdc42 is controversial [6, 8, 68-71]. It is also extremely unlikely that the spreading

defect of Vav1/3 deficient platelets is due to impairment of activation of Rho as there is no evidence of stress fibre formation in platelets spread under these conditions. Under physiological conditions the limited spreading response initiated by αIIbβ3 is reinforced by G protein coupled agonists such as ADP and thrombin leading to Rac activation and Rac-dependent full spreading [10, 55]. In the present study we have shown that Vav proteins are not required for G protein coupled receptor signalling leading to Rac activation or actin polymerisation, suggesting that this process would occur normally in Vav deficient mice.

It is well established that Vav family proteins have GEF-independent functions thought to be due to their role as adaptor proteins and stabilising signalling complexes [21, 23, 27, 28]. Indeed, Vav family proteins have been shown to be required for normal formation of the LAT-Gads-SLP-76-PLCγ2 signalosome downstream of the T cell receptor [23]. It is likely that this function of Vav proteins is responsible for their critical role in the regulation of PLCγ isoforms by ITAM receptors, including GPVI. Demonstration of inhibition of PLCγ2 downstream of αIIbβ3 in the present study provides strong evidence that this mechanism also applies to the regulation of PLCγ isoforms downstream of integrin receptors.

In summary, the data presented here demonstrate an important role for Vav family proteins in signalling by the platelet integrin αIIbβ3 and for normal regulation of PLCγ2, intracellular Ca^{2+} mobilisation and platelet spreading on fibrinogen. These results add to previous studies that demonstrate a pivotal role for Vav family proteins in the regulation of PLCγ2 by distinct classes of surface glycoproteins, including integrins, ITAM and lectin receptors.

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Figure Legends:

Figure 1: (A) Washed platelets from wild type (left panel) or Vav1/3$^{-/-}$ (right panel) mice were incubated over fibrinogen-coated coverslips in the presence of 2U/ml apyrase and 10μM indomethacin for 45 minutes at 37ºC and subsequently imaged by differential interference contrast (DIC) microscopy. A representative field of view is shown. (B) The mean ± the s.e.m number of adherent platelets per 13,500μm^2 were counted and plotted as a bar chart. (C) The mean ± s.e.m. number of filopodia per platelet were counted in 50 platelets chosen at random and plotted as a bar chart. (D) The surface area of 301 wild type and 319 Vav1/3$^{-/-}$ platelets on fibrinogen was calculated by imaging slides as above with a graticule standard and using Image J software to measure surface area. Surface areas are plotted as a frequency distribution. (E) Washed platelets from WT or Vav1/3$^{-/-}$ mice were stained with FITC-conjugated rat IgG (shaded) or rat anti-mouse αIIb, β3 or αIIbβ3 (non-shaded). Results are from one experiment that is representative of two others.

Figure 2: (A) Washed platelets were incubated over fibrinogen-coated petri dishes in the presence of 2U/ml apyrase and 10μM indomethacin for 45 minutes at 37ºC. Basal (non-adherent) and fibrinogen stimulated (adherent) cells were lysed in NP40 lysis buffer and PLCγ2 and Syk were immunoprecipitated. Immunoprecipitates were separated by SDS PAGE and western blotted with 4G10 anti-phosphotyrosine antibody. Membranes were subsequently stripped and reprobed for PLCγ2 and Syk respectively. (B) Washed platelets were labelled with the Ca^{2+} reporter dye Oregon Green BAPTA 1-AM and incubated over fibrinogen-coated coverslips in the presence of 2U/ml apyrase and 10μM indomethacin and imaged in real time using the FITC

channel on a fluorescent microscope. The fluorescence intensity of a representative platelet is shown. Spikes in fluorescence indicative of transient cytoplasmic Ca^{2+} elevation are indicated by arrows. (C) The number of Ca^{2+} transients in 30 platelets chosen at random from each genotype were counted and the average per platelet was plotted as a bar chart $\pm$ s.e.m. Results are from one experiment that is representative of two others.

Figure 3: (A) Washed platelets were incubated over fibrinogen-coated petri dishes in the presence of 2U/ml apyrase and 10μM indomethacin for 45 minutes at 37ºC. Basal (non-adherent) and fibrinogen stimulated (adherent) cells were lysed in Rac assay lysis buffer. An aliquot of lysate was taken into Laemmli buffer for total Rac analysis by western blot. Remaining lysates were incubated with GST-PAK for 1 hour and active Rac was precipitated. Precipitates were separated by SDS PAGE and western blotted for Rac. (B) Washed platelets in suspension in the presence of 2U/ml apyrase and 10μM indomethacin were stimulated with 1U/ml thrombin for 60 seconds and lysed in Rac assay lysis buffer and active Rac precipitated and western blotted as above. (C) Platelets were stimulated with 1U/ml thrombin for 60 seconds, fixed and F-actin stained with FITC-phalloidin. Specifically bound FITC-phalloidin was eluted and quantified in a spectrofluorimeter. The amount of F-actin is expressed as the percentage of thrombin induced F-actin formation in wild type cells $\pm$ s.e.m for triplicate samples. (D) Washed platelets from wild type (left panel) or Vav1/3$^{-/-}$ (right panel) mice were stimulated with 1U/ml thrombin and immediately incubated over fibrinogen-coated coverslips for 45 minutes at 37ºC in the presence of 2U/ml apyrase and 10μM indomethacin and subsequently imaged by differential interference contrast (DIC) microscopy. A representative field of view is shown. (E) The surface area of 300 wild type and 300 Vav1/3$^{-/-}$ platelets on fibrinogen was calculated by imaging slides as above with a graticule standard and using Image J software to measure surface area. Surface areas are plotted as a frequency distribution. Results are from one experiment that is representative of two others.

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Table 1: (A) Washed platelets from the genotypes of mice indicated were incubated over fibrinogen-coated coverslips in the presence of 2U/ml apyrase and 10μM indomethacin for 45 minutes at 37ºC. Slides were imaged by differential interference contrast (DIC) microscopy and the surface area of the platelets was calculated with a graticule standard and using Image J software. The surface areas of $Vav1^{-/-}$, $Vav3^{-/-}$, $Vav1/3^{-/-}$ and $Vav1/2/3^{-/-}$ cells were compared to wild type surface areas by Tukey test, * indicates statistical significance, $p<0.01$. $Vav1^{-/-}$, $Vav3^{-/-}$ and $Vav1/2/3^{-/-}$ surface areas were compared to $Vav1/3^{-/-}$ in Tukey test, ^ indicates statistical significance, $p<0.01$. (B) Platelets were stimulated with 1U/ml thrombin before adding to coverslips and imaging as above. Surface areas were compared in student t test and were not significantly different, $p<0.01$.

(A)

Genotype	Mean surface area (μm^2)
WT	12.6 ± 0.22 (n=301)
$Vav1^{-/-}$	10.2*,^ ± 0.16 (n=299)
$Vav3^{-/-}$	11.3*,^ ± 0.16 (n=289)
$Vav1/3^{-/-}$	9.3* ± 0.13 (n=319)
$Vav1/2/3^{-/-}$	9.44* ± 0.13 (n=330)

*$p<0.01$ relative to WT

^$p<0.01$ relative to $Vav1/3^{-/-}$

(B)

Genotype and treatment	Mean surface area (μm^2)
WT + Thrombin	19.3 ± 0.41 (n=300)
$Vav1/3^{-/-}$ + Thrombin	17.9 ± 0.41 (n=300)

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Figure 1

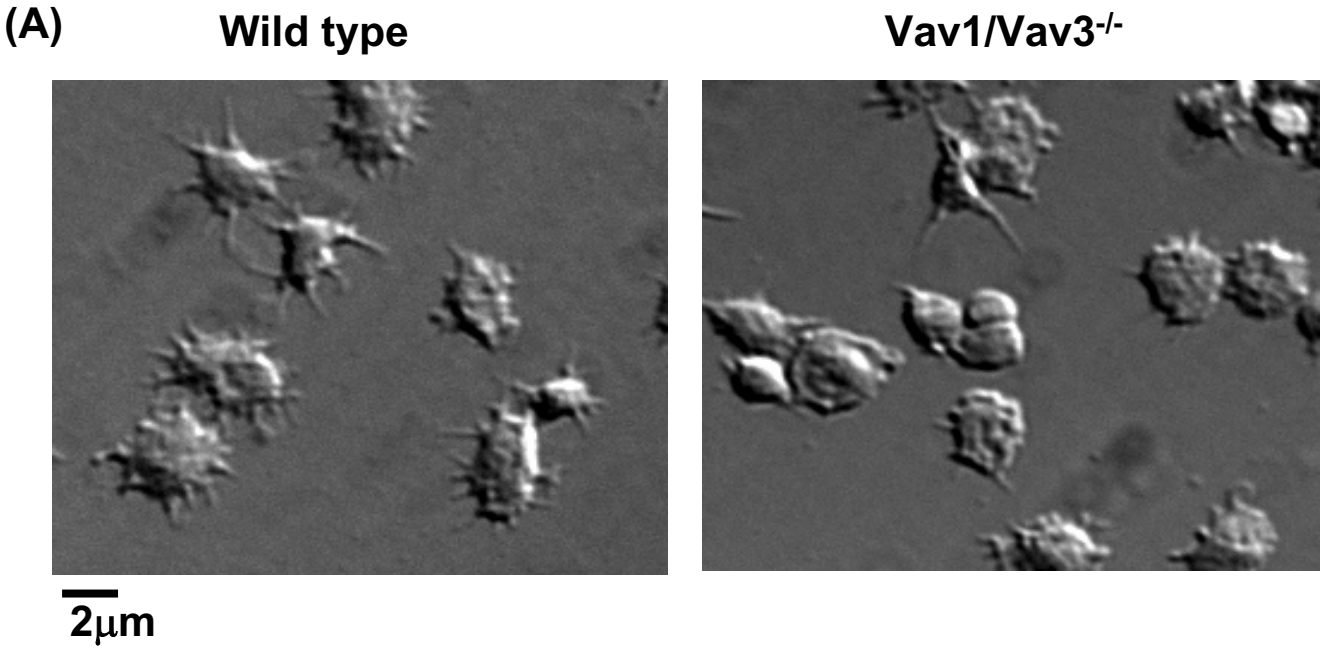

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Figure 1 continued

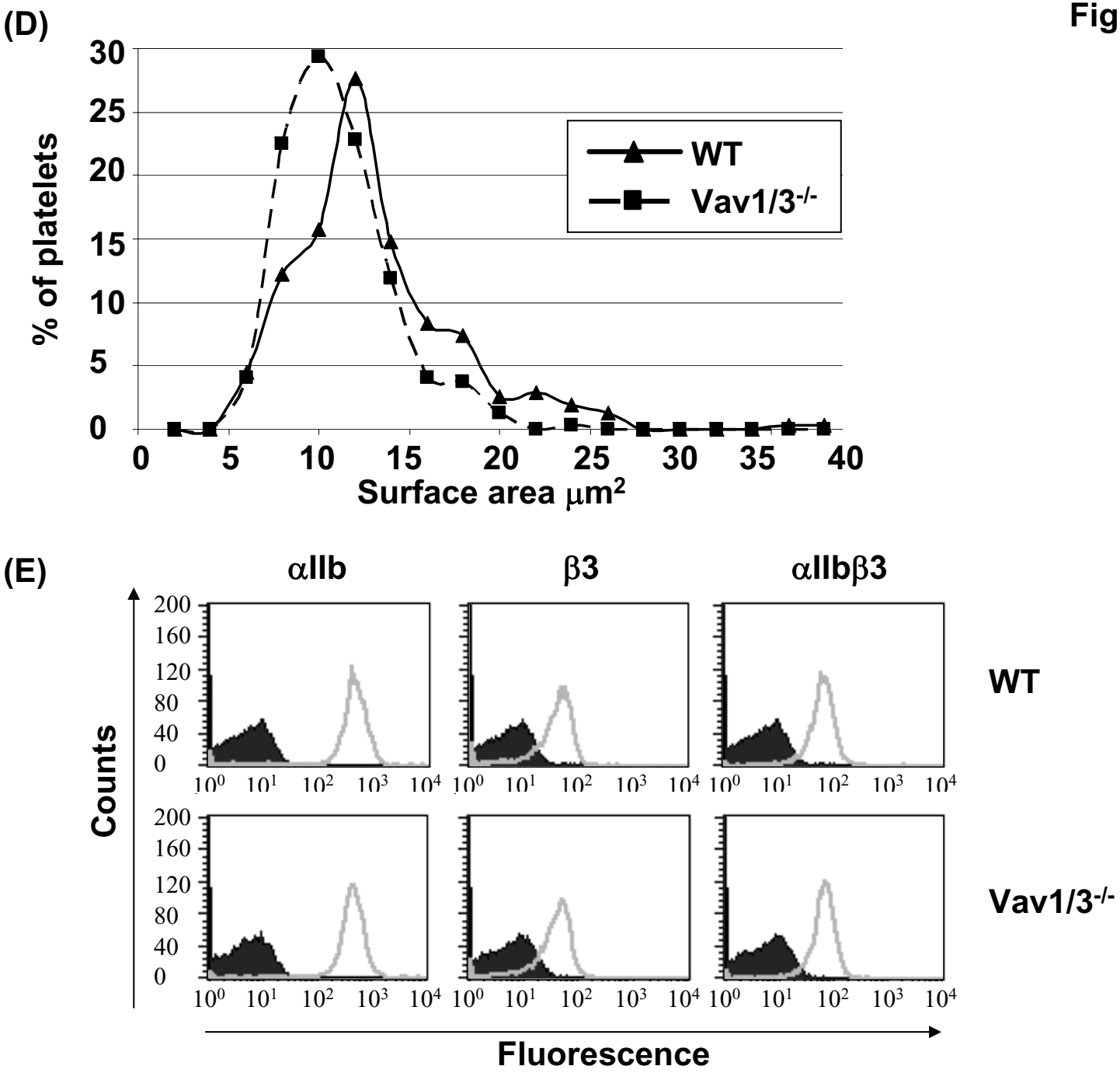

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Figure 2

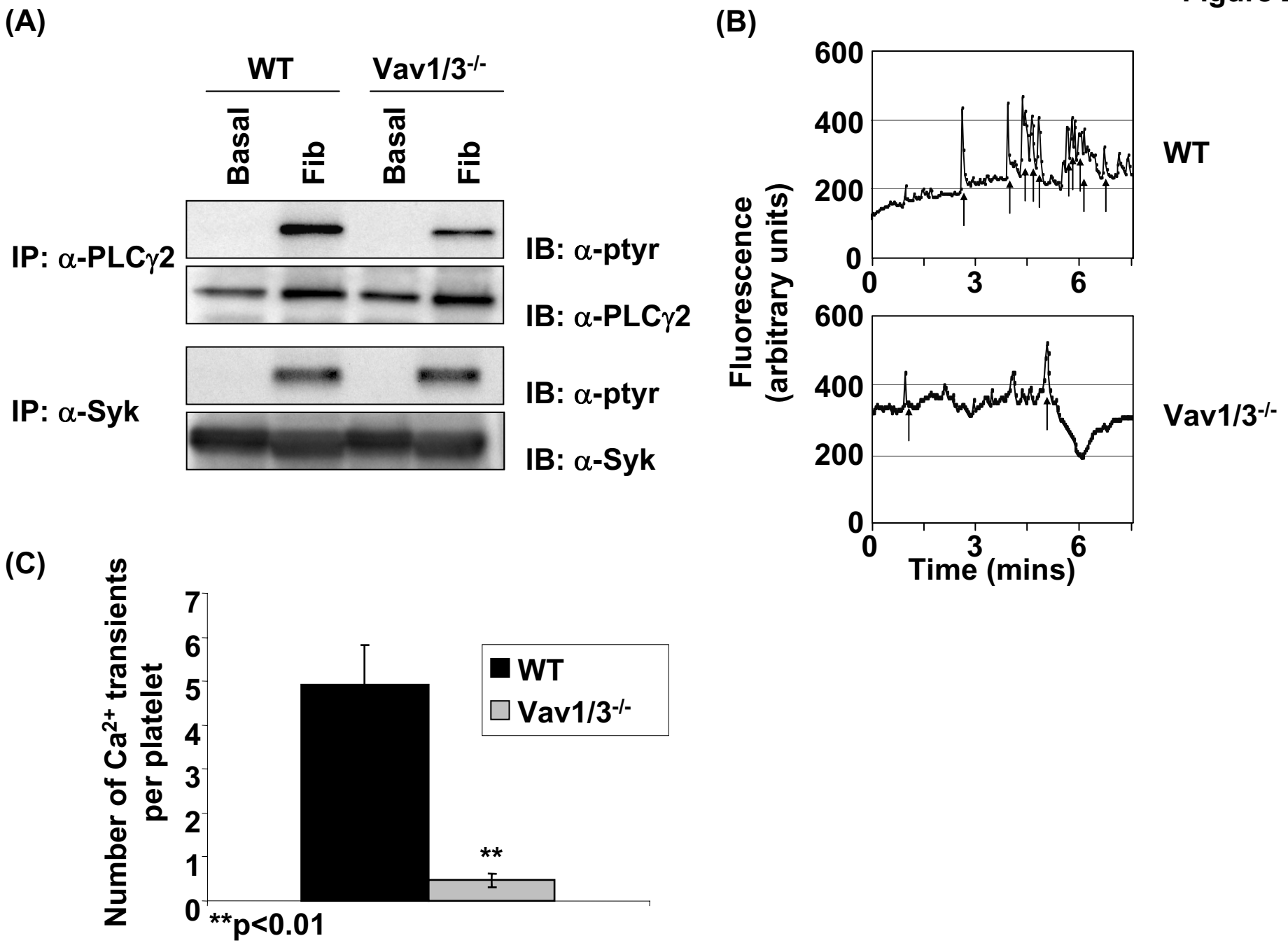

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Figure 3

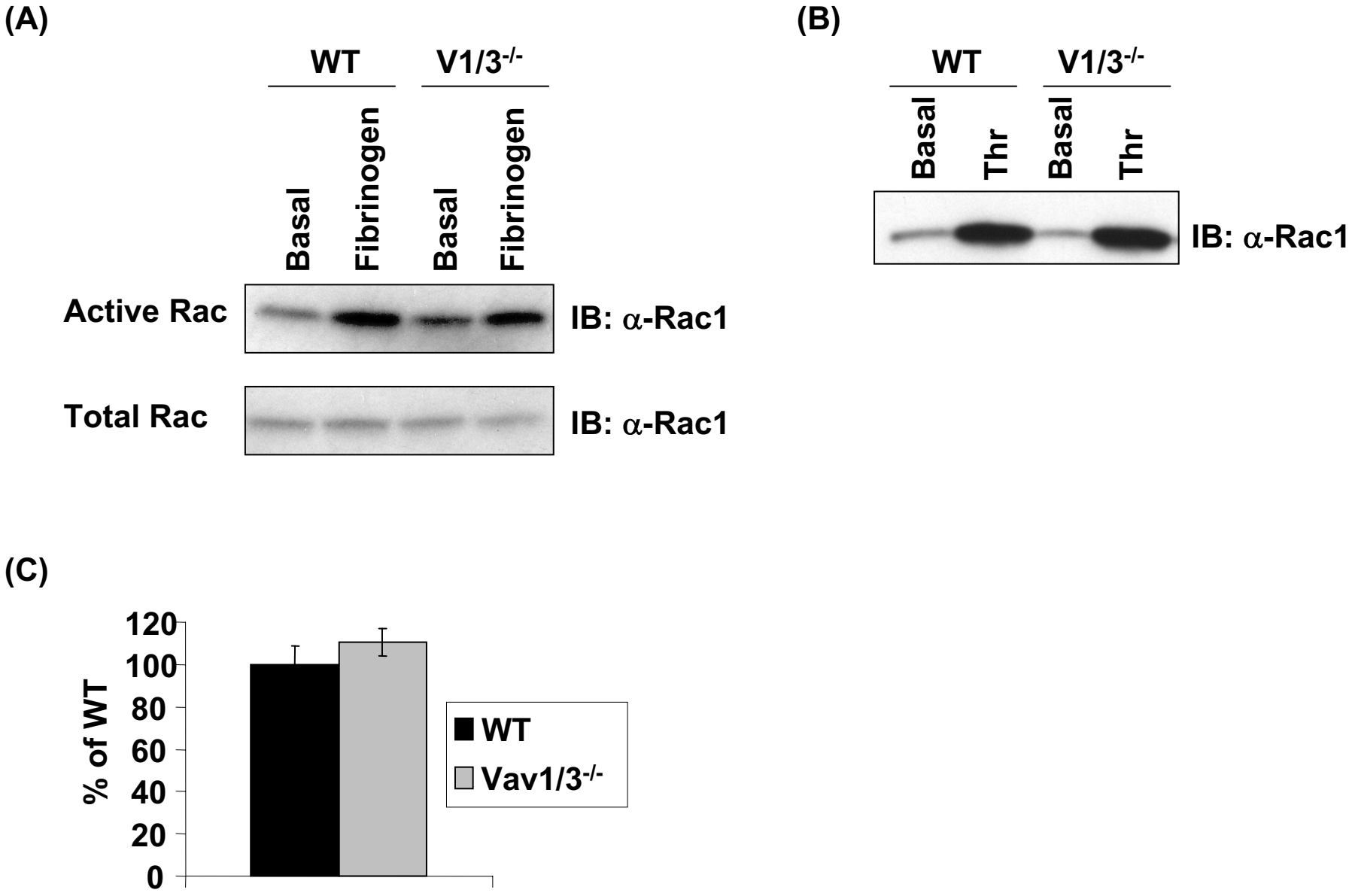

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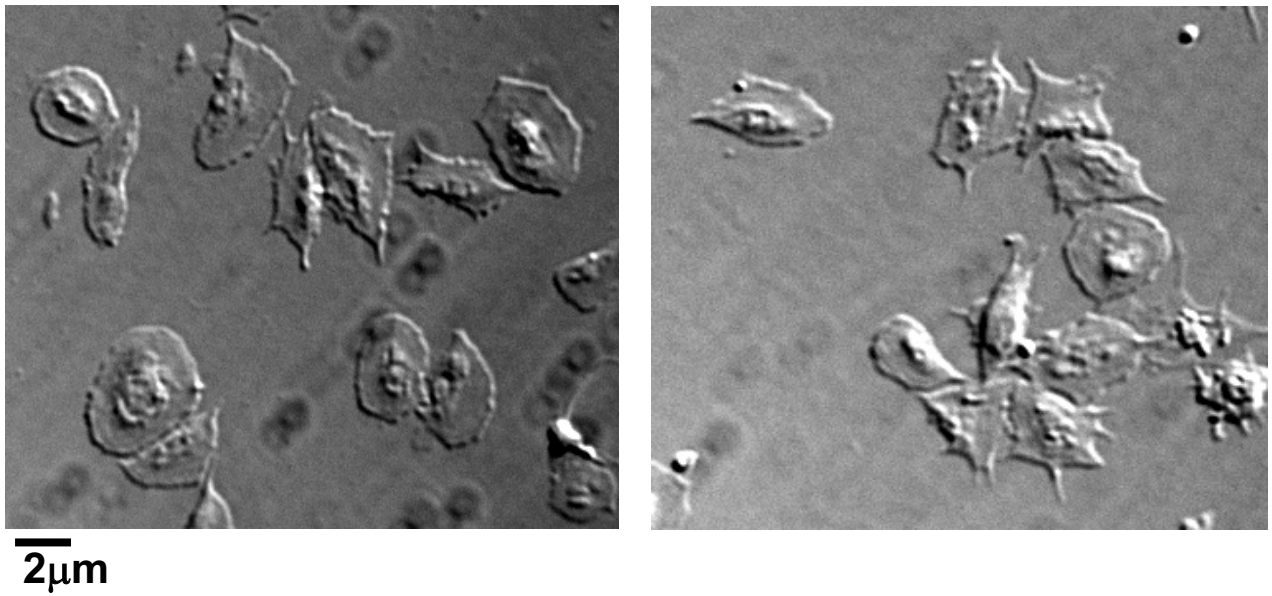
Figure 3 continued

(D)



(E)