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The PI 3-kinase PI3KC2 α regulates mouse platelet membrane structure and function independently of membrane lipid composition

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Abstract

PI3KC2 α is a phosphoinositide 3-kinase with a recently reported function in platelets; PI3KC2 α -deficient mouse platelets have altered membrane structure and impaired function. Yet, how these membrane changes cause platelet dysfunction remains unknown. Here, focused ion beam-scanning electron microscopy of PI3KC2 α -deficient platelet ultrastructure reveals a specific effect on the internal membrane structure, while liquid chromatography-tandem mass spectrometry profiling of 294 lipid species shows unaltered lipid composition. Functionally, PI3KC2 α -deficient platelets exhibit impaired thrombosis specifically under conditions involving membrane tethering. These studies indicate that the structural changes in PI3KC2 α -deficient platelets are limited to the membrane, occur without major changes in lipid composition, and selectively impair cell function during thrombus formation. These findings illustrate a unique mechanism that may be targetable for anti-thrombotic benefit.

Introduction

The phosphoinositide 3-kinases (PI3Ks) are a family of lipid kinases that catalyse the phosphorylation of the 3' hydroxyl group of the inositol ring of phosphoinositides to produce

the 3-phosphorylated phosphoinositides (3-PPIs) – important second messengers for a wide range of processes in cells [1, 2]. There are eight mammalian PI3K isoforms, divided into three classes based on structural and functional similarities: four class I, three class II, and one class III PI3K. The class I PI3Ks are comfortably the most widely studied and have well-defined roles in range of cells, including platelets [3]. More recently, mouse genetic models have been used to uncover the importance of the sole class III PI3K, Vps34 [4, 5], and one of the class II PI3Ks, PI3KC2 α [6-8], in platelet function. PI3KC2 α in particular regulates the thrombotic function of mouse platelets by a unique and as-yet-undefined mechanism that may involve the structure of the cell membrane. Specifically, we used an inducible shRNA-based approach to deplete PI3KC2 α protein in the platelets of adult mice to show that PI3KC2 α regulates the structure of the platelet internal membrane system (the open canalicular system; OCS) [6, 7]. Here, transmission electron microscopy (TEM) revealed that PI3KC2 α -deficient platelets exhibit dilatations of the channels of the OCS. Perhaps surprisingly, platelets from PI3KC2 α -deficient mice displayed normal function in all *in vitro* function tests performed (aggregation, granule secretion, integrin activation, and adhesion and spreading on activating surfaces), yet have impaired platelet thrombotic function in both *in vitro* and *in vivo* models [6, 7]. A subsequent study confirmed this role for PI3KC2 α in platelet structure and function in a distinct mouse model involving heterozygosity of a kinase-inactivating point mutation in the PI3KC2 α active site [8]. These intriguing findings suggest PI3KC2 α links regulation of the platelet internal membrane structure to the cell's prothrombotic function. However, the mechanism by which this occurs remains unknown.

One potential mechanism by which alterations in the platelet membrane structure might impair platelet function is provided by the observation that PI3KC2 α -deficient platelets exhibit reduced cytoskeletal co-localisation of key proteins that link the cell membrane with the cytoskeleton, most notably spectrin and myosin [8]. If the reduction in these proteins is sufficient to impair communication between the membrane and cytoskeleton, one prediction is that this may lead to impaired filopodia formation in activated platelets. Yet how such an impact would result in the observed selective impairment of platelet function in the setting of thrombosis – and not a global impairment of platelet function in, for example, standard assays of aggregation, granule secretion, or platelet spreading [6], remains unclear.

Given this uncertainty regarding the mechanism by which PI3KC2 α regulates platelet membrane structure and function, we have further examined the structural changes to the platelet membrane induced by PI3KC2 α -deficiency and have investigated how these structural changes affect the function of the platelet membrane. Here, we use focused ion beam-scanning electron microscopy (FIB-SEM) [9], as well as traditional scanning electron microscopy (SEM), to perform a detailed, three-dimensional ultrastructural analysis of platelets from PI3KC2 α -deficient mice. This analysis indicates PI3KC2 α -deficient platelets exhibit specific changes to the structure of the OCS. These changes occur without major alterations in the lipid composition of these platelets. Surprisingly, the structural changes to the platelet OCS membrane have no impact on the formation of filopodia by platelets activated in suspension, yet markedly impacts on the ability of these platelets to form thrombi in a blood flow environment involving a prominent shear gradient that produces membrane tether formation [10-12]. Together, these findings indicate that PI3KC2 α modulates the structure of the platelet internal membrane (OCS) independently of changes to membrane composition. These structural changes to the OCS appear to compromise platelet membrane function specifically in the setting of thrombus formation. If this regulation of membrane structure by PI3KC2 α is acute, these studies reveal the potential for a novel approach toward thrombosis-specific anti-platelet therapy.

Materials & Methods

Mice

All animal experiments were approved by the Alfred Medical Research and Education Precinct Animal Ethics Committee (approvals E/1465/2014/M and E/1644/2016/M). Mice were backcrossed $\geq$ 5 generations on a C 57BL/6 genetic background (i.e. 98%) and maintained on a 12 h light/dark cycle with food and water *ad libitum*. All genetic studies were littermate controlled and were performed with mice at 6 to 20 weeks of age of either sex. Mice deficient in PI3KC2 α (CMV-rtTA;TRE-GFP-shPI3KC2 α) were generated using an inducible shRNA-based gene-silencing approach, as reported previously [6]. Control mice consisted of either pooled wild-type and monotransgenic littermates or were mice with similar transgenes but with a control shRNA targeting Renilla luciferase (CMV-rtTA;TRE-GFP-

shControl). To induce shRNA-based knockdown of PI3KC2 α expression, mice were placed on a doxycycline diet (600 mg/kg, Speciality Feeds, Australia), for at least 10 days prior to experimentation.

Platelet isolation

Blood was drawn from the inferior vena cava of anaesthetised mice into enoxaparin (40 U/ml, final concentration) using a 25-gauge needle. Platelets were isolated as previously described [6]. Briefly, blood was treated with acid-citrate-dextrose and platelet wash buffer (4.3mM K₂HPO₄, 4.3mM Na₂HPO₄, 24.3mM NaH₂PO₄, 113mM NaCl, 5.5mM D-glucose and 10 mM theophylline; pH 6.5; containing 0.5% BSA, 20 U/ml enoxaparin and 0.01 U/ml apyrase), platelets isolated via centrifugation, and resuspended in Tyrode's buffer containing 0.5% BSA, 1.8 mM Ca²⁺ and 0.02 U/ml apyrase.

Focused ion beam-scanning electron microscopy

Isolated mouse platelets were fixed in 2.5% glutaraldehyde, post-fixed in 1% osmium tetroxide and 1.5% potassium ferrocyanide, incubated in 4% uranyl acetate, then dehydrated through a series of graded ethanol concentrations before being embedded in Epon resin. Samples were imaged using a Helios NanoLab microscope (FEI). Stacks of approximately 1000 images with a field size of 15 μ m x 15 μ m were generated. Samples were milled with the FIB (20 kV) at a thickness of 20 nm per section. Three dimensional models were computed using the imaging software Amira (FEI).

Scanning electron microscopy

Isolated mouse platelets were activated with ADP under constant stirring in the presence of eptifibatide (40 μ g/ml) and then fixed with the addition of glutaraldehyde (2.5%, final concentration). Fixed platelets were seeded on glass coverslips pre-coated with 0.01% poly-L-lysine; coverslips were then rinsed, dehydrated, and coated with a 10 nm layer of platinum/palladium or gold using a sputter coater (Cressington 208-HR). Imaging was performed using a Nova NanoSEM 450 scanning electron microscope (FEI) at 5 kV, and analysis completed using FIJI software.

Platelet lipid composition

Platelet lipid composition analyses were performed as previously described [13, 14]. Briefly, lipids were extracted using a single-phase chloroform:methanol extraction. Isolated mouse platelets in suspension were added to equal volumes of internal standard mix, comprising known concentrations of all lipid classes measured. A 2:1 ratio of chloroform:methanol was added to each sample. Samples were mixed with a rotary mixer for 10 min, sonicated for 30 min, and rested for 20 min at room temperature. Samples were then centrifuged at 16,000g for 10 min and the resulting supernatant removed and dried with nitrogen gas at 40°C. Samples were dissolved in water-saturated butanol and sonicated for 10 min prior to addition of an equal volume of methanol with 10 mM ammonium formate. The lipid extracts were centrifuged at 3350g for 5 min and the supernatant removed for analysis.

Lipid analysis was performed using liquid chromatography electrospray ionisation – tandem mass spectrometry, as previously described in detail [13, 14], using an Agilent 1200 UHPLC coupled to an AB Sciex Q/TRAP 4000 mass spectrometer with a turbo-ion spray source (350 °C) and Analyst 1.5 and Multiquant data systems. Separation by liquid chromatography was performed on a Zorbax C18 column (1.8 µm, 50 x 2.1 mm; Agilent Technologies), again as previously described [13, 14]. In total, 294 lipids were detected and quantitated.

The total lipid concentration for each class measured was determined by summing the concentrations of individual species in that class. Univariate comparisons were conducted comparing lipid classes between PI3KC2α-deficient (CMV-rtTA;TRE-GFP-shPI3KC2α) and littermate control mice, using Welch's *t* test followed by Bonferroni correction to account for multiple comparisons.

Microfluidic assay

Platelet aggregation was performed using a previously described method modified for use in the present study with mouse whole blood [11]. Briefly, a microfluidic device consisting of 200 µm wide channels with inset 40 µm stenosis was fabricated using standard photolithography techniques [10]. Microchannels were selectively coated at the stenosis geometry with human vWF (10 µg/ml - isolated from Biostate® CSL Ltd) for 10 min, blocked with BSA (2 µg/ml) for 10 min, then coated with botrocetin (2.5 µg/ml) for 10 min, before

being flushed with Tyrode's buffer prior to sample perfusion. Hirudin-anticoagulated mouse whole blood was perfused through the microfluidic at 45 $\mu\text{l}/\text{min}$ (equating to a shear rate at the stenosis geometry of $11,500\text{ s}^{-1}$) and platelet deposition at the stenosis monitored in real time via epifluorescence (Nikon Ti-U microscope - Plan Fluor 20x/0.50 objective with Andor Zyla sCMOS detector). Mid-plane platelet aggregate size was analysed in ImageJ following thresholding (Huang's fuzzy image thresholding method). Maximal aggregation, expressed as cross-sectional area (μm^2), was extrapolated from non-linear curve fitting following 300 s perfusion time.

Analyses

Statistical analyses were performed using GraphPad Prism. Statistical significance was defined at $P < 0.05$ and was determined with an unpaired, two-tailed Student's t -test, followed by Bonferroni correction to account for multiple comparisons where appropriate and as indicated in the figure legends.

Results

PI3KC2 α regulates platelet membrane structure

We have previously used 2-dimensional TEM imaging to demonstrate that PI3KC2 α -deficiency results in a dilation of the channels of the OCS within mouse platelets [6]. In order to examine this and any other potential ultrastructural change caused by PI3KC2 α -deficiency in more detail, we used FIB-SEM to create whole-cell, three-dimensional reconstructions of PI3KC2 α -deficient mouse platelets. These reconstructions allow for visualisation and quantitation of various intracellular compartments, which in this case comprised the OCS and plasma membrane, as well as alpha and dense granules. In agreement with our previous 2-dimensional findings, we observed dilation of the OCS in platelets from PI3KC2 α -deficient mice when compared with platelets from littermate controls (Figure 1A). Areas of OCS dilation in PI3KC2 α -deficient platelets were more evident at certain points in a given cell (Figure 1A, red arrows) and the total volume of OCS in platelets isolated from PI3KC2 α -deficient mice was 48% greater than in platelets from littermate control mice (3.6 ± 0.5 vs $5.4 \pm 0.6\%$ of total cell volume for control and PI3KC2 α -deficient cells, respectively; Figure 1B). Despite this OCS dilation, no obvious differences were observed in the spatial distribution of the OCS throughout the cell (Figure 1A). In contrast to the OCS, the total volume of either

alpha or dense granules was not different between control and PI3KC2 α -deficient platelets (Figure 1C).

Given the OCS dilatation observed here and previously [6, 7], we next examined the external aspect of the OCS – its openings at the plasma membrane – via SEM (Figure 2A). There were similar numbers of OCS openings in the visible plasma membrane of cells examined in these studies (Figure 2B). However, in a near-identical finding to the OCS measurements inside platelets, the diameter of OCS openings at the plasma membrane were significantly increased by 46% in platelets from PI3KC2 α -deficient mice versus littermate controls (37 ± 4 vs 54 ± 3 nm, respectively; Figure 2C).

PI3KC2 α does not regulate platelet membrane lipid composition

One of the most important contributors to membrane structure is the lipid content of the membrane. Therefore, we next examined whether the observed structural changes to the OCS were caused by or associated with alterations in the lipid profile of the platelet. We performed a detailed analysis of 294 lipids across the 22 most abundant lipid classes and subclasses using liquid chromatography electrospray ionisation-tandem mass spectrometry. This analysis revealed an unchanged lipid profile in PI3KC2 α -deficient when compared with littermate control platelets (Figure 3), with no significant differences in molar abundance observed in any of the lipid classes analysed.

PI3KC2 α does not regulate filopodia formation in platelets

We next examined whether the structural alteration in the platelet internal membrane impacts on platelet membrane function by imaging filopodia formation in platelets activated in suspension or in the presence of shear forces. First, isolated platelets were stimulated with ADP (in the presence of eptifibatide to prevent aggregation), then fixed and imaged by SEM (Figure 4A). No differences were detected in either the number or length of filopodia between platelets isolated from PI3KC2 α -deficient or littermate control mice (Figure 4B,C). In addition, filopodia formed by platelets flowed over a monolayer of activated platelets were also examined. Again, no differences in tether length or stability (total time of attachment) were observed (Figure 4D,E).

PI3KC2 α regulates platelet adhesion and thrombus formation

Given the impaired arterial thrombus formation previously observed in PI3KC2 α -deficient mice, we examined platelet function in a microfluidic whole blood flow assay known to be dependent on membrane tether formation [10-12]. Mouse blood was perfused through a von Willebrand factor-coated microfluidic channel incorporating a shear force gradient-inducing stenosis [10-12]. Platelet aggregation occurred downstream of the stenosis and the size of these platelet aggregates was quantified using the epifluorescent signal of the GFP-positive platelets (Figure 5). The extent of platelet deposition was significantly lower in blood from PI3KC2 α -deficient mice ($838 \pm 314 \mu\text{m}^2$) versus that in blood from control mice ($2194 \pm 485 \mu\text{m}^2$) – a 62% reduction (Figure 5).

Together, these findings suggest loss of PI3KC2 α leads to a specific structural change in the platelet membrane that occurs independently of membrane lipid composition. This membrane modification is sufficient to selectively impact upon platelet function in the setting of thromboses formed downstream of a shear gradient-inducing stenosis and dependent on membrane tethering.

Discussion

We performed an analysis of the structural and functional consequences of the loss of PI3KC2 α in mouse platelets in order to gain insights into the mechanism by which PI3KC2 α deficiency leads to the previously observed anti-thrombotic phenotype. We showed that PI3KC2 α deficiency leads to an ultrastructural change in platelets that is limited to the internal membrane (OCS) of these cells. Specifically, the OCS in platelets from PI3KC2 α -deficient mice was dilated throughout the cell, including at the plasma membrane. This structural change in the platelet membrane occurred independently of the cell's lipid composition. Yet inspection of platelet membrane function revealed PI3KC2 α deficiency had selective effects under pro-thrombotic conditions: filopodia formation was unaffected, but platelet deposition and aggregation was significantly impaired in an ex vivo whole blood flow thrombosis model.

We and others have previously shown that platelets from PI3KC2 α -deficient mice exhibit an increased surface area occupied by the channels of the OCS in TEM images [6, 8]. Here we add to those findings to show that the enlargements previously reported in 2D TEM images are reflective of a widespread and largely uniform dilation of membrane channels throughout the cell, including at the plasma membrane. Our detailed three-dimensional analysis of PI3KC2 α -deficient platelets revealed this structural change was specific to the OCS: its volume was significantly increased in PI3KC2 α -deficient platelets while the volumes of alpha and dense granules were unaffected, despite the occasional change in granule number or shape between individual cells. Furthermore, the size of the openings of the OCS at the plasma membrane were significantly larger in PI3KC2 α -deficient platelets. Despite this OCS dilation throughout the cell, no gross change in the spatial distribution of the OCS across the cell was observed.

Despite these obvious changes in the structure of the cell's membrane, it remains unclear how PI3KC2 α -deficiency leads to such changes. It has previously been shown that PI3KC2 α -deficiency reduces the basal levels of PI(3)P in platelets [8]. Yet, this small change in a low abundance lipid appears unlikely to directly cause the structural changes in the platelet membrane observed here. Given that the biggest predictor of any membrane structure is its lipid composition, here we expanded on these previous studies by performing a comprehensive analysis of the lipidome of both PI3KC2 α -deficient and wild-type platelets. This more broad-based analysis included all of the most abundant membrane lipid species and revealed the gross lipid profile of PI3KC2 α -deficient platelets was indistinguishable to that of wild-type platelets. These studies indicate the observed internal membrane dilatation is unlikely driven by compositional changes to the membrane. This is, to our knowledge, the most comprehensive lipidomic analysis of the mouse platelet. On this, it is interesting to note some potential differences in the relative abundance of some lipids between mouse platelets and similar lipidomic analyses of human platelets [15]. For example, while phospholipids are the predominant component in both mouse and human platelet membranes, there is a notably higher abundance of sterol lipids and a relative paucity of glycerolipids in mouse platelets when compared with human [15]. Whether these differences are important in terms of membrane function remains to be determined.

Previous studies have demonstrated an in vivo anti-thrombotic phenotype conferred by loss of PI3KC2 α [6, 8]. Given that platelets from these PI3KC2 α -deficient mice have a structural defect that is specific to the OCS – a reserve of membrane thought to be utilised for platelet shape change and spreading during activation and aggregation [16] – we examined platelet membrane function in both static and blood flow environments. We found that platelet membrane function is affected specifically in the setting of thrombus formation. Here, filopodia production in suspension-activated platelets or under flow were unaffected by loss of PI3KC2 α , yet in a microfluidic assay incorporating a high shear gradient-inducing stenosis, PI3KC2 α -deficiency led to a marked reduction in platelet deposition. We utilised this microfluidic blood flow assay because of its incorporation of a pronounced stenosis that has been shown to cause a substantial shear gradient (from very high at the point of stenotic narrowing, to very low immediately downstream of the stenosis) [10-12]. Importantly, in vivo studies of platelet behaviour following exposure to such a shear gradient environment (via a vascular stenosis) have shown that initial platelet deposition occurs largely independently of cellular activation by soluble agonists [12]. Indeed, such shear gradients appear to drive initial platelet deposition via the formation of membrane tethers [10-12]. As a result, the significant reduction in platelet deposition in this system using blood from PI3KC2 α mice may suggest that PI3KC2 α is important for the earliest stages of platelet adhesion, where membrane tethers, presumably derived from the OCS, are pulled from platelets without detectable cell activation via an interaction between von Willebrand factor and the platelet adhesion receptor GPIb α [17]. This hypothesis fits our previous observations in which PI3KC2 α -deficient platelet adhesion and thrombus formation is differentially affected under conditions in which platelet activation is substantial, i.e. on a collagen-coated surface upon which marked platelet activation occurs [6]. Alternatively, PI3KC2 α may be involved in the subsequent propagation of platelet aggregates, since incoming platelets that tether to the surface of forming thrombi are discoid and demonstrate low levels of activation markers and minimal calcium flux, in sharp contrast to the initial core of the thrombus where platelets are generally fully activated [12, 18]. The lack of effect of PI3KC2 α deficiency on platelet filopodia production in response to cell activation may suggest that the involvement of PI3KC2 α is most prevalent where changes in shear stress sustain and drive thrombus propagation, potentially in the absence of conspicuous agonist-induced activation. The

surprising lack of effect of PI3KC2 α deficiency in standard assays of agonist-induced in vitro platelet function [6, 8] supports this hypothesis.

The intriguing link between the PI3KC2 α -dependent modulation of platelet internal membrane structure and impaired platelet function specifically in the setting of blood flow suggests targeting PI3KC2 α and/or the platelet internal membrane system may have utility as an anti-thrombotic strategy. However, it remains unknown whether this function of PI3KC2 α translates to human platelets. In addition, the relevance of changes in platelet membrane structure to human platelet function are completely unexplored. There are a number of clinical syndromes in which abnormalities in the OCS are observed, including some with either bleeding or thrombotic consequences [19-21]. Yet platelets from patients with these conditions demonstrate a number of structural abnormalities in addition to OCS changes, making it difficult to determine how much, if any, contribution the OCS defect has to the clinical phenotype. The development of PI3KC2 α inhibitors would be a useful step forward in determining the role of this enzyme in human platelets and in addressing any validity of this target as an anti-thrombotic approach. If PI3KC2 α function is conserved in human platelets and acute inhibition of this enzyme is able to reproduce the phenotype observed in mouse platelets, this opens the possibility of manipulating the platelet membrane structure via PI3KC2 α as a novel, thrombosis-specific, anti-platelet strategy.

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Declarations of interest

The authors declare no competing financial interests.

Author contributions

MVS performed research, analysed data and wrote the manuscript; RJB, MJM and J-YR performed research; AE, CG, PJM, HHN and WSN contributed to study design, data analysis and interpretation of results; JRH designed the study, interpreted results and wrote the manuscript.

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Figure legends

Figure 1. PI3KC2 α regulates internal platelet membrane structure. (A) Representative 3-dimensional whole-cell FIB-SEM reconstructions of a platelet from a CMV-rtTA;TRE-GFP-shPI3KC2 α bi-transgenic mouse (C2 α -def) and a monotransgenic littermate (control) mouse, showing the OCS (blue), alpha granules (yellow), dense granules (black), and plasma membrane (light blue). Bottom panels show reconstructions with individual components only for clarity. Note dilation of the OCS in the PI3KC2 α -deficient platelet (red arrows). (B) Quantitation of the volume of each organelle measured (as a percentage of the total platelet volume). Data are mean $\pm$ SEM from n=6 platelets. *, $P < 0.05$ (unpaired, two-tailed, Student's *t* test).

Figure 2. PI3KC2 α regulates external platelet membrane structure. (A) Representative SEM images of resting platelets isolated from CMV-rtTA;TRE-GFP-shPI3KC2 α bi-transgenic mice (C2 α -def) or their wild-type and monotransgenic littermates (control), showing OCS surface openings (red arrows and the focus of the increased magnification images). (B) Number and (C) size of OCS openings. Data are mean $\pm$ SEM from n=112-116 platelets from n=4 mice per genotype. **, $P < 0.01$ (unpaired, two-tailed, Student's *t* test).

Figure 3. PI3KC2 α does not regulate platelet lipid composition. Concentration of the 22 most abundant lipid classes in platelets isolated from CMV-rtTA;TRE-GFP-shPI3KC2 α bi-transgenic mice (C2 α -def) or their wild-type and monotransgenic littermates (control), via LC-ESI-MS/MS. Lipid classes are phosphatidylcholine (PC), free cholesterol (COH), alkylphosphatidylcholine (PC(O)), alkenylphosphatidylethanolamine (PE(P)), cholesterol ester (CE), phosphatidylethanolamine (PE), sphingomyelin (SM), phosphatidylinositol (PI), lysophosphatidylcholine (LPC), alkenylphosphatidylcholine (PC(P)), diacylglycerol (DG), alkylphosphatidylethanolamine (PE(O)), triacylglycerol (TG), ceramide (Cer), lysophosphatidylethanolamine (LPE), lysoalkylphosphatidylcholine (LPC(O)), dihydroceramide (dhCer), phosphatidylserine (PS), monohexosylceramide (MHC), lysophosphatidylinositol (LPI), trihexosylceramide (THC) and dihexosylceramide (DHC). Molar abundance of each class is the total of the individual species within each class. Data are mean $\pm$ SEM from n=10-13 mice per genotype. No significant differences were observed ($P>0.05$ by unpaired, two-tailed, *t*-test with Bonferroni correction for multiple comparisons).

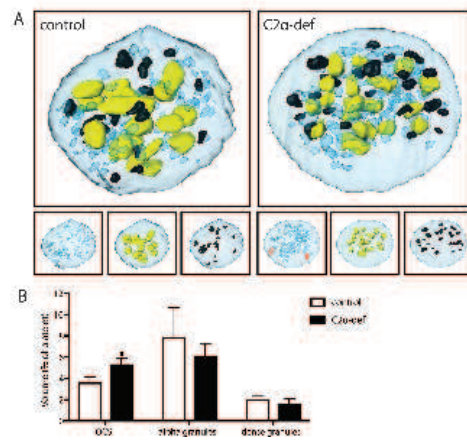
Figure 4. PI3KC2 α does not regulate filopodia formation. (A) Representative SEM images of platelets isolated from CMV-rtTA;TRE-GFP-shPI3KC2 α bi-transgenic mice (C2 α -def) or monotransgenic littermates (control) and activated in suspension with 5 or 50 μ M ADP (in the presence of eptifibatide to prevent aggregation). All scale bars are 1 μ m. (B) Number and (C) length of filopodia per platelet. Data are mean $\pm$ SEM from n=119-122 platelets from n=6 mice per genotype. No significant differences were observed in any comparison ($P>0.05$ by unpaired, two-tailed, *t* test). (D) Length and (E) stability (total adhesion time) of filopodia from C2 α -def or control platelets flowed over a monolayer of wild-type mouse platelets. Data are mean $\pm$ SEM from n=100 platelets from n=5 mice per genotype. No significant differences were observed in any comparison ($P>0.05$ by unpaired, two-tailed, *t* test).

Figure 5. PI3KC2 α regulates platelet adhesion and deposition driven by a shear gradient. (A) DIC image showing the geometry of the microfluidic flow cell used in the whole blood flow assay. The channel has a width of 200 μ m narrowing to 40 μ m at the stenosis, with side-wall entry and exit angles of 80°. (B) Representative fluorescence images following perfusion of blood taken from CMV-rtTA;TRE-GFP-shPI3KC2 α (C2 α -def) or CMV-rtTA;TRE-GFP-shControl (control) mice through the flow cell shown in (A), with platelet aggregates seen forming

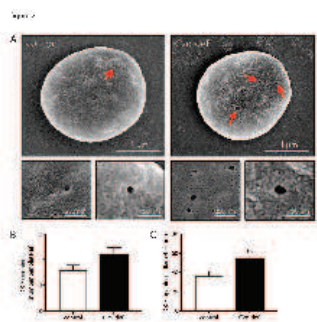
downstream of the stenosis. Arrows indicate direction of flow. Scale bars, 10 μm . (C) Maximal platelet aggregation measured in these experiments (expressed as aggregate area). Data are mean $\pm$ SEM from $n=5-6$ mice per genotype. *, $P<0.05$ (unpaired, two-tailed, Student's t test).

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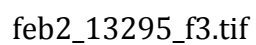
Figure 1

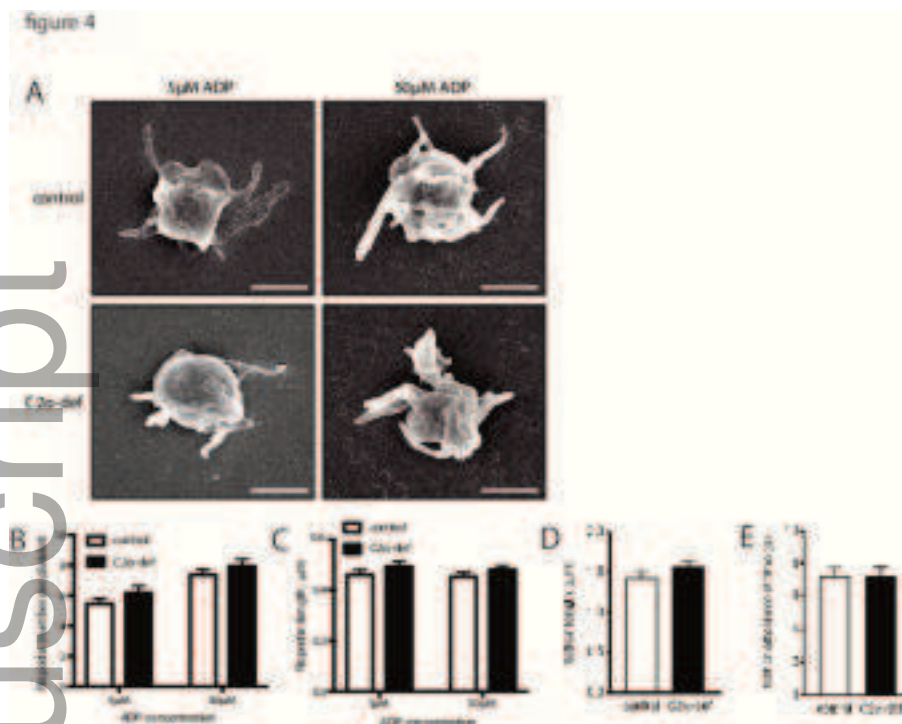


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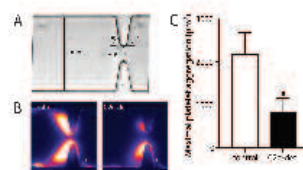
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Figure 5



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