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Loss of PIKfyve in platelets causes a lysosomal disease leading to inflammation and thrombosis in mice

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PIKfyve is essential for the synthesis of phosphatidylinositol-3,5-bisphosphate [PtdIns(3,5)P₂] and for the regulation of endolysosomal membrane dynamics in mammals. PtdIns(3,5)P₂ deficiency causes neurodegeneration in mice and humans, but the role of PtdIns(3,5)P₂ in non-neural tissues is poorly understood. Here we show that platelet-specific ablation of PIKfyve in mice leads to accelerated arterial thrombosis, and, unexpectedly, also to inappropriate inflammatory responses characterized by macrophage accumulation in multiple tissues. These multiorgan defects are attenuated by platelet depletion *in vivo*, confirming that they reflect a platelet-specific process. PIKfyve ablation in platelets induces defective maturation and excessive storage of lysosomal enzymes that are released upon platelet activation. Impairing lysosome secretion from *PIKfyve*-null platelets *in vivo* markedly attenuates the multiorgan defects, suggesting that platelet lysosome secretion contributes to pathogenesis. Our findings identify PIKfyve as an essential regulator for platelet lysosome homeostasis, and demonstrate the contributions of platelet lysosomes to inflammation, arterial thrombosis and macrophage biology.

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Phosphoinositides are minor components of membrane phospholipids, yet they are essential for the regulation of diverse cellular processes, including signal transduction, cytoskeletal control and membrane trafficking¹. Phosphoinositide metabolism is tightly modulated by specific lipid kinases and phosphatases. Altering phosphoinositide turnover by dysregulating these enzymes can lead to a variety of human diseases².

PtdIns(3,5)P₂ is a phosphoinositide of low abundance that is synthesized from PtdIns(3)P on the endosomal compartments in mammalian cells by the lipid kinase PIKfyve (also known as FAB1)³. PIKfyve forms a protein complex with other regulatory proteins, such as the PtdIns(3,5)P₂ 5-phosphatase Fig4 (also known as SAC3)^{4,5} and the docking protein Vac14 (also known as ArPIKfyve)^{6,7}. The PIKfyve complex and its product PtdIns(3,5)P₂ are essential regulators of membrane homeostasis, and of vesicle trafficking and cargo transport along the endosomal–lysosomal pathway^{8,9}.

Recently, physiological functions of PtdIns(3,5)P₂ have been elucidated using genetically engineered mice that lack different components of the PIKfyve complex¹⁰. *PIKfyve*-null mice are embryonically lethal¹¹, but mice expressing residual PIKfyve activity are viable and develop defects within multiple organs, such as in the nervous, cardiopulmonary and hematopoietic systems¹². Similarly, *Fig4*-null mice or *Vac14*-null mice develop several defects, including neurodegeneration, hypopigmentation and early lethality^{13,14}. Notably, homozygous *Fig4* mutations were also identified in patients who have the neurodegenerative diseases Charcot–Marie–Tooth syndrome 4J and amyotrophic lateral sclerosis^{14,15}, demonstrating a role for PtdIns(3,5)P₂ in neural development. Although studies showed that PtdIns(3,5)P₂ deficiency causes defects in multiple cellular pathways including those required for endolysosomal trafficking in yeast and mammalian cell cultures, the physiological consequences of PtdIns(3,5)P₂ in non-neural cells, such as those of the hematopoietic system, are not well understood.

Platelets are hematopoietic cells that are crucial for haemostatic plug formation in response to vascular injury. This process has been shown to require a series of key platelet activation events that are tightly regulated by several phosphoinositides¹⁶. However, the regulatory role of PtdIns(3,5)P₂ in platelets remains unknown. Platelets are anucleated, but they store numerous biologically active substances in their secretory organelles, which include alpha granules, dense granules and lysosomes^{17,18}. Platelet granules are generated in megakaryocytes from the endosomal–lysosomal system^{19–22} by, as yet, poorly understood mechanisms.

In this study, we investigated whether PIKfyve has an essential role in platelets and in megakaryocytes using mice lacking PIKfyve specifically in these cell types. Given the role of PIKfyve-mediated PtdIns(3,5)P₂ production in the regulation of the endosomal–lysosomal pathway, we hypothesized that PIKfyve was essential for the biogenesis and function of platelet granules. We show that PIKfyve is critical for proper lysosomal homeostasis in platelets, but it is not necessary for the biogenesis of alpha granules or dense granules. Remarkably, *PIKfyve*-null platelets are defective in the maturation, storage and release of lysosomal enzymes. Unexpectedly, these platelet lysosomal defects induce aberrant inflammatory responses in macrophages that impair the development, body mass, fertility and survival of mice.

Results

Loss of PIKfyve in platelets causes multiorgan defects. To investigate the role of PIKfyve in platelets and in megakaryocytes, we generated mice lacking PIKfyve kinase activity specifically in

these cell types. The *PIKfyve* gene was conditionally targeted with loxP sites flanking the exons corresponding to the kinase activation loop to generate floxed alleles of *PIKfyve* (*PIKfyve*^{fl}, Fig. 1a). Mice expressing *PIKfyve*^{fl} were crossed with transgenic mice expressing the Cre recombinase that is controlled by the platelet-factor-4 promoter (*Pf4-Cre*), which restricts the expression of Cre recombinase exclusively within megakaryocytes and their derived platelets²³.

PIKfyve^{fl/fl} *Pf4-Cre* mice developed several morphological abnormalities relative to their littermate *PIKfyve*^{fl/+} mice, *PIKfyve*^{fl/fl} mice and *PIKfyve*^{fl/+} *Pf4-Cre* mice. At about 3 weeks of age, *PIKfyve*^{fl/fl} *Pf4-Cre* mice exhibited mild growth delay and body hair loss (Supplementary Fig. 1a). Over time, they gradually developed coarse facial features, abdominal distention, a generalized increase in the bulk of their soft tissues and body weight gain (Fig. 1b,c). Despite this weight gain, body composition analysis by dual-energy X-ray absorptiometry showed that *PIKfyve*^{fl/fl} *Pf4-Cre* mice had reduced body fat, but increased lean body mass, as compared with their littermate controls (Fig. 1d). In addition, they also had decreased bone mineral density (Supplementary Fig. 1b). As *PIKfyve*^{fl/fl} *Pf4-Cre* mice aged, they remained infertile (Supplementary Fig. 1c) and their general body functions deteriorated. The vast majority died before 28 weeks of age (Fig. 1e).

PIKfyve^{fl/fl} *Pf4-Cre* mice accumulate vacuolated macrophages.

To elucidate the cause of multiorgan defects in *PIKfyve*^{fl/fl} *Pf4-Cre* mice, histological and immunohistochemical analyses were performed in mice of 3–28 weeks of age. Unlike their control littermates, *PIKfyve*^{fl/fl} *Pf4-Cre* mice exhibited massive organomegaly (Supplementary Fig. 2a). This phenotype reflected progressive tissue infiltration by engorged cells with multiple cytoplasmic vacuoles that had a ‘foamy’ appearance (Fig. 2a and Supplementary Fig. 2b, arrowheads). Although the vacuoles of these cells did not stain with classical dyes for proteins or lipids, their cytoplasm strongly stained with Alcian blue and periodic acid Schiff, suggesting an excessive storage of acidic polysaccharides in the vacuolated cells (Fig. 2b and Supplementary Fig. 3a). In their livers and lungs, the vacuolated cells accumulated in a perivascular distribution and displaced the normal tissue (Fig. 2a), suggesting that the vacuolated cells originated from the blood or vasculature. By immunohistochemical analysis, the vacuolated cells stained with the macrophage-specific marker F4/80 (Fig. 2c), but not with the markers of other cell lineages, including GPIb (a megakaryocyte-specific marker). The F4/80-positive vacuolated macrophages in the livers of *PIKfyve*^{fl/fl} *Pf4-Cre* mice were approximately three- to fourfold larger than the F4/80-positive macrophages in the livers of the control mice (Fig. 2d). In addition, tissues infiltrated with the vacuolated macrophages were often associated with mononuclear cell infiltrates that stained with the lymphocyte marker CD45R (Supplementary Fig. 3b), suggesting a local inflammatory response. These results demonstrate that the loss of PIKfyve in platelets causes the invasion of normal tissues with inflammatory vacuolated macrophages.

***PIKfyve*-null platelets induce macrophage pathology.** Cells from PtdIns(3,5)P₂-deficient organisms have been shown to form large cytoplasmic vacuoles^{9,10}. However, because our genetic approach was designed to only ablate PtdIns(3,5)P₂ synthesis in platelets and in megakaryocytes, we did not expect to see the robust cytoplasmic vacuolation in the macrophages of *PIKfyve*^{fl/fl} *Pf4-Cre* mice. Therefore, we further addressed whether these unexpected macrophage abnormalities resulted from aberrant PIKfyve disruption in macrophages, or by nonautonomous effects

of *PIKfyve* disruption in platelets. Immunoblotting analysis showed that *PIKfyve* protein was absent in platelets, but present in the liver, spleen and lungs of *PIKfyve^{fl/fl} Pf4-Cre* mice. *PIKfyve* protein was also present in all tissues of the control mice (Fig. 3a and Supplementary Fig. 4a). These results strongly suggest that the *PIKfyve* protein was ablated only in the megakaryocyte lineage of *PIKfyve^{fl/fl} Pf4-Cre* mice.

Although *Pf4-Cre* expression has been shown to be restricted to megakaryocytes and platelets^{23–26}, we next re-evaluated *Pf4-Cre* expression in *PIKfyve^{fl/fl} Pf4-Cre* mice by crossing them to mice expressing a Cre-dependent LacZ reporter (*Rosa26-LacZ*)²⁷. Tissue staining of *PIKfyve^{fl/fl} Pf4-Cre/Rosa26-LacZ* mice with X-gal showed β -galactosidase expression only in megakaryocytes and in platelets (Fig. 3b and Supplementary Fig. 4b, arrows), but not in the vacuolated macrophages or in the other cells (Fig. 3b, arrowheads). This genetic evidence excludes the possibility that *Pf4-Cre* induced *PIKfyve* ablation in the macrophages of *PIKfyve^{fl/fl} Pf4-Cre* mice. Together, these results demonstrate that the macrophage infiltration and enlargement observed in *PIKfyve^{fl/fl} Pf4-Cre* mice are indirectly induced by *PIKfyve* ablation in megakaryocytes and/or in platelets.

To further address whether *PIKfyve*-null platelets contribute to the robust macrophage response leading to the multiorgan failure in *PIKfyve^{fl/fl} Pf4-Cre* mice, we studied the effects of platelet depletion using monoclonal GP1b α antibodies^{28,29}. Platelet depletion was initiated at 8–10 weeks of age. At that point, the *PIKfyve^{fl/fl} Pf4-Cre* mice had already developed body hair loss, some weight gain and tissue infiltration of their vacuolated macrophages. Control mice and *PIKfyve^{fl/fl} Pf4-Cre* mice were either untreated or administered GP1b α antibodies twice weekly

via intraperitoneal injections for 6 weeks. As reported previously, the injection of GP1b α antibodies efficiently induced acute thrombocytopenia in both sets of mice^{28,29}. However, repetitive injections of GP1b α antibodies were less effective at inducing stable thrombocytopenia over time likely because of the reactive thrombocytopoiesis associated with complete platelet depletion with antiplatelet antibodies³⁰. Nevertheless, the *PIKfyve^{fl/fl} Pf4-Cre* mice that were treated with GP1b α antibodies showed a significant delay in weight gain and attenuated hair loss as compared with the untreated *PIKfyve^{fl/fl} Pf4-Cre* mice (Fig. 3c,d). Together, our findings demonstrate that platelets are the primary contributors in the development of the inflammatory macrophage response and multiorgan defects in *PIKfyve^{fl/fl} Pf4-Cre* mice.

We also investigated whether the macrophage response in *PIKfyve^{fl/fl} Pf4-Cre* mice could be rescued or transferred by hematopoietic stem cell (HSC) transplantation. Lethally irradiated wild-type mice of 8–12 weeks of age were reconstituted with a mixture of donor cells that were half derived from *PIKfyve^{fl/fl} Pf4-Cre* HSCs and half derived from wild-type HSCs. Thirty-five weeks later, a necropsy showed that infiltration of vacuolated macrophages was readily apparent in the liver, lung, colon and bone marrow of the irradiated wild-type recipient mice (Supplementary Fig. 5b,e, arrowheads). Conversely, lethally irradiated *PIKfyve^{fl/fl} Pf4-Cre* mice of 8–12 weeks of age were reconstituted exclusively with wild-type donor HSCs. At about 8 weeks after transplantation, the characteristic hair loss and weight gain were significantly attenuated. A necropsy at 35 weeks after transplantation confirmed that the infiltration of the vacuolated macrophages in multiple tissues was completely reversed (Supplementary Fig. 5c,f). Together, these results further

Figure 1 | Platelet-specific ablation of *PIKfyve* causes multiorgan abnormalities in mice. (a) Schematic representation of genetic targeting of *PIKfyve*. The target exons 37 and 38 (red bars) were targeted with lox P recombination sites (yellow arrows) to generate *PIKfyve*-floxed alleles (*PIKfyve^{fl}*). Mice expressing *PIKfyve^{fl}* were then crossed with *Pf4-Cre* mice to induce homologous recombination of the *PIKfyve^{fl}* (*PIKfyve* Post-Cre). (b) General appearance of control and *PIKfyve^{fl/fl} Pf4-Cre* littermates at 24 weeks of age. Note the characteristic coarse facial features, body hair loss and severely distended body morphology. (c) Body weight of control mice and *PIKfyve^{fl/fl} Pf4-Cre* mice. The average numbers of mice were eight per time point and per group. (d) Percent of total body fat in the control ($n=3$) and *PIKfyve^{fl/fl} Pf4-Cre* ($n=3$) mice at 12–24 weeks of age. (e) Survival curves of female and male control ($n=40$) and *PIKfyve^{fl/fl} Pf4-Cre* ($n=40$) mice. * $P<0.05$, ** $P<0.01$, *** $P<0.001$. All error bars indicate mean $\pm$ s.d. Student's t -test.

Figure 2 | Inflammatory vacuolated macrophages infiltrate multiple tissues in the *PIKfyve^{fl/fl} Pf4-Cre* mice. (a) Representative histological sections of the lungs and livers stained with haematoxylin and eosin (H&E) from control mice and *PIKfyve^{fl/fl} Pf4-Cre* mice. Scale bar, 100 μm. Insets show increased magnification of boxed areas in **a**. Scale bar, 20 μm. Arrowheads indicate the vacuolated cells. (b) Lung sections stained with Alcian blue. Scale bar, 100 μm. Inset shows high magnification of the boxed area. (c) Liver structures of control mice and *PIKfyve^{fl/fl} Pf4-Cre* mice were stained for macrophages with anti-F4/80 by immunohistochemistry. Scale bar, 100 μm. Insets in the upper right corner of each image show higher magnification images of boxed areas. Scale bar, 20 μm. (d) Cell diameters of F4/80+ cells in the livers of control mice ($n=5$) and *PIKfyve^{fl/fl} Pf4-Cre* mice ($n=5$). *** $P<0.001$. All error bars indicate mean $\pm$ s.d. Student's t -test.

demonstrate that the phenotype of *PIKfyve^{fl/fl} Pf4-Cre* mice can be either rescued or transferred by hematopoietic progenitor cells.

Megakaryopoiesis and thrombopoiesis do not require *PIKfyve*.

We next studied the effects of *PIKfyve* ablation in megakaryocytes of *PIKfyve^{fl/fl} Pf4-Cre* mice. Fibroblasts and neurons of $\text{PtdIns}(3,5)\text{P}_2$ -deficient organisms typically develop large vacuoles^{9,10}. Histological analysis by light microscopy of bone marrow sections showed that *PIKfyve*-null megakaryocytes were morphologically indistinguishable from control megakaryocytes (Supplementary Fig. 6a). However, analysis by transmission electron microscopy of cultured megakaryocytes derived from *PIKfyve^{fl/fl} Pf4-Cre* bone marrow revealed multiple enlarged cytoplasmic vacuoles (Fig. 4a). Despite this abnormality, flow cytometric analysis showed no significant differences in the number of megakaryocytes in the bone marrow of control or *PIKfyve^{fl/fl} Pf4-Cre* mice (Fig. 4b). Moreover, *PIKfyve*-null

megakaryocytes had normal ploidy, implying that the loss of *PIKfyve* did not affect megakaryocyte maturation (Fig. 4c). Finally, counts of platelets and leukocytes as well as haemoglobin level were similar between control and *PIKfyve^{fl/fl} Pf4-Cre* mice (Fig. 4d–f). Thus, *PIKfyve^{fl/fl} Pf4-Cre* mice normally develop megakaryocytes *in vivo* and produce normal numbers of platelets, and they also normally develop other blood cells.

PIKfyve regulates trafficking of platelet lysosomal cargoes.

We next investigated the effects of *PIKfyve* ablation in the morphology and functions of platelets in *PIKfyve^{fl/fl} Pf4-Cre* mice. Transmission electron microscopy demonstrated that in contrast to the *PIKfyve*-null megakaryocytes derived from the bone marrow, the ultrastructure of *PIKfyve*-null platelets was indistinguishable from that of control platelets (Supplementary Fig. 6b). To test whether the loss of *PIKfyve* impacted granule content, we assessed the abundance of representative components

Figure 3 | Platelets drive the multiorgan defects in the *PIKfyve^{fl/fl} Pf4-Cre* mice. (a) The levels of PIKfyve protein were determined in the platelets and liver of the indicated mice by immunoblotting. Note the absence of the PIKfyve protein in the platelets, but its presence in the liver of *PIKfyve^{fl/fl} Pf4-Cre* mice ($n = 3$). Tissues from the wild-type mice, the *PIKfyve^{fl/+} Pf4-Cre* mice and the *PIKfyve^{fl/fl}* mice were used as positive controls. β -Actin was used as a loading control. (b) X-gal staining of the lung tissues from *PIKfyve^{fl/fl} Rosa26-LacZ* mice and *PIKfyve^{fl/fl} Pf4-Cre Rosa26-LacZ* mice. Scale bar, 20 μ m. The arrow indicates the platelet clumps in the lung vessels expressing LacZ (blue stain). Arrowheads indicate the vacuolated macrophages that did not stain blue. (c) Representative general morphology of 15-week-old control mice and *PIKfyve^{fl/fl} Pf4-Cre* mice after 6 weeks of either no intervention (untreated) or biweekly injections of anti-GP1b α antibodies (+ GP1b α Ab). (d) Body weight change of the control mice and *PIKfyve^{fl/fl} Pf4-Cre* mice after 6 weeks of no intervention or treatment with anti-GP1b α antibodies ($n = 3$ for each group). * $P < 0.05$. All error bars indicate mean $\pm$ s.d. Student's t -test.

by immunoblotting. *PIKfyve*-null platelets expressed normal levels of the alpha granule components von Willebrand factor and platelet factor 4 (Supplementary Fig. 6c,d). In contrast, *PIKfyve*-null platelets exhibited increased levels of the lysosomal protein LAMP-1 (lysosomal-associated membrane protein 1; Fig. 5a) and increased activity of the lysosomal enzyme β -hexosaminidase (Fig. 5b). These results suggest that *PIKfyve*-null platelets store normal levels of alpha granule components, but store excessive levels of lysosomal components.

To test whether PIKfyve regulates granule secretion, we stimulated platelets *ex vivo* and monitored granule release by surface expression of granule markers or release of mediators into the medium. Following thrombin stimulation, *PIKfyve*-null platelets translocated the α -granule protein P-selectin to their cell surface as efficiently as control platelets (Supplementary Fig. 7a). Likewise, ATP secretion from dense granules was similar in control and *PIKfyve*-null platelets (Supplementary Fig. 7b). However, the releasates from *PIKfyve*-null platelets showed increased activity of the lysosomal enzyme, β -hexosaminidase (Fig. 5c). Further analysis showed that the *PIKfyve*-null platelets released a normal percentage of their total cellular β -hexosaminidase following agonist stimulation (Supplementary Fig. 7c). These data indicate that *PIKfyve*-null platelets release higher levels of β -hexosaminidase than the control platelets because they contain excessive amounts of this lysosomal enzyme, and not because they secrete it more efficiently. Consistent with this finding, β -hexosaminidase activity was found to also be elevated in the plasma of *PIKfyve^{fl/fl} Pf4-Cre* mice (Fig. 5c). Together, these findings demonstrate that *PIKfyve*-null platelets store and release excessive levels of lysosomal cargo proteins.

To more comprehensively analyse how the loss of PIKfyve affects the contents secreted upon platelet activation, a quantitative proteomics analysis was performed. By tandem liquid chromatography-mass spectrometry (LC-MS/MS), we identified the proteins released from the control platelets and the *PIKfyve*-null platelets following thrombin stimulation. We then used the spectral counting of the identified peptides to quantitatively compare the proteins released from the control platelets and the *PIKfyve*-null platelets. Using a cutoff of 2.0-fold as a significant difference between them, we identified 17 proteins that were increased and 38 proteins that were decreased in the releasates of *PIKfyve*-null platelets relative to wild-type platelets (Supplementary Tables 1 and 2). Among those proteins that were more abundant in the *PIKfyve*-null platelet releasates, several lysosomal enzymes stood out including cathepsin D, cathepsin B and maltase (Supplementary Table 1). Remarkably, levels of the cathepsin D were 20-fold higher in *PIKfyve*-null platelet releasates than in control releasates (Supplementary Table 1). Further analysis of the sequence coverage and MS spectra of the tryptic peptides for cathepsin D demonstrated that the releasates of *PIKfyve*-null platelets contained predominantly the procathepsin D—the immature and inactive proenzyme form of cathepsin D—but undetectable levels of mature cathepsin D (Supplementary Fig. 8a–c). To further validate these findings, we analysed the expression of cathepsin D in platelet lysates by immunoblotting. Consistent with the proteomics data, both cathepsin D and procathepsin D were undetectable in the lysates of wild-type platelets, and procathepsin D was highly elevated in *PIKfyve*-null platelets (Fig. 5d). These results suggest that cathepsin D expression is upregulated in *PIKfyve*-null platelets, but that the intracellular maturation of procathepsin D is defective in *PIKfyve*-null platelets. Consequently, cathepsin D activity was negligible in both the lysates and releasates of both wild-type and the *PIKfyve*-null platelets (Supplementary Fig. 7d). Together, these data demonstrate that PIKfyve ablation in platelets results in a defective lysosomal pathway reflected

Figure 4 | PIKfyve is not necessary for the development of megakaryocytes nor for platelet production. (a) Electron micrographs of megakaryocytes grown in culture in the presence of thrombopoietin for 7 days. Note multiple enlarged vacuoles in the cytoplasm of *PIKfyve^{fl/fl} Pf4-Cre* mice as compared with the control mice. Scale bar, 10 μ m. (b) CD41+ megakaryocyte counts of control *PIKfyve^{fl/fl} Pf4-Cre* mice ($n=3$ for each group). (c) Megakaryocyte ploidy ($n=3$ for each group). (d–f) Counts of platelets (d), WBC (e) and Hb (f) of control mice and *PIKfyve^{fl/fl} Pf4-Cre* mice ($n=3$ for each group). All error bars indicate mean $\pm$ s.d. Student's *t*-test.

by abnormal storage, maturation and release of lysosomal enzymes.

Loss of PIKfyve in platelets accelerates arterial thrombosis.

Platelets have long been known to contain primary lysosomes and release their lysosomal enzymes in response to stimuli^{31,32}, but the physiologic role of platelet lysosome secretion has not been clearly delineated. Thus, we tested whether the lysosomal abnormalities observed in *PIKfyve*-null platelets correlated with any functional abnormalities. Washed platelets from control or *PIKfyve^{fl/fl} Pf4-Cre* mice formed aggregates to a similar degree in response to different agonists (Supplementary Fig. 6e), suggesting that activation of the dominant platelet integrin, α IIb β 3, is intact in the *PIKfyve*-null platelets. In addition, Akt phosphorylation was normally induced upon stimulation (Fig. 5f), suggesting that the intracellular signalling pathway in *PIKfyve*-null platelets is also preserved. To determine the effect of PIKfyve ablation in platelets during thrombosis *in vivo*, the carotid arteries of mice were analysed for occlusive thrombus formation in response to a chemical injury with ferric chloride. At 12–16 weeks of age, *PIKfyve^{fl/fl} Pf4-Cre* mice formed occluding thrombi significantly faster than their control littermates (3.8 ± 1.6 min versus 6.1 ± 1.2 min, Fig. 5e). Since *PIKfyve*-null platelets showed normal aggregation *ex vivo*, we speculated that the excessive secretion of the platelet lysosomal contents from *PIKfyve*-null platelets could contribute to endothelial damage and systemic inflammation leading to accelerated thrombus formation. Consistent with this possibility, plasma levels of vWF, a marker of endothelial damage and systemic inflammation³³, were significantly elevated in the plasma of *PIKfyve^{fl/fl} Pf4-Cre* mice when measured by immunoblotting (Fig. 5g) or enzyme-linked immunosorbent assay (ELISA; Fig. 5h). Together, these results suggest that excessive lysosomal secretion from *PIKfyve*-null platelets is associated with endothelial damage and systemic inflammation, perhaps underlying excessive thrombosis in *PIKfyve^{fl/fl} Pf4-Cre* mice.

Impaired release of platelet lysosomes reverts the pathology.

If the pathological macrophage inflammatory response in *PIKfyve^{fl/fl} Pf4-Cre* mice reflects the excessive stimulus-induced secretion of lysosomal enzymes, we hypothesized that attenuating the release of platelet lysosomes would comparably attenuate the inflammatory responses. To test this hypothesis, *PIKfyve^{fl/fl} Pf4-Cre* mice were crossed with *pallid* mice, a model of Hermansky–Pudlak syndrome type 9 (ref. 34). *Pallid* mice lack an essential subunit of BLOC-1, a complex required for the biogenesis of lysosome-related organelles such as pigment cell melanosomes and platelet dense granules^{35,36}. Importantly, stimulus-dependent secretion of lysosomal contents is impaired (but not ablated) in platelets from *pallid* mice³⁷. In contrast to *PIKfyve^{fl/fl} Pf4-Cre* mice that developed persistent dorsal alopecia and weight gain, *PIKfyve^{fl/fl} Pf4-Cre/pallid* mice maintained hair growth until death and exhibited attenuated weight gain (Fig. 6a,b; note that because the *pallid* mutation also impairs melanosome biogenesis, *pallid* mice exhibit hypopigmented fur). As expected, platelet and plasma levels of β -hexosaminidase were lower in *PIKfyve^{fl/fl} Pf4-Cre/pallid* mice than in *PIKfyve^{fl/fl} Pf4-Cre* mice (Fig. 6c,d). Together, these data suggest that the release of platelet lysosomes contributes to the pathogenesis of multiorgan defects in *PIKfyve^{fl/fl} Pf4-Cre* mice.

Discussion

In this study, we show that PIKfyve kinase activity is critical in limiting the pathological inflammatory and thrombotic functions of platelet lysosomes. Our data indicate that PIKfyve kinase activity in platelets is essential for the normal maturation of cathepsin D and for the proper storage and release of lysosomal enzymes. Unexpectedly, dysregulated lysosomal homeostasis and enhanced lysosomal release from *PIKfyve*-null platelets drive an aberrant inflammatory response in macrophages. This leads to chronic inflammation and arterial thrombosis that affect the development, body mass, fertility and survival of mice.

Figure 5 | *PIKfyve* is critical for the maturation, storage and release of lysosomal enzymes in platelets. (a) Immunoblot of platelet lysates from the control mice and *PIKfyve^{fl/fl} Pf4-Cre* mice showing the expression of EEA-1 and LAMP-1. β -Actin was used as the loading control. (b) Quantification of protein bands shown in a ($n=3$ for each group). (c) β -hexosaminidase activity in the platelet lysates, platelet releasates, and plasma of control mice ($n=3$) and *PIKfyve^{fl/fl} Pf4-Cre* mice ($n=3$). (d) Immunoblot of platelet lysates from control mice and *PIKfyve^{fl/fl} Pf4-Cre* mice showing the expression of Procathepsin D. β -actin was used as the loading control. (e) Time to occlusion of carotid arteries upon vascular injury with 7.5% of FeCl_3 in the control mice ($n=9$) and the *PIKfyve^{fl/fl} Pf4-Cre* mice ($n=8$). (f) Immunoblot of platelet lysates from control platelets and *PIKfyve^{fl/fl} Pf4-Cre* platelets showing the Akt phosphorylation in resting condition or stimulated with 1 U ml^{-1} of thrombin for 10 min. (g) SDS agarose electrophoresis showing the multimer pattern of plasma vWF from the control mice ($n=3$) and the *PIKfyve^{fl/fl} Pf4-Cre* ($n=3$) mice. (h) Plasma levels of vWF from the control mice ($n=3$) and the *PIKfyve^{fl/fl} Pf4-Cre* ($n=3$) mice were quantified by ELISA. * $P < 0.05$. All error bars indicate mean $\pm$ s.d. Student's t -test.

Our study shows that *PIKfyve*-null platelets excessively accumulate lysosomal components, which include lysosomal membrane proteins as well as lysosomal enzymes such as β -hexosaminidase and procathepsin D. Procathepsin D is targeted from the trans-Golgi network (TGN) to endosomes primarily via the mannose-6-phosphate receptor (M6PR) pathway and is further processed to active cathepsin D in acidic lysosomes³⁸. The M6PR must then recycle to the TGN for another round of transport. The excessive accumulation and release of procathepsin D in *PIKfyve*-null platelets suggests that

PIKfyve is critical for procathepsin D trafficking from the TGN to endosomes. This conclusion is consistent with a published study showing that $\text{PtdIns}(3,5)\text{P}_2$ deficiency leads to the defective retrograde trafficking of M6PR from endosomes to the TGN³⁹, which would ultimately exhaust the M6PR from the TGN and result in default secretion of procathepsin D. Similarly, the fibroblasts of *Vac14*-null mice develop defective steady-state localization of M6PR, and they also accumulate the immature forms of cathepsin D¹³. Since $\text{PtdIns}(3,5)\text{P}_2$ is also required for acidification of the lysosome-like vacuole in yeast⁹ and because

Figure 6 | Impaired secretion of platelet lysosomes attenuates the phenotypes of *PIKfyve*^{fl/fl} *Pf4-Cre* mice. **(a)** General appearance of *PIKfyve*^{fl/fl} *Pf4-Cre* mice and *PIKfyve*^{fl/fl} *Pf4-Cre/pallid* mice on C57BL/6 background at 17 weeks of age. In the presence of the *pallid* mutation, the hair loss and weight gain are attenuated in the *PIKfyve*^{fl/fl} *Pf4-Cre/pallid* mice. Note that the *pallid* mice have an underlying defect in melanosome biogenesis causing hypopigmented fur. **(b)** Body weight of the *PIKfyve*^{fl/fl} *Pf4-Cre* mice, and the sex-matched and age-matched *PIKfyve*^{fl/fl} *Pf4-Cre/pallid* mice. The average numbers of mice were five per time point and per group. The dashed line indicates the mean ratio of body weight of the control mice. **(c,d)** Analysis of β -hexosaminidase activity in the platelet lysate **(c)** and plasma **(d)** of the *PIKfyve*^{fl/fl} *Pf4-Cre* mice ($n = 3$) and the *PIKfyve*^{fl/fl} *Pf4-Cre/pallid* ($n = 3$) mice. * $P < 0.05$, ** $P < 0.01$. All error bars indicate mean $\pm$ s.d. Student's *t*-test.

the low pH in lysosomes is essential for the proteolytic maturation of procathepsin D⁴⁰, an abnormal lysosomal pH could also contribute to the defective processing of procathepsin D in the *PIKfyve*-null platelets. In addition, wild-type platelets contain and secrete only minor amounts of procathepsin D whereas *PIKfyve*-null platelets excessively store and release procathepsin D. This suggests that PtdIns(3,5)P₂ deficiency might additionally feed back on lysosomal enzyme biosynthesis, perhaps by activating the CLEAR (coordinated lysosomal expression and regulation) gene network^{41,42}.

Interestingly, overexpression and secretion of procathepsin D have been found in several human cancers, including breast and lung carcinomas⁴³. In these tumour cells, alterations of pathways that are M6PR dependent and independent have both been suggested^{44,45}. In addition, excessive release of the lysosomal enzymes are also seen in the lysosomal storage disorders, Mucopolysaccharidosis II and Mucopolysaccharidosis III⁴⁶. In these diseases, an inability to generate the M6P recognition signal on the lysosomal enzymes impairs their delivery from the TGN to the lysosome via the M6PR pathway resulting in the excessive release of lysosomal enzymes extracellularly⁴⁷. Thus, the absence of *PIKfyve* in platelets results in a phenotype that is reminiscent of several human disorders with impaired trafficking from the TGN to the lysosome.

Similar to *PIKfyve* deficiency, the deficiency of the inositol phosphatase and *PIKfyve* chaperone protein called *Fig4* results in

the reduced intracellular PtdIns(3,5)P₂ concentrations. Notably, *Fig4*-deficient mice develop enlarged endolysosomal vacuoles and excessive accumulation of lysosomal proteins, reminiscent of lysosomal storage diseases⁴⁸. Likewise, mice lacking another *PIKfyve*-binding partner called *Vac14* have decreased synthesis of PtdIns(3,5)P₂ and develop excessive vacuolation of late endosomal and lysosomal compartments¹³. Together with our findings, these studies further support that the depletion of PtdIns(3,5)P₂ by impairing any components of the *PIKfyve* complex could lead to the features that are reminiscent of those found in lysosomal storage disorders.

It is notable that lysosomal storage diseases are frequently associated with inappropriate immune responses that result in chronic inflammation. For example, tissue infiltration of reactive macrophages can lead to visceral organomegaly in both Gaucher and Niemann-Pick diseases^{49,50}. Likewise, microglial activation is frequently associated with the neuropathology in Mucopolysaccharidoses⁵¹. Similar to these findings, we unexpectedly observed that the ablation of *PIKfyve* in platelets causes inflammatory responses that are associated with robust macrophage defects, which lead to organomegaly in mice. Using several lines of evidence, we demonstrated that this macrophage phenotype is non-cell-autonomous, showing that *PIKfyve*-null platelets can induce aberrant responses in normal macrophages. Interestingly, this inflammatory response has not been described in mice lacking *Fig4* or *Vac14*. This could be explained because these mice do not survive long enough to fully develop the robust tissue accumulation of macrophages seen in *PIKfyve*^{fl/fl} *Pf4-Cre* mice. However, a similar non-cell-autonomous phenomenon has also been observed in *Fig4*-null mice, which have a neurodegenerative disorder¹⁴. The genetic restoration of *Fig4* exclusively in the neuronal cells of *Fig4*-null mice not only reverts the dysfunctions of neuronal cells, but it also reverts the dysfunctions of the adjacent oligodendrocytes including hypomyelination and maturation defects⁵². Together with our findings, these studies indicate that the depletion of PtdIns(3,5)P₂ in a single cell type may have indirect implications in other cell types. Furthermore, they also show that our findings are consistent with the broad secondary defects shown in lysosomal storage disorders.

Megakaryocytes and platelets have long been known to possess lysosomes that contain different hydrolases including proteases and glycosidases^{31,53}. Upon platelet activation *in vivo*, platelets release not only alpha granules and dense granules, but they also release their lysosomal contents⁵⁴. The physiopathological role of platelet lysosome secretion is not well understood. However, it has been speculated that the release of platelet lysosomal enzymes may be required for the remodelling of extracellular matrix during wound healing⁵⁵. In addition, the pathological release of lysosomal enzymes may also cause the local injury of the vascular wall leading to atherosclerosis and the focal thickening of arteries⁵⁶. Thus, proper regulation of the platelet lysosomal secretion would be essential to maintain a normal response to wound healing, and also to avoid the inflammatory and thrombotic defects that could result from the excessive platelet lysosomal secretion. Consistent with this concept, this study shows that the excessive storage and secretion of lysosomal enzymes from platelets lacking *PIKfyve* result in the accelerated arterial thrombosis and inflammation observed in *PIKfyve*^{fl/fl} *Pf4-Cre* mice. Thus, our studies further support that the proper regulation of platelet lysosomal storage and secretion have a crucial role in limiting the proinflammatory and prothrombotic functions of platelets.

Our study demonstrates the novel finding that *PIKfyve* kinase activity is a critical regulator of the lysosomal pathway in mouse platelets. This work also provides evidence that in addition to the

effects mediated by the release of platelets alpha and dense granules, secretion of platelet lysosomes may be a previously unrecognized initiator of vascular damage and inflammatory responses. These findings could have important implications and applications in the diagnosis and in the design of novel targets for clinical disorders mediated by platelets.

Methods

Animal models. All animals were maintained on standard chow and tap water under specific pathogen-free conditions. All animal procedures were approved by the Institutional Animal Care and Use Committees at the University of Pennsylvania. Targeting of the *PIKfyve* gene was generated using C57BL/6 mice (inGenious Targeting Laboratory). A bacterial artificial chromosome derived from C57BL/6 (RPC123) genomic DNA was used to construct the targeting vector. The targeting vector was designed to excise exons 37 and 38 of the mouse *PIKfyve* gene that encodes the activation loop of the kinase domain. The 7.55-kb long homology arm extended 5' to exon 37, and the 1.5-kb short homology arm extended 3' to exon 38. A *pGK-gb2 loxP/FRT* neocassette was inserted on the 3' side of exon 38, and the single loxP site was inserted at the 5' side of exon 37. The resulting 10.6-kb fragment was subcloned into an ~2.4-kb backbone vector (pSP72, Promega) containing an ampicillin selection cassette to generate a final targeting construct of 14.8 kb. C57BL/6 embryonic stem cells (ES) were electroporated with targeting vectors. Clones resistant to the G418 antibiotic were selected and screened by Southern blot and PCR to identify recombinant ES clones. The selected clones were microinjected into C57BL/6 blastocysts. Resulting chimeras with black coat colour were mated with wild-type C57BL/6 mice to generate *PIKfyve* F1 heterozygous offspring. F1 heterozygous mice were crossed with a *FLP* transgenic mouse to excise the neomycin cassette that was flanked by FRT sites. The resulting F2 mosaic offspring were crossed with wild-type mice to generate F3 heterozygotes that were missing the neomycin cassette. F3 heterozygotes were crossed to generate mice carrying the *PIKfyve*-floxed allele (*PIKfyve^{fl/fl}*). *PIKfyve^{fl/fl}* mice were crossed with transgenic mice expressing Cre recombinase under the control of the platelet factor 4 promoter (*Pf4-Cre*) to induce tissue-restricted Cre recombination in the megakaryocyte lineage. For some experiments, *PIKfyve^{fl/fl}* mice were crossed with a B6;129S4-Gt(*ROSA*)26Sor^{tm1Sor}/J (*Rosa26-LacZ*) reporter strain and then with a *Pf4-Cre* transgenic mouse. For other experiments, *PIKfyve^{fl/fl}* mice were crossed with B6-Cg-*Pldn^{pa/j}* (*pallid*) mice, and their offspring were paired with the *Pf4-Cre* transgenic mice to generate *PIKfyve^{fl/fl}* *Pf4-Cre/pallid* mice. For all studies, both female and male mice were used. Sex-matched littermates were used as controls for all studies if not indicated otherwise.

PCR genotyping. Genomic DNA isolated from mouse tail biopsies was used to genotype mice by PCR. The recombinant ES cells were identified with 5'-CCAGAGGCCACTTGTGTAGC-3' and A3: 5'-CAGCCAGTGTCTCGCATACAAAGG-3' primers. The insertion of neomycin cassette in F1 mice was identified with 5'-CCAGGCCACTTGTGTAGC-3' and 5'-GGATGAAGTGCCTTGTATCTGCAGC-3' primers. The 3rd LoxP integration was identified with 5'-CCATTGCCTGGCTTGAACAGAG-3' and 5'-GAACTCTCCCGCTAGTACAGC-3' primers. PCR-generated products were either 310 bp corresponding to the wild-type alleles or 370 bp corresponding to the targeted allele (LoxP integrated). *Pf4-Cre* transgene was identified with 5'-CCATACAGCACACCTTTTG-3' and 5'-TGCACAGTCAGAGTT-3' primers. *Rosa26/LacZ* transgene was identified with 5'-AAAGTCGCTCTGAGTTGTTAT-3', 5'-GCGAAGAGTTTGTCTCAACC-3' and 5'-GGAGCGGGAGAAATGGATATG-3'.

Body composition analysis. Mice were anaesthetized with sodium pentobarbital (90 mg kg⁻¹) and scanned by dual-energy X-ray absorptiometry at the Mouse Metabolism Core at the University of Pennsylvania.

Histochemistry and immunohistochemistry. Necropsies were performed on female and male control and *PIKfyve^{fl/fl}* *Pf4-Cre* mice between 3 weeks and 28 weeks of age. They included macroscopic observations, body and organ weight measurements, complete blood counts, serum chemistry analysis and microscopic examination of the brain, thymus, lymph nodes, heart, lung, small intestine, large intestine, liver, spleen, pancreas, kidney, ovaries, testis, skin, muscle, fat and bone marrow. Fresh postmortem tissues were fixed overnight in 10% formalin, paraffin-embedded and sectioned. Paraffin sections were deparaffinized with xylene and ethanol, and stained with haematoxylin/eosin, periodic acid Schiff and Alcian blue. For immunohistochemistry, paraffin sections were deparaffinized with xylene and ethanol, and antigen retrieved in 0.01 M citrate buffer by microwaving for 30 min. They were stained with the following primary antibodies: F4/80 (Serotec; 1:100), CD41 (BD Pharmingen; MWReg30; 1:50) and CD45R/B220 (BD Pharmingen; RA3-6B2; 1:500). Sections were counterstained with haematoxylin. For X-gal staining, frozen tissue sections were fixed with 0.5% glutaraldehyde for 10 min, and stained overnight with the chromogenic substrate for β -galactosidase, X-gal (Cell Center, University of Pennsylvania). Sections were then counterstained with haematoxylin or haematoxylin/eosin as indicated.

Immunoblotting. Tissues were harvested and homogenized in RIPA buffer with a protease inhibitor cocktail (Roche). The protein concentrations were determined using the BCA Protein Assay (Pierce). Proteins were separated on SDS-PAGE gradient gels (Invitrogen) and then transferred onto the polyvinylidene difluoride membrane (Invitrogen). The membrane was blotted with the indicated primary antibodies against: PIKfyve-N-terminal (Sigma-Aldrich; P0054; 1:400), LAMP-1 (Developmental Studies Hybridoma Bank; 1:1,000), EEA-1 (Santa Cruz; 1:1,000), Cathepsin D (Calbiochem; 1:1,000), vWF (DAKO; 1:1,000), PF4 (Bethyl; 1:1,000), Akt (Cell Signaling Technology; 1:1,000), pAkt (Cell Signaling Technology; 1:1,000) and β -actin (Cell Signaling Technology; 1:1,000). This was followed by blotting with horseradish peroxidase-conjugated secondary antibodies and developing with enhanced chemiluminescence substrate (GE Healthcare). Full blots are shown in the Supplementary Figs 8 and 9.

Megakaryocyte and platelet preparation. Megakaryocytes were derived from the bone marrow by flushing mouse femurs and cultured in the presence of 1% thrombopoietin (TPO, R&D Systems) in IMDM supplemented with 10% FBS²⁶. After 4 days, cultured megakaryocytes were isolated by sedimentation through a 1.5%/3% BSA step gradient. To prepare washed platelets, blood was collected from the inferior vena cava of mice into 0.1 volume of acid citrate dextrose buffer. Platelet-rich plasma was separated by centrifugation at 200 g for 10 min and incubated with 1 μ M prostaglandin E1 for 10 min at room temperature. Platelets were then isolated by centrifugation at 800 g for 10 min and resuspended in HEPES-Tyrod buffer pH 7.4.

Megakaryocyte number and ploidy analysis. To measure the number of megakaryocytes in the bone marrow, isolated megakaryocytes from the bone marrow were stained with CD41 antibody conjugated with Alexa 488 (BD Biosciences), and cells were counted on the FACS Calibur flow cytometer (BD Biosciences) discriminating size to subtract out platelets. To determine the megakaryocyte ploidy, cells were stained with propidium iodide (Sigma) and the relative DNA content was measured. FlowJo software was used to analyse all flow cytometry data.

P-selectin expression analysis. Platelets were diluted to a density of 5×10^6 cells ml⁻¹, stimulated with thrombin at indicated concentrations and then incubated with a phycoerythrin-conjugated P-selectin antibody (BD Pharmingen). Fluorescence values were analysed on a FACS Calibur flow cytometer (BD Biosciences).

Platelet aggregation and ATP secretion. Platelets were diluted to a density of 2.5×10^6 cells ml⁻¹ in HEPES-Tyrod buffer pH 7.4. Platelet aggregation in response to agonist stimulation was assessed by changes in light transmission detected by a dual-channel lumi-aggregometer (Chrono-Log) with stirring at 37 °C. Platelet secretion of ATP was assessed concurrently by the luciferin/luciferase reaction after adding chrono-lume no. 395 (Chrono-Log) to the aggregometer cuvette.

Transmission electron microscopy. Megakaryocytes grown in culture were harvested on day 6 and fixed in glutaraldehyde. Ultrathin sections were obtained and collected on hexagonal copper grids. Sections were stained with Reynold's lead citrate and examined using transmission electron microscopy at the Electron Microscopy Core Facility at the University of Pennsylvania.

Platelet depletion in vivo. To study the effects of platelet depletion in the control mice and the *PIKfyve^{fl/fl}* *Pf4-Cre* mice, female and male mice at 8–10 weeks of age were administered with GPIIb/IIIa antibody (Emfret) at 2 μ g g⁻¹ twice weekly by intraperitoneal injections for a total of 6 weeks. Blood counts were monitored weekly by collecting blood from the retro-orbital sinus. Body weight was monitored twice weekly. Necropsies were performed at the completion of 6 weeks of antibody treatment.

Radiation bone marrow chimeras. Bone marrow cells were isolated from wild-type mice expressing either CD45.1 or CD45.2 congenic markers or from *PIKfyve^{fl/fl}* *Pf4-Cre* mice expressing CD45.2 congenic markers. Bone marrow cells were depleted of T cells and red blood cells by using magnetic beads bound to antibodies against Ter119, CD3, CD4 and CD8. To study the phenotype rescue effect, *PIKfyve^{fl/fl}* *Pf4-Cre* recipient mice expressing CD45.2 congenic markers were irradiated with 900 rads and administered by retro-orbital injection with 2×10^6 bone marrow cells from wild-type mice expressing CD45.1 markers. To study the phenotype transfer effect, wild-type recipient mice expressing CD45.1 markers were irradiated with 900 rads, and administered by retro-orbital injection with 1×10^6 bone marrow cells from wild-type mice expressing thy 1.1 marker and 1×10^6 bone marrow cells from *PIKfyve^{fl/fl}* *Pf4-Cre* mice expressing CD45.2 markers. Transplanted mice were maintained in sterile water with sulfamethoxazole/trimethoprim for 3 weeks. Mice were killed and analysed for engraftment and histology analyses at about 33–35 weeks after transplantation.

β -Hexosaminidase and cathepsin D activity assays. The β -hexosaminidase activity was determined using a chromogenic substrate⁵⁷. Mouse platelets or plasma were mixed with 10 mM substrate *p*-nitrophenyl-*N*-acetyl- β -D-glucosaminide (Sigma) in citrate-phosphate buffer pH 4.5. Platelet extracts were incubated for 16 h at 37 °C and plasma was incubated for 1 h at 37 °C. The enzymatic reaction was stopped by the addition of 0.5 M NaOH. The absorbance of released *p*-nitrophenolate was read at 405 nm in an ELISA plate reader. Cathepsin D activity was determined using a fluorogenic substrate⁵⁸. The reaction mixture contained 10 μ l of platelet lysate solution, 10 μ l of 200 μ M of substrate Bz-Arg-Gly-Phe-Phe-Pro-4MeO β NA (Calbiochem) and 80 μ l of 50 mM sodium acetate buffer pH 4.0. Reaction mixtures were incubated for 30 min at 37 °C. The enzymatic reaction was stopped by adding 5% trichloroacetic acid. The release of 2-naphthylamine was measured in a spectrofluorometer at emission wavelength of 410 nm and excitation wavelength of 335 nm.

FeCl₃-induced carotid artery thrombosis assay. Mice were anaesthetized using sodium pentobarbital (90 mg kg⁻¹). A midline incision was made in the neck, and the carotid artery was exposed and dissected away from surrounding tissue. The artery was placed on a Doppler flow probe (Transonic Systems, Ithaca, NY) to monitor blood flow through the vessel. After a baseline flow measurement was recorded, vascular injury was initiated by placing a 1 mm² piece of filter paper saturated with 7.5% FeCl₃ (Sigma) on the surface of the artery for 2 min. The filter paper was then removed and the artery rinsed with saline, followed by the continuous recording of blood flow in the vessel for 20 min post injury. The time to vessel occlusion was recorded.

Plasma vWF analysis. For analysis of plasma vWF multimer distribution, mouse plasma (10 μ l) was denatured at 60 °C for 20 min with 10 μ l of sample buffer (70 mM Tris, pH 6.8, 2.4% SDS, 0.67 M urea, 4 mM EDTA, 10% glycerol, 0.01% bromophenol blue). Proteins were fractionated on 1% agarose gel on ice and transferred to a nitrocellulose membrane. The membrane was blotted with vWF IgG antibody (Dako; 1:5,000) to detect the vWF multimers. For quantification analysis of plasma vWF antigen, we performed ELISA. A microtitre plate coated with rabbit anti-vWF IgG (Dako; 1:2,000) was incubated with diluted mouse plasma for 1 h at room temperature. After washing with phosphate-buffered saline, bound vWF was determined by peroxidase-conjugated rabbit anti-vWF IgG (Dako; 1:2,000). A pre-mixed TMB (3,3',5,5'-tetramethylbenzidine) solution was used for colour development. The absorbance at 450 nm was determined by an ELISA plate reader.

Mass spectrometry analysis. To prepare platelet releasates for proteomics analysis, washed platelets (5 $\times$ 10⁸ cells ml⁻¹) were stimulated with 1 U ml⁻¹ thrombin for 10 min at 37 °C. Activated platelets were centrifuged at 13,000 g for 10 min at 4 °C and the supernates were isolated from the pellets. The concentrations of proteins from the platelet supernates were measured using the BCA protein assay (Pierce). Proteins (40 μ g) were digested with trypsin in solution. Tryptic digests were analysed by LC-MS/MS on a hybrid LTQ-Orbitrap Elite mass spectrometer (ThermoFisher Scientific, San Jose, CA) coupled with a nano LC Ultra (Eksigent). Peptides were separated by reverse phase (RP)-HPLC on a nanocapillary column, 75 μ m id $\times$ 15 cm Reprosil-pur 3 μ M (Dr Maisch, Germany) in a Nanoflex chip system (Eksigent). Mobile phase A consisted of 1% methanol (Fisher)/0.1% formic acid (Thermo) and mobile phase B of 1% methanol/0.1% formic acid/80% acetonitrile. Peptides were eluted into the mass spectrometer at 300 nl min⁻¹ with each RP-LC run comprising a 90 min gradient from 10 to 25% B in 65 min, 25–40% B in 25 min. The mass spectrometer was set to repetitively scan *m/z* from 300 to 1,800 (*R* = 240,000 for LTQ-Orbitrap Elite) followed by data-dependent MS/MS scans on the twenty most abundant ions, with a minimum signal of 5,000, dynamic exclusion with a repeat count of 1, repeat duration of 30 s, exclusion size of 500 and duration of 60 s, isolation width of 2.0, normalized collision energy of 33 and waveform injection and dynamic exclusion enabled. FTMS full scan AGC target value was 1e6, while MSn AGC was 1e4, respectively. FTMS full scan maximum fill time was 500 ms, while ion trap MSn fill time was 50 ms; microscans were set at 1. FT preview mode; charge state screening and monoisotopic precursor selection were all enabled with rejection of unassigned and 1+ charge states.

For database search, MS/MS raw files were searched against a mouse protein sequence database including isoforms from the Uniprot Knowledgebase (taxonomy:10090 AND keyword: 'Complete proteome [KW-0181]') using MaxQuant version 1.4.1.2 with the following set parameters: fixed modifications, Carbamidomethyl (C); decoy mode, revert; MS/MS tolerance ITMS, 0.5 Da; false discovery rate for both peptides and proteins, 0.01; minimum peptide length, 6; modifications included in protein quantification, acetyl (protein N-term), oxidation (M); peptides used for protein quantification, razor and unique. The numbers of peptide spectral matches to each protein were used for quantitative comparison between samples as described below.

For statistical analysis of mass spectrometry data, we adapted a method proposed by Choi⁵⁹ to use the number of peptide spectral matches (PSMs) in each protein to determine the likelihood that the protein was differentially regulated across the control mice and the knockout mice. The idea is to develop a control

model and a knockout model, and, for each protein, to test whether the knockout model describes its expression data significantly better than the control model. To do so, we estimate a Bayes Factor $B_p = \frac{P(M^K | Data)}{P(M^C | Data)}$ for each protein. We assume that the number of PSMs for protein *p* in sample *i* can be modelled as a Poisson distribution with mean λ_{pi} , since each element x_{pi} in the data matrix **X** is count data. To combat the limited number of biological replicates in each treatment group, we pooled information across replicates and estimate each λ_{pi} using a control and knockout hierarchical log-linear model, denoted by:

$$M^C : \log(\lambda_{pi}^C) = \log(L_p) + \log(N_i) + a_0 + c_p \quad (1)$$

$$M^K : \log(\lambda_{pi}^K) = \log(L_p) + \log(N_i) + a_0 + c_p + T_i k_p \quad (2)$$

where L_p is the length of protein *p*, N_i is the mean number of spectral counts in sample *i*,

$$T_i = \begin{cases} 0 & \text{if sample } i \text{ is a control mouse} \\ 1 & \text{if sample } i \text{ is a knockout mouse} \end{cases}$$

a_0 is a model constant, c_p is a protein's native expression term and k_p is a protein's knockout expression term. We give the model a well-defined structure by putting priors on $a_0 \sim N(0, \delta_a^2)$, $c_p \sim N(0, \delta_c^2)$, and $k_p \sim N(0, \delta_k^2)$. To make data-driven estimates of δ_a^2 and δ_k^2 , we place hyper priors on these two parameters, given by $\delta_a^2, \delta_k^2 \sim \text{InvGamma}(0.1, 0.1)$. We let a_0 'float', and thus set $\delta_a^2 = 10^2$. We use these parameters (denoted as a vector by θ) to estimate a protein's Bayes Factor:

$$\begin{aligned} B_p &= \frac{P(M^K | \mathbf{X})}{P(M^C | \mathbf{X})} = \frac{\int P(\mathbf{X} | M^K, \theta^K) P(\theta^K | M^K) d\theta^K P(M^K)}{\int P(\mathbf{X} | M^C, \theta^C) P(\theta^C | M^C) d\theta^C P(M^C)} \\ &= \frac{E_{\theta^K | M^K} [P(\mathbf{X} | M^K, \theta^K)]}{E_{\theta^C | M^C} [P(\mathbf{X} | M^C, \theta^C)]} \quad (\text{assuming we have no prior knowledge of } P(M^K) \text{ and } P(M^C) \text{ for any protein}) \\ &= \frac{\lim_{N \rightarrow \infty} \frac{1}{N} \sum_{n=1}^N P(\mathbf{X} | M^K, \theta_n^K)}{\lim_{N \rightarrow \infty} \frac{1}{N} \sum_{n=1}^N P(\mathbf{X} | M^C, \theta_n^K)} \end{aligned} \quad (3)$$

where the transition states $\theta_n^K \rightarrow \theta_{n+1}^K$ are determined using Metropolis Hastings and Gibbs Sampling Markov Chain Monte Carlo techniques. Specifically, at iteration *n* with parameter vector θ^K (we explain using the knockout model, since $M^C \subset M^K$),

$$P(a_{0i} | \cdot) xP(\mathbf{X} | a_{0i}, \cdot) P(a_{0i}) = N(a_{0i}; 0, \delta_a^2) \prod_i \prod_p \text{Poisson}(x_{pi} | a_{0i}, \cdot) \quad (4)$$

$$P(c_{pn} | \cdot) xP(\mathbf{X} | c_{pn}, \cdot) P(c_{pn}) = N(c_{pn}; 0, \delta_c^2) \prod_i \text{Poisson}(x_{pi} | c_{pn}, \cdot) \quad (5)$$

$$P(k_{pn} | \cdot) xP(\mathbf{X} | k_{pn}, \cdot) P(k_{pn}) = N(k_{pn}; 0, \delta_k^2) \prod_i \text{Poisson}(x_{pi} | k_{pn}, \cdot) \quad (6)$$

$$P(\delta_{ca}^2 | \cdot) xP(\{c_p\}_{p=1}^G | \delta_{ca}^2) P(\delta_{ca}^2) = \text{InvGamma}\left(0.1 + \frac{G}{2}, 0.1 + \frac{\sum_{p=1}^G c_p^2}{2}\right) \quad (7)$$

$$P(\delta_{kn}^2 | \cdot) xP(\{k_p\}_{p=1}^G | \delta_{kn}^2) P(\delta_{kn}^2) = \text{InvGamma}\left(0.1 + \frac{G}{2}, 0.1 + \frac{\sum_{p=1}^G k_p^2}{2}\right). \quad (8)$$

where *G* is the number of proteins.

To determine the set of proteins that significantly changed in the knockout set with respect to the control set, we imposed a strict cutoff and the requirement that each protein have a Bayes Factor of at least 8 and a fold change of at least 2.0.

Statistical analysis. GraphPad Prism 5 was used to analyse data. All data are presented as mean $\pm$ s.d. Two-tailed Student's *t*-tests were used for the comparison of two groups. A log-rank test was performed for Kaplan–Meier survival analysis. *P*-values of <0.05 (*), <0.01 (**) and <0.001 (***) were used to ascertain statistical significance.

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Authors contributions

S.H.M. planned and performed experiments, data analysis and presentation of most of the experiments and wrote the manuscript; A.S. and T.J.S. carried out experiments; L.Z., Y.W., M.J.R., J.F.G., S.Z., L.L. and R.M. provided technical assistance; C.M. and S.H.S. performed the proteomics experiments and analysis; J.K.C. contributed to the data analyses; G.K. and M.S.M. provided critical reagents and contributed to the data analyses; C.S.A. supervised the project and wrote the manuscript.

Additional information

Supplementary Information accompanies this paper at <http://www.nature.com/naturecommunications>

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