ROLE OF PIKFYVE IN PLATELET LYSOSOMAL HOMEOSTASIS

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DEDICATION

To my best friend and husband, Soo-Wan, for making me smile every day.

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ABSTRACT

ROLE OF PIKFYVE IN PLATELET LYSOSOMAL HOMEOSTASIS

Sang Hee Min

Charles S. Abrams

PIKfyve is a lipid kinase that is essential for the synthesis of phosphatidylinositol-3,5-bisphosphate [PtdIns(3,5)P2], and for the regulation of membrane dynamics within the endolysosomal system in mammals. Depletion of intracellular pools of PtdIns(3,5)P2 in humans and in mice is associated with neurodegeneration and early lethality. However, the biological role of PtdIns(3,5)P2 in non-neural tissues is not well understood. Platelets are hematopoietic cells that function in a variety of physiological responses. Essential to many of these functions is the activation-dependent release of effectors from distinct storage granules - alpha granules, dense granules, and lysosomes - that derive from the endolysosomal system. In this work, we show that platelet-specific ablation of the PIKfyve gene in mice results in accelerated arterial thrombosis, but also unexpectedly to multiorgan defects that impair development, body mass, fertility, and survival by inducing inappropriate inflammatory responses characterized by macrophage accumulation in multiple tissues. Platelet depletion *in vivo* significantly impairs the progression of multiorgan defects in these mice, confirming that these defects reflect a platelet-specific process. Although *PIKfyve-null* platelets generate and release normal amounts of alpha granule and dense granule contents, they develop defective maturation and excessive storage of lysosomal enzymes, which are released upon platelet activation. Remarkably, impairing the secretion of lysosomes from *PIKfyve-deficient* platelets *in vivo* significantly attenuates the multiorgan defects in mice, suggesting that platelet

lysosome secretion contributes to pathogenesis. Together, these results demonstrate that PIKfyve is an essential regulator for the biogenesis of platelet lysosomes, and highlight the previously unrecognized and important pathological contributions of platelet lysosomes in inflammation, arterial thrombosis, and macrophage biology.

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CHAPTER 1: INTRODUCTION [1]

1.1) Phosphoinositides: overview

Phosphatidilinositol (PtdIns) is a class of membrane phospholipid, which is composed of an inositol head group, a glycerol backbone, and two fatty acid tails. PtdIns is modified by transient phosphorylation of its inositol head group, which generates various phosphorylated forms of PtdIns, known as phosphoinositides (Hokin and Hokin 1953, Hokin 1996). PtdIns and phosphoinositides are very minor components of the membrane phospholipids, and yet they are essential in the regulation of diverse cellular processes, which include membrane trafficking, cytoskeletal dynamics, membrane transports, and nuclear events in many different cells (Di Paolo and De Camilli 2006).

A total of seven phosphoinositides have been identified that derive from PtdIns following the reversible phosphorylation of the hydroxyls situated in the D3, D4 and D5 positions of the inositol head group. These seven phosphoinositides are: PtdIns(3)P, PtdIns(4)P, PtdIns(5)P, PtdIns(3,5)P2, PtdIns(3,4)P2, PtdIns(4,5)P2, and PtdIns(3,4,5)P3 (Figure 1). The turnover of phosphoinositides occurs on the cytosolic leaflet in the cellular membrane or on the membrane of intracellular organelles by specific phosphoinositide-metabolizing enzymes, which include lipid kinases, lipid phosphatases,

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and phospholipases (Heck, Mellman et al. 2007, Krauss and Haucke 2007). The individual phosphoinositide-metabolizing enzymes have distinct expression and regulation patterns and contribute to the spatiotemporal organization of specific phosphoinositides (Krauss and Haucke 2007). Consequently, the different phosphoinositides are rapidly and reversibly generated on the membranes of distinct cellular compartments.

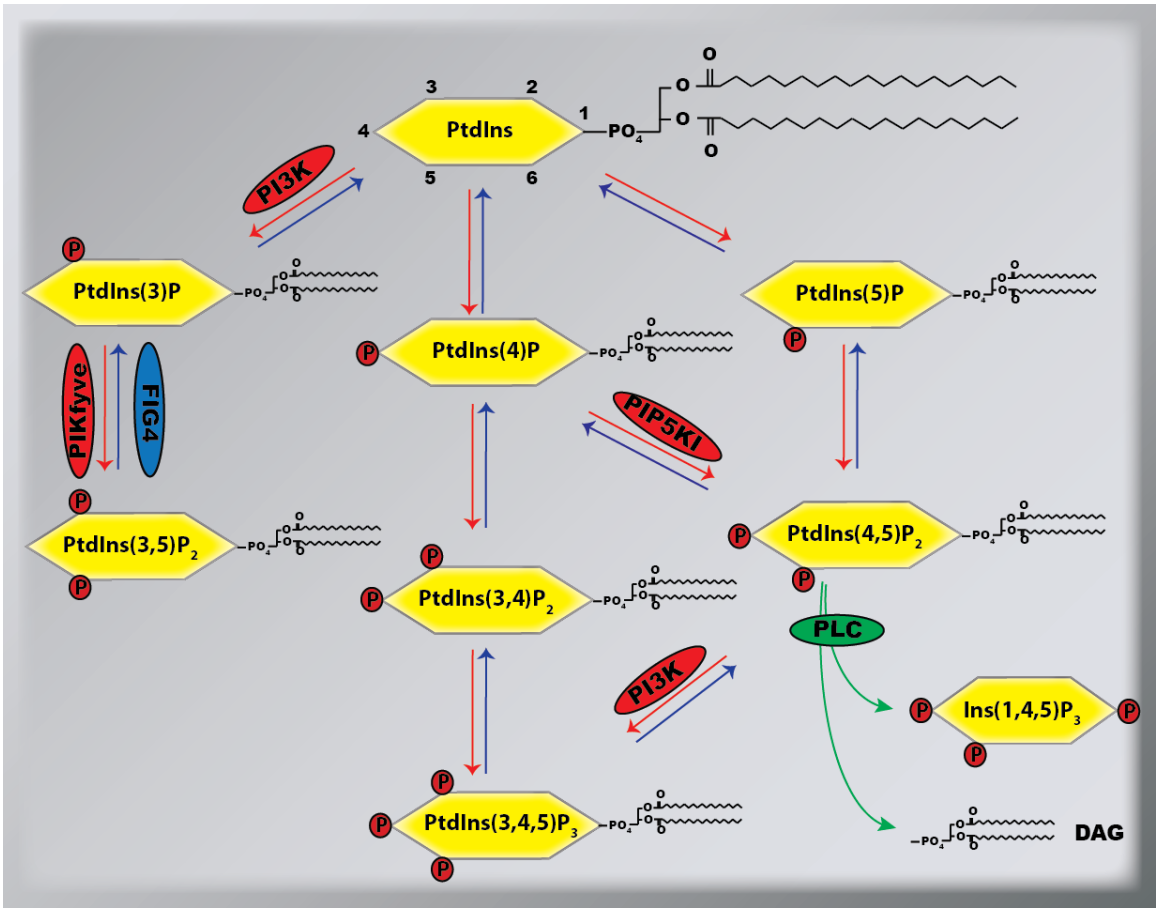

Figure 1.1. Metabolism of phosphoinositides by phosphoinositide-metabolizing enzymes. Shown is the relationship between different phosphoinositides and their metabolizing lipid kinases (red arrows), lipid phosphatases (blue arrows) and phospholipase C (green arrows).

Once synthesized, the individual phosphoinositides can be recognized by selective subsets of proteins containing specific phosphoinositide-binding domains, such as PH (pleckstrin homology), FYVE (Fab1, YOTB, Vac1, EEA1 homology), PX (phox homology), PROPPINs (β-propellers that bind phosphoinositides), ENTH (epsin N-terminal homology), and ANTH (AP180 N-terminal homology) (Lemmon 2008). For example, PtdIns(4,5)P2 on the plasma membrane binds to ENTH domain of Epsin and indirectly recruit Epsin binding proteins such as AP2 to bind clathrin coats and form clathrin-coated vesicles. This recruitment of a specific effector protein to a phosphoinositide confined in a specific cellular compartment provides each of the individual phosphoinositides with unique functions in cells.

The essential physiological functions of phosphoinositides have been demonstrated by many human disorders or abnormal phenotypes in mice associated with mutations in the phosphoinositide-metabolizing enzymes (Nicot and Laporte 2008, McCrea and De Camilli 2009).

1.2) Phosphoinositide organization during platelet plug formation

Platelet plug formation is essential during hemostasis, but when perturbed, it can lead to pathological bleeding or thrombosis (Jackson 2011). Thus, this is a tightly controlled process requiring activation of platelets under carefully modulated intracellular signaling transduction (Jackson 2007, Stalker, Newman et al. 2012). When there is a vascular injury, platelets tether to collagen or to von Willebrand factor (vWF) and initiate

an intracellular signaling cascade that leads to firm and stable adhesion to the subendothelium (Ruggeri and Mendolicchio 2007). This is followed by integrin activation on the platelet surface, and subsequently aggregation between platelets (Jackson 2007). Further stabilization of the platelet plug and prevention of platelet disaggregation requires additional amplification of the platelet signaling pathways. Over the past few decades, accumulating evidence indicates that phosphorylated phosphatidylinositols are crucial components in this complex network of platelet signaling.

The role of PtdIns(4,5)P2 signaling in platelet biology

PtdIns(4,5)P2 synthesis by PIP5KI

PtdIns(4,5)P2 is a predominant phosphoinositide in the cellular membrane. PtdIns(4,5)P2 is synthesized either from PtdIns(4)P by the D5-OH kinase activity of PIP5KI (Hay, Fisette et al. 1995) or from PtdIns(5)P by the D4-OH kinase activity of phosphatidylinositol 5-phosphate 4-kinase type II (PIP5KII or PIP4K) (Rameh, Tolias et al. 1997, Clarke, Wang et al. 2010). Studies comparing the relative labeling rate of the D4- and D5- hydroxyl positions of the inositol ring suggest that the catalytic activity of the D5 position is more efficient (King, Hawkins et al. 1989, Stephens, Hughes et al. 1991). In addition, the relative abundance of PtdIns(4)P is much greater than PtdIns(5)P (Stephens, Hughes et al. 1991, Roberts, Clarke et al. 2005), suggesting that PIP5KI phosphorylation of PtdIns(4)P is the major source of PtdIns(4,5)P2 synthesis. In agreement with these findings, while the loss of PIP4K does not reduce the synthesis of

PtdIns(4,5)P2 in mammalian cells, the loss of PIP5KI significantly decreases the intracellular pool of PtdIns(4,5)P2. This demonstrates that PIP5KI is the predominant kinase in the generation of PtdIns(4,5)P2.

PIP5KI isoforms and splice variants in platelets

PIP5KI exists in three different isoforms (α, β and γ). Intriguingly, all three PIP5KI isoforms can synthesize PtdIns(4,5)P2 from PtdIns(4)P (Ishihara, Shibasaki et al. 1996, Loijens and Anderson 1996, Ishihara, Shibasaki et al. 1998). This in turn raises the question why PIP5KI exists in more than one isoform? Recently, work by our group as well as by others addressed this question using genetically engineered mice lacking each isoform of PIP5KI in platelets. Platelets contain all three isoforms of PIP5KI, but the murine PIP5KI-β and PIP5KI-γ are the most abundant forms (Wang, Chen et al. 2008, Wang, Litvinov et al. 2008). Platelets lacking PIP5KI-β have defective synthesis of PtdIns(4,5)P2 as well as defective Ins(1,4,5)P3 formation in the first one minute after stimulation with thrombin (Wang, Chen et al. 2008). Consequently, these platelets display defective aggregation *ex vivo* and impaired formation of an occluding thrombus *in vivo* (Wang, Chen et al. 2008). Remarkably, platelets lacking both PIP5KI-α and PIP5KI-β have a complete loss of Ins(1,4,5)P3 formation, even though they still contain PIP5KI-γ (Wang, Chen et al. 2008). This suggests that PIP5KI-γ synthesizes a pool of PtdIns(4,5)P2 that is not required for the synthesis of second messengers such as Ins(1,4,5)P3.

As predicted, platelets lacking PIP5KI-γ can still produce normal amounts of Ins(1,4,5)P3 upon stimulation with thrombin, despite having impaired synthesis of PtdIns(4,5)P2 (Wang, Litvinov et al. 2008). But in contrast to cells lacking PIP5KI-α or PIP5KI-β, megakaryocytes or platelets lacking PIP5KI-γ have a significant defect in anchoring their cell membranes to the underlying cytoskeleton (Wang, Litvinov et al. 2008, Wang, Zhao et al. 2013). This suggests that PIP5KI-γ synthesis of PtdIns(4,5)P2 does not contribute to Ins(1,4,5)P3 formation, but rather directly affects the membrane anchoring of the cytoskeleton. Additional evidence suggests that PIP5KI-γ synthesizes a discrete pool of PtdIns(4,5)P2 that interacts directly with actin-binding proteins (such as talin) (Di Paolo, Pellegrini et al. 2002, Ling, Doughman et al. 2002). Once bound to PtdIns(4,5)P2, these actin-regulating proteins are enabled to anchor the cell membrane to the underlying cytoskeleton (Gilmore and Burridge 1996).

These studies demonstrate that although all PIP5KI isoforms can synthesize PtdIns(4,5)P2, they have non-redundant functional roles in platelets. How the different isoforms of PIP5KI promote unique physiological effects is unclear, but several mechanisms are proposed. First, although PIP5KI-α and PIP5KI-β have homologous primary sequences, PIP5KI-γ is structurally different (Loijens and Anderson 1996, Wang, Chen et al. 2008). This suggests that individual PIP5KI isoforms could be regulated differently. Second, spatially distinct localization of individual PIP5KI isoforms may generate discrete pools of PtdIns(4,5)P2. Several studies indicate that different PIP5KI isoforms are localized in distinct cellular compartments in tissue culture cells. For instance, PIP5KI-α is more predominant in membrane ruffles (Doughman, Firestone et al. 2003), while PIP5KI-β concentrates close to the endosomes (Padron, Wang et al. 2003),

and PIP5KI-γ is targeted to focal adhesions and nerve terminals (Wenk, Pellegrini et al. 2001, Di Paolo, Pellegrini et al. 2002, Ling, Doughman et al. 2002). In addition, our data indicate that PIP5KI-α and PIP5KI-β, but not PIP5KI-γ can synthesize the pool of PtdIns(4,5)P2 utilized by PLC to generate Ins(1,4,5)P3 (Wang, Chen et al. 2008, Wang, Litvinov et al. 2008). From these studies, one could hypothesize that the pools of PtdIns(4,5)P2 from different PIP5KI isoforms are highly compartmentalized and targeted by selective downstream effector molecules, providing unique functions to each of the PIP5KI isoforms. However, it is also conceivable that only PIP5KI-α and PIP5KI-β can interact with auxiliary proteins that in turn regulate the activity of downstream effectors such as PLC. Nevertheless, the exact mechanisms by which different PIP5KI isoforms fulfill unique niches within platelets need to be further elucidated.

As mentioned above, several groups of researchers have speculated that PIP5KI-γ might generate PtdIns(4,5)P2 that binds to talin and positions it to participate in signaling pathways that emanate towards or in response to integrin activation (Gilmore and Burridge 1996, Di Paolo, Pellegrini et al. 2002, Ling, Doughman et al. 2002, Calderwood and Ginsberg 2003). PIP5KI-γ has two predominant splice variants that differ by the inclusion of or exclusion of 27 amino acids encoded by the terminal exon of the PIP5K-γ gene (Di Paolo, Pellegrini et al. 2002, Ling, Doughman et al. 2002). These two splice variants are the longer 90 kDA (p90) splice form and the shorter 87 kDa (p87) splice form. These additional 27 amino acids allow the p90 splice variant to bind to talin, a protein that is important for both integrin activation and for linking integrins to the underlying cytoskeleton (Di Paolo, Pellegrini et al. 2002, Ling, Doughman et al. 2002, Calderwood and Ginsberg 2003). Consequently, platelets and megakaryocytes lacking

the p90 splice variant of PIP5KI-γ have defective anchoring of their integrins to the underlying cytoskeleton (Wang, Zhao et al. 2013). This effect depends on the kinase activity of PIP5KI-γ, which implies that the local synthesis of PtdIns(4,5)P2 by the p90 splice variant plays an important role in maintaining the integrity of the connection between the cell membrane and actin. Remarkably, platelets lacking this p90 splice variant of PIP5KI-γ have no obvious defect in the activation of integrins or in cell adhesion. In contrast, platelets lacking both p87 and p90 PIP5KI-γ do have a defect in shear-resistant adhesion (Wang, Zhao et al. 2013). These data suggest that although both the p87 and p90 PIP5KI splice variants can synthesize PtdIns(4,5)P2, they have non-redundant functions in platelet cytoskeletal dynamics and in stable adhesion. This study further provides evidence that PIP5KI has a variety of intracellular functions. Not only does PIP5KI produce isolated pools of PtdIns(4,5)P2, it also, because of its individual isoforms and splice variants, can directly interact and regulate distinct signaling proteins.

Regulation of PIP5KI by small GTPases

Several studies suggest that the localization and kinase activity of PIP5KI isoforms are regulated by small GTPases of the Rho family. For instance, J. Hartwig and colleagues demonstrated that thrombin-mediated Rac1 activation induces the synthesis of PtdIns(4,5)P2 by PIP5KI-α, which in turn leads to actin filament assembly (Hartwig, Bokoch et al. 1995). The S. Offermanns group showed that exposure of platelets to thromboxane A2 stimulates Rho and Rho kinase, which thereby activate PIP5KI (Gratacap, Payrastre et al. 2001). We have shown that PIP5KI is rapidly translocated

from the cytosol to actin-rich compartments upon platelet activation, but this process is impaired when the Rho GTPase is inhibited (Chatah and Abrams 2001, Yang, Carpenter et al. 2004). Together, these works suggest that Rho family GTPases regulate the trafficking of PIP5KI into the membrane cytoskeleton. Once PIP5KI is positioned next to its substrate, PtdIns(4)P, PIP5KI is poised to generate PtdIns(4,5)P2.

The role of second messengers Ins(1,4,5)P3 and DAG in platelet biology

Phospholipase C (PLC) hydrolyzes PtdIns(4,5)P2, thereby cleaving the bond between the glycerol and phosphate moieties to generate the second messengers DAG and IP_3 (Figures 3A) (Eyster 2007). PLCs are classified into six subfamilies based on sequence homology. They are: PLC-β, PLC-γ, PLC-δ, PLC-ε, PLC-ζ and PLC-η. Many of these PLC isoforms have multiple variants, leading to a very large and diverse family of enzymes (Rhee and Choi 1992). Although all PLC isoforms can hydrolyze PtdIns(4,5)P2 to generate DAG and Ins(1,4,5)P3, each PLC isoform has distinct cellular expressions, subcellular localizations, and specific modes of activation (Rhee and Bae 1997). This diversity further contributes to the temporo-spatial metabolism of PtdIns(4,5)P2 signaling.

Ins(1,4,5)P3 is a soluble second messenger that can diffuse through the cytosol and bind to Ins(1,4,5)P3 receptors (Berridge and Irvine 1989), which in turn function as Ca^{2+} channels in the platelet dense tubular systems (DTS). This triggers Ca^{2+} release from the DTS to the cytosol, which in turn modifies the cytosolic concentrations of $Ca^{2+,}$ and regulates diverse downstream signaling effects that include the release of platelet

secretory granules (Brass and Joseph 1985). In contrast to Ins(1,4,5)P3 generated by PLC-mediated hydrolysis of PtdIns(4,5)P2, the second messenger DAG simultaneously generated by this process functions as a signaling molecule that is essential for the localization and activation of different isoforms of protein kinase C (PKC) (Lapetina, Reep et al. 1985). In turn, PKC has regulatory functions in many cellular processes, which include proliferation, apoptosis, survival, and migration (Lapetina, Reep et al. 1985, Griner and Kazanietz 2007). In platelets, DAG-mediated activation of PKC also leads to the phosphorylation of a 47kDa protein called pleckstrin, which is a protein that is important in platelet secretion (Lian, Wang et al. 2009).

Human platelets contain PLC-β and PLC-γ. PLC-β has a pleckstrin homology (PH) domain that binds to the $G_{\beta\gamma}$ subunit of the heterotrimeric G protein on the plasma membrane. This process activates the catalytic domains of PLC-β. Conversely, after collagen stimulation, PLC-γ binds to the PtdIns(3,4,5)P3 on the plasma membrane, where it can be directly phosphorylated by receptor tyrosine kinases (RTK). Upon platelet activation, all PLC isoforms induce the rapid hydrolysis of PtdIns(4,5)P2 into DAG and Ins(1,4,5)P3. Patients who have a deficiency of PLC-β, have a mild bleeding disorder (Lee, Rao et al. 1996, Yang, Sun et al. 1996). *Ex vivo*, platelets from these patients, or mice engineered to lack PLC-β isoforms, exhibit abnormal platelet aggregation and secretion (Lian, Wang et al. 2005). This is due to diminished PLC-β–mediated Ins(1,4,5)P3 production, intracellular Ca^{2+} mobilization, and pleckstrin phosphorylation (Lee, Rao et al. 1996, Yang, Sun et al. 1996, Lian, Wang et al. 2005).

The role of PtdIns(3,4,5)P3 signaling in platelet functions

PI3K isoforms

Phosphatidylinositol 3-kinases (PI3Ks) can phosphorylate the D3-OH group of the inositol ring within phosphoinositides to generate PtdIns(3,4,5)P3, PtdIns(3,4)P2, and PtdIns(3)P (Cantley 2002). There exist three classes of PI3K isoforms (I, II and III) (Vanhaesebroeck and Waterfield 1999). The class I PI3K are critical for the phosphorylation of PtdIns(4,5)P2 to generate the second messenger PtdIns(3,4,5)P3 (also known as PIP_3) (Anderson and Jackson 2003). This class of PI3K is subdivided into class IA (PI3K-α, PI3K-β and PI3K-δ) and IB (PI3K-γ). The class IA PI3K is a heterodimer that is composed of a catalytic subunit (p110-α, p110-β, and p110-δ) bound to a regulatory subunit (p85-α, p85-β, p55-γ, p55-α and p50-α) that possesses an SH2 (Src homology 2) motif. Since SH2 domains bind to tyrosine phosphorylated residues on other proteins, the regulatory subunit enables class IA PI3K to bind to tyrosine phosphorylated receptor tyrosine kinases or to adaptor molecules (Gibbins, Briddon et al. 1998). This process unfolds the PI3K complex, and enables it to phosphorylate PtdIns(4,5)P2 to generate PtdIns(3,4,5)P3. In contrast, the single member of the class IB PI3K family (PI3K-γ) is composed of the catalytic subunit p110-γ and the p101 regulatory domain. This complex becomes enabled to synthesize PtdIns(3,4,5)P3 after p101 binds to the $G_{\beta\gamma}$ subunit of heterotrimeric G-proteins. However, recent studies suggest that PI3K-β can also be activated by G protein coupled receptors (GPCRs) (Tang and Downes 1997, Hazeki, Okada et al. 1998, Kubo, Hazeki et al. 2005) (Guillermet-Guibert, Bjorklof et al. 2008). Regardless of the mechanism, activation of all Type I PI3Ks lead to the synthesis of PtdIns(3,4,5)P3, which in turn can bind to and activate many downstream effector

molecules, including the protein kinase, Akt. In addition to class I PI3K isoforms that synthesize PtdIns(3,4,5)P3, there are class II PI3Ks that phosphorylate PtdIns(4)P to synthesize PtdIns(3,4)P2, and there are class III PI3Ks that phosphorylate the D-3 position of PtdIns to form PtdIns(3)P. While the role of class I PI3K in platelet adhesion by activation of αIIbβ3 has been extensively studied (Jackson, Yap et al. 2004, Gratacap, Guillermet-Guibert et al. 2011), the function of class II and class III PI3Ks in platelets is not well established.

The role of class I PI3K isoforms in platelet plug formation

Human and mouse platelets contain all isoforms of class I PI3K including α, β, and γ, and even small amounts of PI3K-δ (Vanhaesebroeck, Welham et al. 1997, Zhang, Vanhaesebroeck et al. 2002, Watanabe, Nakajima et al. 2003, Jackson, Yap et al. 2004). The binding of collagen to the platelet GPVI receptor leads to the phosphorylation of the receptor's FcRγ chain, which allows it to bind to the SH2 domain of PI3K-α, PI3K-β and PI3K-δ (Falet, Barkalow et al. 2000, Watanabe, Nakajima et al. 2003). Conversely, activation of the $P2Y_{12}$ receptor leads to the release of the $G_{\beta\gamma}$ subunit from heterotrimeric G-proteins that binds the p101 subunit of PI3K-γ and activates this lipid kinase complex (Heraud, Racaud-Sultan et al. 1998, Trumel, Payrastre et al. 1999) (Kauffenstein, Bergmeier et al. 2001). Activation of all type I PI3Ks lead to the synthesis of PtdIns(3,4,5)P3, which in turn activate the protein kinase, Akt (Chen, De et al. 2004, Woulfe, Jiang et al. 2004) in platelets. In addition, PtdIns(3,4,5)P3 also facilitates the activation of PLC-γ (Gilio, Munnix et al. 2009). Together, these signaling events have

been shown to contribute to the activation of the small GTPase Rap1b, which is an activator of integrin $\alpha IIb\beta_3$ (Canobbio, Stefanini et al. 2009).

Initial studies using PI3K inhibitors that block all isoforms of PI3K such as wortmannin and LY294002 suggested that PI3K is crucial for platelet adhesion and aggregation under high shear (Yap, Anderson et al. 2002, Kasirer-Friede, Cozzi et al. 2004). However, the exact contribution of individual isoforms of PI3K during platelet activation remained largely unknown. Over the past few years, studies using isoform-specific targeted mouse models and PI3K inhibitors provided strong evidence that the different isoforms of class I PI3K have distinct cellular and physiological functions in diverse cell types including in platelets. For instance, it is now established that PI3K-α is crucial for cell growth and survival. This has implications in oncogenesis, and targeting of PI3K-α is being investigated as a strategy for chemotherapeutics (Stephens, Williams et al. 2005). Conversely, PI3K-γ plays an essential role in innate immunity and inflammatory responses (Camps, Ruckle et al. 2005, Ruckle, Schwarz et al. 2006) while PI3K-δ is crucial for adaptive immunity involving B and T cells and mast cells (Okkenhaug and Vanhaesebroeck 2003, Ali, Bilancio et al. 2004). In platelets, PI3K-δ knockout mice and kinase-deficient PI3K-δ knockin mice have only a minor defect in the GPVI-mediated activation of integrin αIIbβ3(Senis, Atkinson et al. 2005). This implies that other PI3K isoforms are the major mediators of collagen-induced platelet adhesion and aggregation. Remarkably, *in vivo* inhibition of PI3K-β impairs the formation of stable αIIbβ3-mediated platelet adhesion and prevents thrombus formation in mice, even though it does not increase the bleeding time (Vanhaesebroeck, Ali et al. 2005). This finding suggests that drugs that inhibit PI3K-β might not cause significant hemorrhage

when given to patients for brief periods of time, such as during cardiac catheterization (Jackson and Schoenwaelder 2006). Similarly, mice lacking PI3K-γ have defective platelet aggregation upon stimulation with the platelet agonist ADP. Moreover, these mice are less prone to form thrombi in response to intravenous injections with ADP. It should be noted that these mice do not have spontaneous bleeding, again suggesting that PI3K-γ might be a viable target for inhibiting thrombosis formation without inducing excessive hemorrhage (Hirsch, Bosco et al. 2001, Lian, Wang et al. 2005). Additional studies support that the functional role of PI3K-β and PI3K-γ in platelets are both required for ADP- or thromboxane A2- induced activation of the Rap1b (Canobbio, Stefanini et al. 2009). However, only PI3K-β (and not PI3K-γ) was necessary for collagen-stimulated signaling pathways that lead to Akt phosphorylation, Rap1b activation, and platelet aggregation (Canobbio, Stefanini et al. 2009). Together, these studies suggest a story that is similar to what is seen in PIP5KI isoforms. Even though all PI3K class I isoforms can produce PtdIns(3,4,5)P3, the individual isoforms have non-redundant roles in platelets.

1.3) PtdIns(3,5)P2 and trafficking to the lysosome

Trafficking to the lysosome

Lysosomes are organelles essential for intracellular degradation and recycling. However, recent studies indicate that lysosomes also play a critical role in cellular metabolism, signaling, repair and secretion (Settembre, Fraldi et al. 2013). To carry out these functions, lysosomes receive substrates from endocytosis, phagocytosis and

autophagy. In addition, newly synthesized soluble and membrane-bound lysosomal proteins are delivered from the TGN (trans-golgi network) to the lysosome (Saftig and Klumperman 2009).

Endocytosis involves the internalization of substrates from the plasma and its delivery to the lysosome passing through a series of endosomal intermediates (Luzio, Parkinson et al. 2009). In this dynamic process, membranes and proteins are constantly outgoing and incoming to undergo a gradual remodeling process called maturation (Huotari and Helenius 2011). The different endosomal intermediates resulting from this maturation process include early endosomes, and multivesicular bodies/late endosomes. In multivesicular bodies, the internalized cargo destined for degradation is sorted into intraluminal vesicles (Luzio, Piper et al. 2009). The more mature forms of multivesicular bodies are called late endosomes. These endosomal intermediates can be distinguished by their content, molecular make-up, morphology, pH and kinetics of endocytic tracers (Saftig and Klumperman 2009). Subsequently, the limiting membranes of late endosomes fuse with lysosomes to form an intermediate structure called hybrid organelles. This process is called endosome-lysosome fusion (Luzio, Pryor et al. 2007). This fusion process requires well-coordinated activities of different protein complexes involved in the biogenesis of late endosomes, endosome-lysosome tethering and endosome-lysosome fusion. These protein complexes include the ESCRT (endosomal sortin complex required for transport), HOPS (homotypic fusion and vacuole protein sorting) and trans-SNARE (soluble N-ethylmaleimide-sensitive factor-attachment protein receptor) complexes (Luzio, Gray et al. 2010). The resulting hybrid organelles display markers of endosomes and lysosomes. Eventually, the lysosome is recycled from the hybrid organelle by a

maturation process that includes condensation of content, removal of non-lysosomal membrane proteins and recycling of SNAREs (Luzio, Pryor et al. 2007).

Lysosomes can receive substrates from the extracellular or intracellular sources. During endocytosis, extracellular substrates are internalized and their luminal contents are delivered to the lysosome for degradation. During autophagy, the intracellular organelles are internalized to form autophagosomes, which fuse with lysosomes to form autolysosomes for the degradation of their luminal contents. To carry out these degradative functions, it is essential that lysosomes receive the necessary soluble hydrolytic enzymes and membrane-bound lysosomal proteins delivered through the biosynthetic pathway.

The delivery of newly synthesized proteins from the TGN to the lysosome can be direct or indirect (Saftig and Klumperman 2009). The direct pathway is used by many of the hydrolases. They are synthesized in the ER, become labeled with mannose-6-phosphate (M6P) in the cis-Golgi complex and subsequently bind to mannose-6-phosphate-receptor (M6PR) in the TGN (Kornfeld 1987). M6PR recruits adaptor proteins such as AP1 (adaptor protein-1) or GGA proteins that bind to clathrin to form clathrin-coated vesicles. These vesicles enter the endosomes where the M6PR-bound hydrolase is released as a result of low pH and M6PR is recycled to TGN. The released hydrolases are subsequently transported to the lysosome. The direct pathway is also used by some lysosomal membrane proteins (LMP) but they do not require binding to M6PR.

The indirect pathway is less understood and involves trafficking from the TGN to the plasma membrane by a default secretory pathway, followed by an endocytic pathway

to culminate in the lysosome. Many LMP use this pathway. LMP are synthesized in the ER, trafficked to the TGN where they are secreted to the plasma membrane by a default secretory pathway. On the plasma membrane, these proteins bind to AP2 (adaptor protein-2) and are endocytosed to be sorted to the lysosome (Janvier and Bonifacino 2005). Unlike other hydrolases, β-glucocerebrosidase (βGC) follows the indirect pathway by binding to LIMP2. βGC is not labeled by M6P in cis-Golgi and it binds to LIMP2 in the TGN to continue on the indirect pathway via the plasma membrane.

PIKfyve complex and PtdIns(3,5)P2 metabolism

PtdIns(3,5)P2 is a phosphoinositide of low abundance that represents only about 0.0001% of total lipids in human erythrocytes (Lemmon 2008). This is about 500 fold lower in comparison to PtdIns(4,5)P2 level. The presence of PtdIns(3,5)P2 was first detected in 1997 in yeast and mammalian cells (Dove, Cooke et al. 1997) (Whiteford, Brearley et al. 1997). PtdIns(3,5)P2 is synthesized from PtdIns(3)P on the endosomal compartments in mammalian cells by the PtdIns(3)P 5-kinase PIKfyve (also known as FAB1 in yeast) (Shisheva 2008). Currently, PIKfyve is thought to be the exclusive enzyme for synthesis of PtdIns(3,5)P2. This notion is supported by genetic studies in yeast showing a complete ablation of PtdIns(3,5)P2 synthesis in Fab1-null mutants (Cooke, Dove et al. 1998).

Although PIKfyve is the exclusive kinase that synthesizes PtdIns(3,5)P2, the kinase activity of PIKfyve is tightly regulated by other proteins that form a complex with PIKfyve. These proteins include the PtdIns(3,5)P2 5-phosphatase Fig4 (also known as

SAC3) (Gary, Sato et al. 2002, Sbrissa, Ikonomov et al. 2007) and the docking protein VAC14 (also known as ArPIKfyve) (Jin, Chow et al. 2008, Sbrissa, Ikonomov et al. 2008). Although Fig4 can dephosphorylate PtdIns(3,5)P2 to PtdIns(3)P *in vitro*, the ablation of Fig4 *in vivo* impairs the PIKfyve-mediated synthesis of PtdIns(3,5)P2, which demonstrates the essential role of Fig4 in the stabilization of PIKfyve complex for its proper kinase activity (Chow, Zhang et al. 2007, Ikonomov, Sbrissa et al. 2009). Similarly, VAC14 is essential for the kinase activity of PIKfyve complex and mice lacking VAC14 have defective synthesis of PtdIns(3,5)P2 (Zhang, Zolov et al. 2007, Jin, Chow et al. 2008).

PtdIns(3,5)P2 effector proteins

PtdIns(3,5)P2 may execute its cellular functions by interacting with proteins containing PtdIns(3,5)P2-specific binding domain. ATG18 in yeast (also known as SVP1) is a multi-WD40 protein that fold as β-propeller (PROPPIN) that binds PtdIns(3,5)P2 with high affinity (Kd ~500nM) and specificity (Dove, Piper et al. 2004). In mammals, four homologues of ATG18 have been described. These include WIPI-1, WIPI-2, WIPI-3 and WIPI-4 (Behrends, Sowa et al. 2010). WIPI-1 binds to PtdIns(3)P and PtdIns(3,5)P2 (Jeffries, Dove et al. 2004, Proikas-Cezanne, Waddell et al. 2004). Depletion of WIPI-1 and WIPI-2 lead to increased autophagosome number, suggesting their essential role in autophagy suppression (Proikas-Cezanne and Robenek 2011). It is currently unknown whether WIPI-3 and WIPI-4 bind to phosphoinositides and carry out any functions in membrane trafficking.

Although several other proteins have been proposed to be effector proteins of PtdIns(3,5)P2, they bind PtdIns(3,5)P2 with significantly lower affinities compared to Atg18. These candidate proteins include Tup1 (WD40 domain) (Han and Emr 2011), Raptor (WD40 domain) (Bridges, Ma et al. 2012), SNX1 and SNX 2 (PX domain) (Carlton, Bujny et al. 2004, Carlton, Bujny et al. 2005), Cti 6 (PHD domain), clavesin (Sec14 domain) (Katoh, Ritter et al. 2009), and class II formins (PTEN domain) (van Gisbergen, Li et al. 2012). In addition, PtdIns(3,5)P2 also binds and activates the ion channels TRPML1 and RyR1 (Dong, Shen et al. 2010, Wang, Zhang et al. 2012), but the lipid binding domain has not been identified in these proteins.

PtdIns(3,5)P2 functions

The cellular functions of Fab1 have been initially studied in yeast mutants lacking Fab1 or its kinase activity. Fab1 mutant cells exhibit enlarged vacuoles and they are defective in the retrograde trafficking from the vacuole to the late endosome, sorting into the multivesicular bodies, and acidification of the vacuole (Gary, Wurmser et al. 1998). Similarly, in mammalian cells , PIKfyve ablation by pharmacologic inhibition and siRNA knockdown decreases the intracellular synthesis of PtdIns(3,5)P2 and cells exhibit cytoplasmic vacuolation that express markers of early and late and/or lysosomes (Ikonomov, Sbrissa et al. 2001, Rutherford, Traer et al. 2006, Jefferies, Cooke et al. 2008, de Lartigue, Polson et al. 2009). Some studies using siRNAs-mediated knockdown of PIKfyve demonstrated that PIKfyve is essential in the recycling of M6PR from endosomes to the TGN (Rutherford, Traer et al. 2006, Zhang, Zolov et al. 2007, de

Lartigue, Polson et al. 2009). Similarly, fibroblasts from Vac14 knockout mice are deficient in the synthesis of PtdIns(3,5)P2 and they have impaired endosome-to-TGN trafficking (Zhang, Zolov et al. 2007). However, other studies proposed that PIKfyve is critical for the anterograde fusion of late endosomes with lysosomes. (Ikonomov, Sbrissa et al. 2003, Rusten, Rodahl et al. 2006, Jefferies, Cooke et al. 2008). Consistent with these findings, a study using PIKfyve inhibitor and siRNA knockdown showed that salmonella containing vacuoles failed to fuse with the late endosome/lysosome for its maturation (Kerr, Wang et al. 2010). This view is further supported by several studies showing that PtdIns(3,5)P2-deficient cells accumulate autophagosomes because they fail to mature into autophagolysosomes (Martin, Harper et al. 2013). These studies indicated that 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 (Dove, Dong et al. 2009, McCartney, Zhang et al. 2014) .

Recently, the physiological functions of PtdIns(3,5)P2 have been elucidated using genetically engineered mice that lack different components of the PIKfyve complex (Takasuga and Sasaki 2013). *PIKfyve-null* mice are embryonically lethal (Ikonomov, Sbrissa et al. 2011), but mice expressing residual PIKfyve activity are viable and develop defects within multiple organs, such as in the nervous, cardiopulmonary, and hematopoietic systems (Zolov, Bridges et al. 2012). Similarly, *Fig4-null* mice or *Vac14-null* mice develop several defects, including neurodegeneration, hypopigmentation, and early lethality (Chow, Zhang et al. 2007, Zhang, Zolov et al. 2007). Notably, homozygous *Fig4* mutations were also identified in patients who have the neurodegenerative diseases Charcot-Marie Tooth Syndrome 4J and Amyotrophic Lateral

Sclerosis (Chow, Zhang et al. 2007, Chow, Landers et al. 2009), demonstrating a role for PtdIns(3,5)P2 in neural development. More recently, a study using muscle-specific PIKfyve knockout mouse showed that PIKfyve is essential for glucose homeostasis (Ikonomov, Sbrissa et al. 2013). Furthermore, the loss of PIKfyve only in the intestinal epithelial cells results in an inflammatory bowel phenotype demonstrating that PIKfyve is essential for structural and functional integrity of intestinal epithelial cells (Takasuga, Horie et al. 2013). Together, these studies indicate that PIKfyve has an essential and distinct role in different types of tissues.

Purpose of the thesis research

In this thesis, my overall goal is to understand the biological role of PIKfyve in platelets and in megakaryocytes using mice lacking PIKfyve only in these cell types. Platelets are anucleated, but they store numerous biologically active substances in their secretory organelles, which include alpha granules, dense granules, and lysosomes. Platelet granules are generated in megakaryocytes from the endosomal-lysosomal system (Heijnen, Debili et al. 1998, Youssefian and Cramer 2000, Ambrosio, Boyle et al. 2012, Meng, Wang et al. 2012) by as yet, poorly understood mechanisms. Given the regulatory role of PIKfyve synthesis of PtdIns(3,5)P2 in endosomal-lysosomal homeostasis and trafficking 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.

CHAPTER 2: MATERIALS AND 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 C57BL6 mice (inGenious Targeting Laboratory). A bacterial artificial chromosome (BAC) derived from C57BL/6 (RPCI23) 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 approximately 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 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 color 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}). *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 5'- CAGCCAGTGCTCGCATACAAAGG -3' primers. The insertion of neomycin cassette in F1 mice was identified with 5'-CCAGAGGCCACTTGTGTAGC -3' and 5'- GGATGAAGTGCCTTGTTATCTGCAG -3' primers. The 3rd LoxP integration was identified with 5'-CCATTGCCTGGCTTAGAACAGAG -3' and 5'-GAACTCTCCCGCGTAGTACAGC-3' primers. PCR generated products were either 310bp corresponding to the wild-type alleles or 370bp corresponding to the targeted allele (LoxP integrated). *Pf4-Cre* transgene was identified with 5'-CCCATACAGCACACCTTTTG-3' and 5'-TGCACAGTCAGCAGGTT-3' primers. *Rosa26/LacZ* transgene was identified with 5'-

AAAGTCGCTCTGAGTTGTTAT-3', 5'-GCGAAGAGTTTGTCCTCAACC-3' and 5'-GGAGCGGGAGAAATGGATATG-3'.

Body composition analysis

Mice were anesthesized with sodium pentobarbital (90 mg per kg) and scanned by dual energy X-ray absorptiometry (DEXA) 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 hematoxylin/eosin, PAS, and Alcian Blue. For immunohistochemistry, paraffin sections were deparaffinized with xylene and ethanol, and antigen retrieved in 0.01M citrate buffer by microwaving for 30 minutes. 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 hematoxylin. For X-gal staining, frozen tissue sections

were fixed with 0.5% glutaraldehyde for 10 minutes, and stained overnight with the chromogenic substrate for β-galactosidase, X-gal (Cell Center, University of Pennsylvania). Sections were then counterstained with hematoxylin or hematoxylin/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; 1:1000), EEA-1 (Santa Cruz; 1:1000), Cathepsin D (Calbiochem; 1:1000), vWF (DAKO; 1:1000), PF4 (Bethyl; 1:1000), Akt (Cell Signaling Technology; 1:1000), pAkt (Cell Signaling Technology; 1:1000), and b-actin (Cell Signaling Technology; 1:1000). This was followed by blotting with horseradish peroxidase-conjugated secondary antibodies and developing with enhanced chemiluminescence substrate (GE Healthcare).

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 IDMD supplemented with 10% FBS (Josefsson, James et al. 2011). 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 minutes and incubated with 1 μM prostaglandin E1 for 10 minutes at room temperature. Platelets were then isolated by centrifugation at 800 g for 10 minutes and resuspended in HEPES-Tyrode 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 analyze 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 analyzed on a FACS Calibur flow cytometer (BD Biosciences).

Platelet aggregation and ATP secretion

Platelets were diluted to a density of 2.5 x 10^6 cells ml^{-1} in HEPES-Tyrode 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 ^oC. Platelet secretion of ATP was assessed concurrently by the luciferin/luciferase reaction after adding chrono-lume no. 395 (Chronolog) 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 GPIbα antibody (Emfret) at 2 μg per g 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 CD 45.2 congenic markers were irradiated with 900 rads and administered by retro-orbital injection with $2x10^6$ bone marrow cells from wild-type mice expressing CD 45.1 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 $1x10^6$ bone marrow cells from wild-type mice expressing thy 1.1 marker and $1x10^6$ bone marrow cells from *PIKfyve*$^{fl/fl}$ *Pf4-Cre* mice expressing CD 45.2 markers. Transplanted mice were maintained in sterile water with sulfamethoxazole/trimethoprim for 3 weeks. Mice were sacrificed, and analyzed 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 substrate (Holmsen and Dangelmaier 1989). 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 hours at 37 ^{o}C, and plasma was incubated

for 1 hour at 37 ^{o}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 substrate(Yasuda, Kageyama et al. 1999). The reaction mixture contained 10 µl of platelet lysate solution, 10 µl of 200 µM of substrate Bz-Arg-Gly-Phe-Phe- Pro-4MeOβNA (Calbiochem), and 80 µl of 50 mM sodium acetate buffer pH 4.0. Reaction mixtures were incubated for 30 minutes at 37 ^{o}C. The enzymatic reaction was stopped by adding 5% TCA. The release of 2-naphthylamine was measured in a spectrofluorometer at emission wavelength 410 nm and excitation wavelength 335 nm.

$FeCl_3$-induced carotid artery thrombosis assay[2]

Mice were anesthetized 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 mm^2 piece of filter paper saturated with 7.5% $FeCl_3$ (Sigma) on the surface of the artery for 2 minutes. 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 minutes post-injury. The time to vessel occlusion was recorded.

[2] This assay was performed in collaboration with Dr. Timothy Stalker, PhD at the University of Pennsylvania.

Plasma vWF analysis

For analysis of plasma vWF multimer distribution, mouse plasma (10 μl) was denatured at 60 ^{o}C for 20 minutes with 10 μl of sample buffer (70mM 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; 1:5000) to detect the vWF multimers. For quantification analysis of plasma vWF antigen, we performed ELISA. A microtiter plate coated with rabbit anti-vWF IgG (Dako; 1:2000) was incubated with diluted mouse plasma for 1 hour at room temperature. After washing with PBS, bound vWF was determined by peroxidase-conjugated rabbit anti-vWF IgG (Dako; 1:2000). A pre-mixed TMB (3,3',5,5'-tetramethylbenzidine) solution was used for color development. The absorbance at 450 nm was determined by an ELISA plate reader.

Mass spectrometry analysis[3]

For the mass spectrometry analysis, platelets were isolated from three control mice and three PIKfyve$^{fl/fl}$ Pf4-Cre mice. To prepare platelet releasates, washed platelets (5×10^{8} cells ml^{-1}) were stimulated with 1 U ml^{-1} thrombin for 10 minutes at 37 ^{o}C. Activated platelets were centrifuged at 13,000g for 10 minutes at 4 ^{o}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 μg) were digested with

[3] The proteomics analysis and its statistical analysis were performed in collaboration with Dr. Steven Seeholzer, PhD and Mr. Chris McKennan at the Proteomics Core in The Children's Hospital of Philadelphia.

trypsin in solution. Tryptic digests were analyzed 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 μm id × 15 cm Reprosil-pur 3 μM (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 minute 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 1800 (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 5000, 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 one. 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.

For quantification and statistical analysis of mass spectrometry data, we adapted a method proposed by Choi(Choi, Fermin et al. 2008) to use a label free model using 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 modeled as a Poisson distribution with mean $\lambda_{p,i}$, since each element $x_{p,i}$ in the PSM 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 $\lambda_{p,i}$ using a control and knockout hierarchical log-linear model, denoted by:

$$M^{\mathrm{C}}: log\left(\lambda_{p,i}^{\mathrm{C}}\right) = log\left(L_p\right) + log(N_i) + a_0 + c_p \quad (1)$$

$$M^{\mathrm{K}}: log\left(\lambda_{p,i}^{\mathrm{K}}\right) = log\left(L_p\right) + log(N_i) + a_0 + c_p + T_i k_p \quad (2)$$

where L_p is the length of protein p, N_i is the mean number of spectral counts in sample i, $T_i = \begin{cases} 0\ if\ \text{sample } i \text{ is a control mouse} \\ 1\ if\ \text{sample } i \text{ is a knockout mouse} \end{cases}$, 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 ${\delta_c}^2$ and ${\delta_k}^2$, we place hyper priors on these two parameters, given by ${\delta_c}^2, {\delta_k}^2 \sim \text{InvGamma}(0.1, 0.1)$. We let a_0 "float", and thus set ${\delta_a}^2 = 10^2$. We use these parameters (denoted as a vector by $\vec{\boldsymbol{\theta}}$) to estimate a protein's Bayes Factor:

$$B_p = \frac{\mathrm{P}(M^{\mathrm{K}}|\mathbf{X})}{\mathrm{P}(M^{\mathrm{C}}|\mathbf{X})} = \frac{\int \mathrm{P}(\mathbf{X}|M^{\mathrm{K}},\vec{\boldsymbol{\theta}}^{\mathrm{K}})\mathrm{P}(\vec{\boldsymbol{\theta}}^{\mathrm{K}}|M^{\mathrm{K}})\, d\vec{\boldsymbol{\theta}}^{\mathrm{K}}}{\int \mathrm{P}(\mathbf{X}|M^{\mathrm{C}},\vec{\boldsymbol{\theta}}^{\mathrm{C}})\mathrm{P}(\vec{\boldsymbol{\theta}}^{\mathrm{C}}|M^{\mathrm{C}}) d\vec{\boldsymbol{\theta}}^{\mathrm{C}}} \frac{\mathrm{P}(M^{\mathrm{K}})}{\mathrm{P}(M^{\mathrm{C}})} = \frac{\mathrm{E}_{\vec{\boldsymbol{\theta}}^{\mathrm{K}}|M^{\mathrm{K}}}[\mathrm{P}(\mathbf{X}|M^{\mathrm{K}},\vec{\boldsymbol{\theta}}^{\mathrm{K}})]}{\mathrm{E}_{\vec{\boldsymbol{\theta}}^{\mathrm{C}}|M^{\mathrm{C}}}[\mathrm{P}(\mathbf{X}|M^{\mathrm{C}},\vec{\boldsymbol{\theta}}^{\mathrm{C}})]}$$ (assuming we have no prior knowledge of $\mathrm{P}(M^{\mathrm{K}})$ and $\mathrm{P}(M^{\mathrm{C}})$ for any protein) $= \frac{\lim_{N\to\infty}\frac{1}{N}\sum_{n=1}^{N}\mathrm{P}(\mathbf{X}|M^{\mathrm{K}},\vec{\boldsymbol{\theta}}_n^{\mathrm{K}})}{\lim_{N\to\infty}\frac{1}{N}\sum_{n=1}^{N}\mathrm{P}(\mathbf{X}|M^{\mathrm{C}},\vec{\boldsymbol{\theta}}_n^{\mathrm{C}})}$

(3)

where the transition states $\vec{\boldsymbol{\theta}}_n \to \vec{\boldsymbol{\theta}}_{n+1}$ are determined using Metropolis Hastings and Gibbs Sampling Markov Chain Monte Carlo techniques. Specifically, at iteration n with parameter vector $\vec{\boldsymbol{\theta}}_n^{\mathrm{K}}$ (we explain using the knockout model, since $M^{\mathrm{C}} \subset M^{\mathrm{K}}$),

$$\mathrm{P}(a_{0_n}|\cdot)\ \alpha\ \mathrm{P}(\mathbf{X}|a_{0_n},\cdot)\mathrm{P}(a_{0_n}) = \mathrm{N}(a_{0_n}; 0, {\delta_a}^2)\prod_i\prod_p \mathrm{Poisson}(x_{p,i}|a_{0_n},\cdot)$$

(4)

$$\mathrm{P}\left(c_{p_n}\middle|\cdot\right)\ \alpha\ \mathrm{P}\left(\overrightarrow{\boldsymbol{X}_{\boldsymbol{p},\cdot}}\middle|c_{p_n},\cdot\right)\mathrm{P}\left(c_{p_n}\right) = \mathrm{N}\left(c_{p_n}; 0, {\delta_c}^2\right)\prod_i \mathrm{Poisson}\left(x_{p,i}\middle|c_{p_n},\cdot\right)$$

(5)

$$\mathrm{P}\left(k_{p_n}\middle|\cdot\right)\ \alpha\ \mathrm{P}\left(\overrightarrow{\boldsymbol{X}_{\boldsymbol{p}_{\cdot}}}\middle|k_{p_n},\cdot\right)\mathrm{P}\left(k_{p_n}\right)=\mathrm{N}\left(k_{p_n};0,{\delta_k}^2\right)\prod_i \mathrm{Poisson}\left(x_{p,i}\middle|k_{p_n},\cdot\right)$$

(6)

$$\mathrm{P}\left({\delta_c}^2{}_n\middle|\cdot\right)\alpha\ \mathrm{P}\left(\{c_p\}_{p=1}^{G}\middle|{\delta_c}^2{}_n\right)\mathrm{P}\left({\delta_c}^2{}_n\right)=\mathrm{InvGamma}\left(0.1+\frac{G}{2},0.1+\frac{\sum_{p=1}^{G}{c_p}^2}{2}\right)$$

(7)

$$\mathrm{P}\left({\delta_k}^2{}_n\middle|\cdot\right)\alpha\ \mathrm{P}\left(\{k_p\}_{p=1}^{G}\middle|{\delta_k}^2{}_n\right)\mathrm{P}\left({\delta_k}^2{}_n\right)=\mathrm{InvGamma}\left(0.1+\frac{G}{2},0.1+\frac{\sum_{p=1}^{G}{k_p}^2}{2}\right)$$

(8)

where G = #proteins.

The fold change for protein 'P' is defined as:

$$\text{Fold change P}=\frac{\left(\frac{SC_{k1}}{N_{k1}}+\frac{SC_{k2}}{N_{k2}}+\frac{SC_{k3}}{N_{k3}}\right)}{\left(\frac{SC_{wt1}}{N_{wt1}}+\frac{SC_{wt2}}{N_{wt2}}+\frac{SC_{wt3}}{N_{wt3}}\right)}$$

where SC_ki is the number of spectral counts for protein 'P' in knockout replicate 'i', N_ki is the average number of spectral counts in knockout replicate 'i', SC_wti is the number of spectral counts for protein 'P' in wild type replicate 'i', and N_wti is the average number of spectral counts in wild type replicate 'i'. To minimize the bias in the number of spectral counts across conditions due to protein amount or machine performance, 6 different mass spectrometry runs were carried out and each run was normalized to the mean number of spectral counts in that run. In addition, to reduce the bias in the number of spectral counts caused by the length of a protein, the spectral counts were also normalized to the length

of protein (longer proteins tend to have more tryptic fragments and thus more spectral counts).

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 required 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 analyze data. All data are presented as mean ± 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

CHAPTER 3: RESULTS

Manuscript title: Loss of PIKfyve in platelets causes a lysosomal disease leading to inflammation and thrombosis in mice.

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Introduction

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 trafficking (Di Paolo and De Camilli 2006). 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 diseases (McCrea and De Camilli 2009).

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)(Shisheva 2008). PIKfyve forms a protein complex with other regulatory proteins, such as the PtdIns(3,5)P2 5-phosphatase Fig4 (also known as SAC3) (Gary, Sato et al. 2002, Sbrissa, Ikonomov et al. 2007) and the docking protein Vac14 (also known as ArPIKfyve) (Jin, Chow et al. 2008, Sbrissa, Ikonomov et al. 2008). 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 (Dove, Dong et al. 2009, McCartney, Zhang et al. 2014) .

Recently, physiological functions of PtdIns(3,5)P2 have been elucidated using genetically engineered mice that lack different components of the PIKfyve complex (Takasuga and Sasaki 2013). *PIKfyve-null* mice are embryonically lethal(Ikonomov, Sbrissa et al. 2011), but mice expressing residual PIKfyve activity are viable and develop defects within multiple organs, such as in the nervous, cardiopulmonary, and hematopoietic systems (Zolov, Bridges et al. 2012). Similarly, *Fig4-null* mice or *Vac14-*

null mice develop several defects, including neurodegeneration, hypopigmentation, and early lethality (Chow, Zhang et al. 2007, Zhang, Zolov et al. 2007). Notably, homozygous *Fig4* mutations were also identified in patients who have the neurodegenerative diseases Charcot-Marie Tooth Syndrome 4J and Amyotrophic Lateral Sclerosis (Chow, Zhang et al. 2007, Chow, Landers et al. 2009), 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 hemostatic 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 phosphoinositides (Min and Abrams 2013). 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(King and Reed 2002, Whiteheart 2011). Platelet granules are generated in megakaryocytes from the endosomal-lysosomal system (Heijnen, Debili et al. 1998, Youssefian and Cramer 2000, Ambrosio, Boyle et al. 2012, Meng, Wang et al. 2012) by as yet, poorly understood mechanisms.

In this study, we investigated whether PIKfyve plays 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.

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. 3.1). 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 platelets (Tiedt, Schomber et al. 2007).

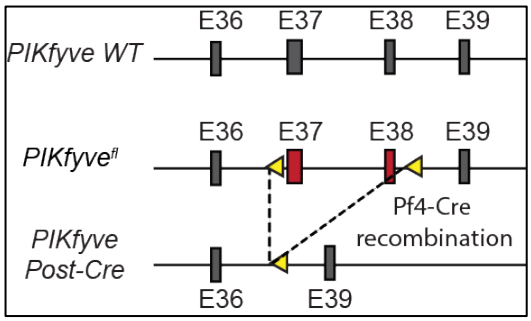

Figure 3.1. Generation of the *PIKfyve*$^{fl/fl}$ *Pf4-Cre* mouse. Schematic representation of genetic targeting of *PIKfyve*. The target exons 37 and 38 (red bars) were targeted with lox P recombination sites (yellow arrows) to generate *PIKfyve* floxed alleles (*PIKfyve*^{fl}). Mice expressing *PIKfyve*^{fl} were then crossed with *Pf4-Cre* mice to induce homologous recombination of the *PIKfyve*^{fl} (*PIKfyve Post-Cre*).

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 (Fig. 3.2a). 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. 3.2b and 3.2c). 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 to their littermate controls (Fig.

3.2d). In addition, they also had decreased bone mineral density (Fig. 3.2e). As *PIKfyve*$^{fl/fl}$ *Pf4-Cre* mice aged, they remained infertile (Fig. 3.2f) and their general body functions deteriorated. The vast majority died before 28 weeks of age (Fig. 3.2g).

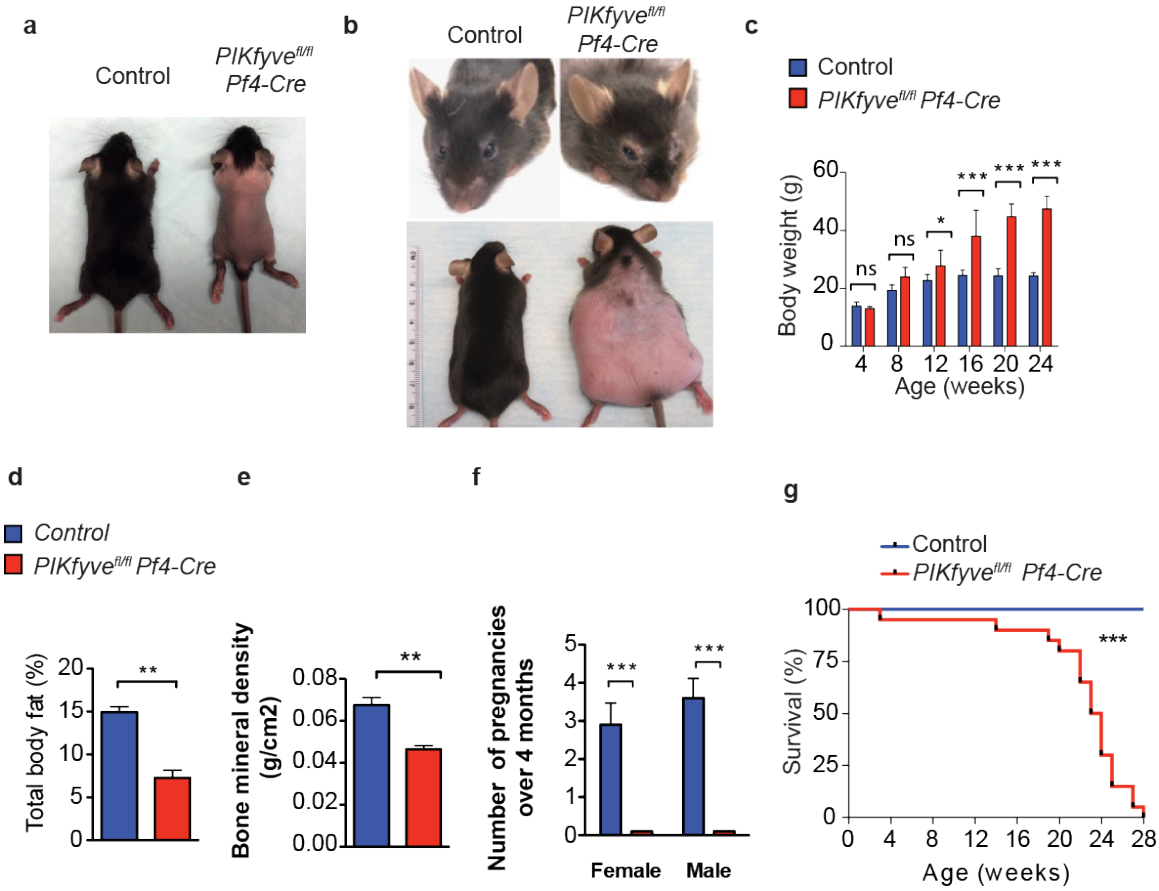

Figure 3.2 Platelet-specific ablation of PIKfyve causes multiorgan abnormalities in mice. (**a)** General appearance of *PIKfyve*$^{fl/fl}$ and *PIKfyve*$^{fl/fl}$ *Pf4-Cre* littermates at 3 weeks of age. Note the characteristic hair loss and small size in the *PIKfyve*$^{fl/fl}$ *Pf4-Cre* mice. (**b**) 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 8 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**) Bone mineral density of the control (n=3) and *PIKfyve*$^{fl/fl}$ *Pf4-Cre* (n=3) mice at 12-24 weeks of age.) (**f**) Number of pregnancies achieved by females (n=10) and by males (n=10) over 4 months of mating period. (**g**) 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 ± S.D. Student's t-test.

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 (Fig. 3.3). This phenotype reflected progressive tissue infiltration by engorged cells with multiple cytoplasmic vacuoles that had a "foamy" appearance (Fig. 3.4, 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 PAS, suggesting an excessive storage of acidic polysaccharides in the vacuolated cells (Fig. 3.5a and 3.5b).

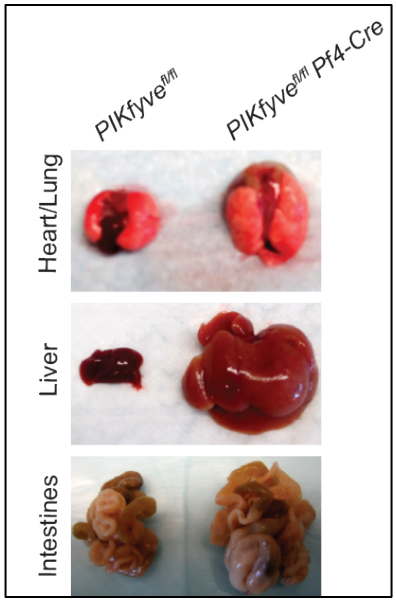

Figure 3.3. *PIKfyve*$^{fl/fl}$ *Pf4-Cre* mice develop multiple organ defects. (a) Representative pictures of the heart, lung, liver, and intestines in the control *PIKfyve*$^{fl/fl}$ mice and in the *PIKfyve*$^{fl/fl}$ *Pf4-Cre* mice at 24 weeks of age.

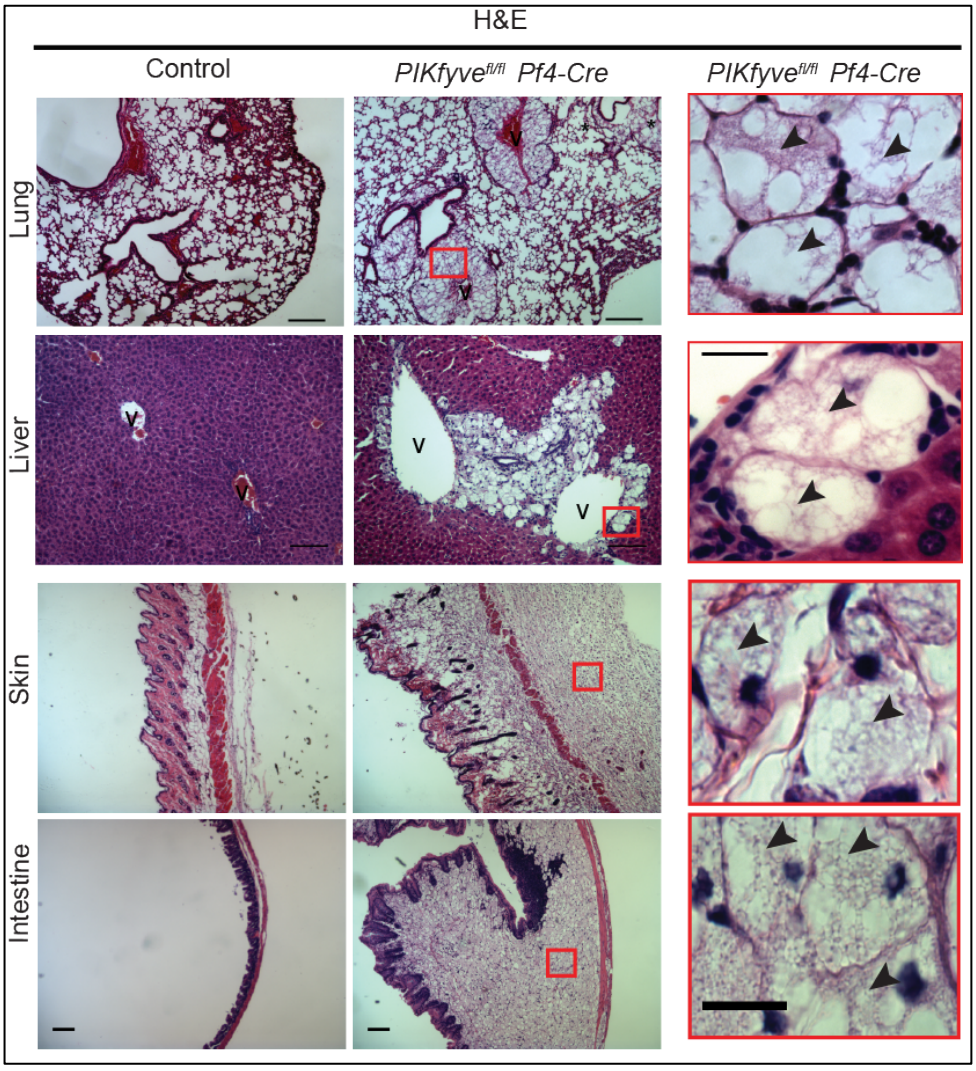

Figure 3.4. Inflammatory vacuolated macrophages infiltrate multiple tissues in the *PIKfyve* $^{fl/fl}$ *Pf4-Cre* mice. Representative histopathology of the lung, liver, skin and intestine from the control *PIKfyve*$^{fl/fl}$ mice and *PIKfyve*$^{fl/fl}$ *Pf4-Cre* mice at 24 weeks of age. Insets show images in the boxed area at higher magnification. Hematoxylin and eosin stain. Scale bar, 100µm, inset, 20µm. All arrowheads indicate the vacuolated cells.

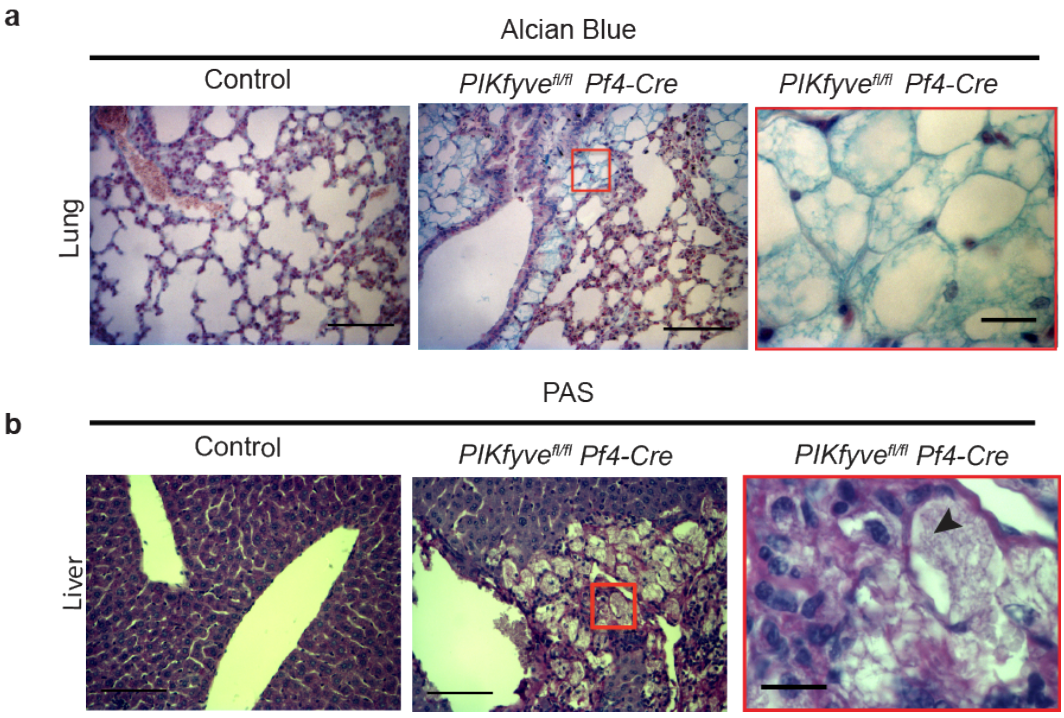

Figure 3.5. The aberrant macrophages with cytoplasmic vacuoles store acidic mucosubstances. (a) Representative lung sections from the control and the *PIKfyve*$^{fl/fl}$ *Pf4-Cre* mice were stained with Alcian Blue. Scale bar, 100μm. Inset shows higher magnification of the boxed area. Scale bar, 20μm. **(b)** Representative liver sections from the control and the *PIKfyve*$^{fl/fl}$ *Pf4-Cre* mice (n=3) were stained with PAS. Scale bar, 100μm. Inset shows higher magnification of boxed area. Scale bar, 20μm.

In their livers and lungs, the vacuolated cells accumulated in a perivascular distribution and displaced the normal tissue, 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. 3.6a), 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 3-4 fold larger than the F4/80-positive macrophages in the livers of the control mice (Fig.3.6b). In addition, tissues infiltrated with the vacuolated macrophages were often

associated with mononuclear cell infiltrates that stained with the lymphocyte marker CD45R (Fig. 3.6c), 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.

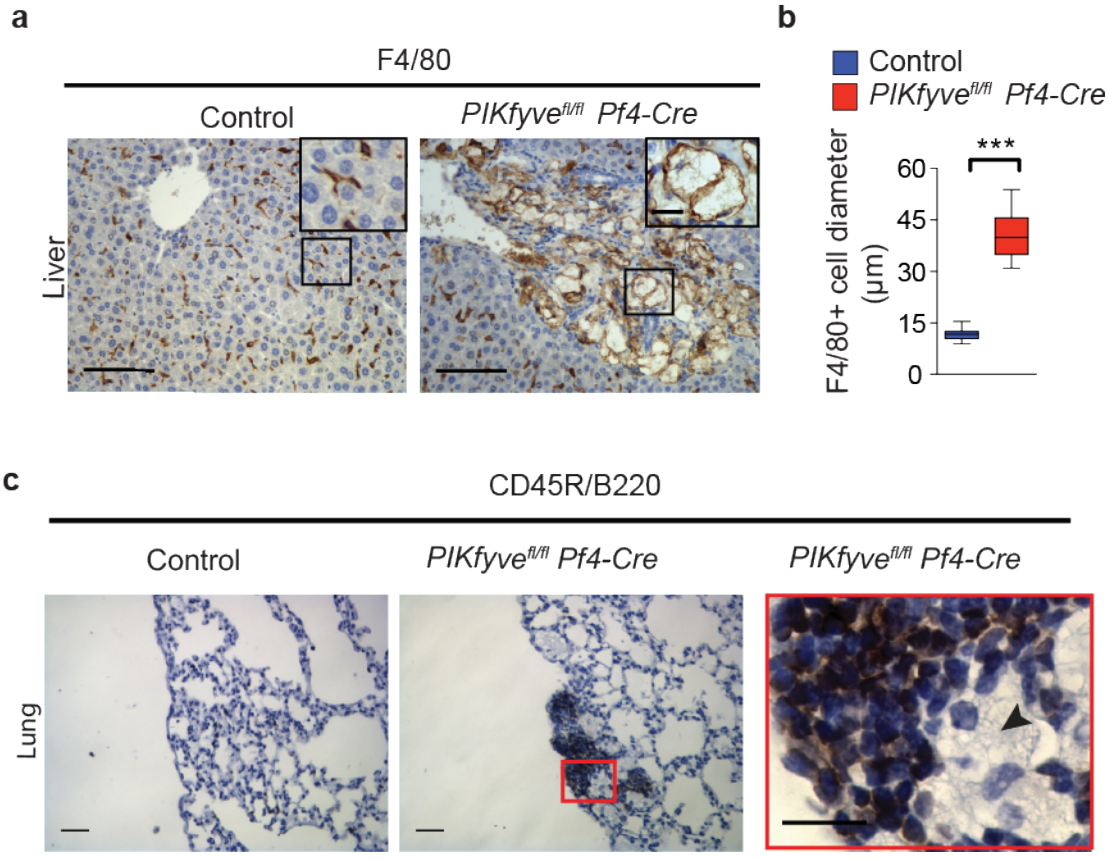

Figure 3.6. The infiltrating vacuolated cells are inflammatory F4/80+ macrophages. **(a)** Liver structures of control mice and *PIKfyve*$^{fl/fl}$ *Pf4-Cre* mice were stained for macrophages with anti-F4/80 antibody by immunohistochemistry. Scale bar, 100μm. Inset shows higher magnification of the boxed area. Scale bar, 20μm. **(b)** Representative lung sections from the control and the *PIKfyve*$^{fl/fl}$ *Pf4-Cre* mice were stained with anti-CD45R/B220 antibody. Scale bar, 100μm. Inset shows higher magnification of the boxed area. Scale bar, 20μm. All arrowheads indicate the vacuolated macrophages.

PIKfyve-null platelets induce macrophage pathology

Cells from PtdIns(3,5)P2-deficient organisms have been shown to form large cytoplasmic vacuoles(Takasuga and Sasaki 2013, McCartney, Zhang et al. 2014). 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 non-autonomous 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. 3.7). These results strongly suggest that the *PIKfyve* protein was ablated only in the megakaryocyte lineage of *PIKfyve*$^{fl/fl}$ *Pf4-Cre* mice.

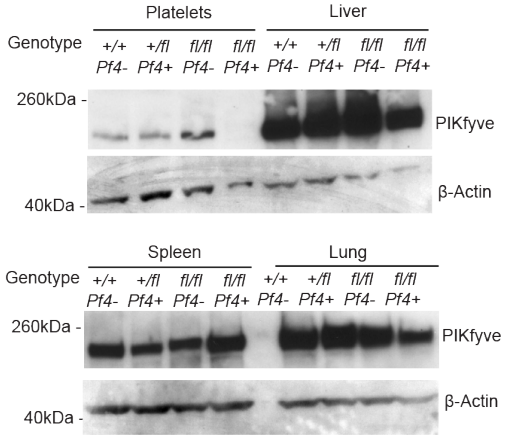

Figure 3.7. PIKfyve expression is absent exclusively in the platelets of *PIKfyve*$^{fl/fl}$ *Pf4-Cre* mice. The levels of PIKfyve protein were determined in the platelets, liver, spleen and lung of the indicated mice by immunoblotting. Note the absence of the PIKfyve protein in the platelets, but its presence in the liver,spleen and lung of *PIKfyve*$^{fl/fl}$ *Pf4-Cre* mice (n=3 per group). 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.

Although Pf4-Cre expression has been shown to be restricted to megakaryocytes and platelets (Tiedt, Schomber et al. 2007, Bertozzi, Schmaier et al. 2010, Carramolino, Fuentes et al. 2010, Josefsson, James et al. 2011), 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*)(Soriano 1999). 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. 3.8a, arrows), but not in the vacuolated macrophages or in other cells (Fig. 3.8b, 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.

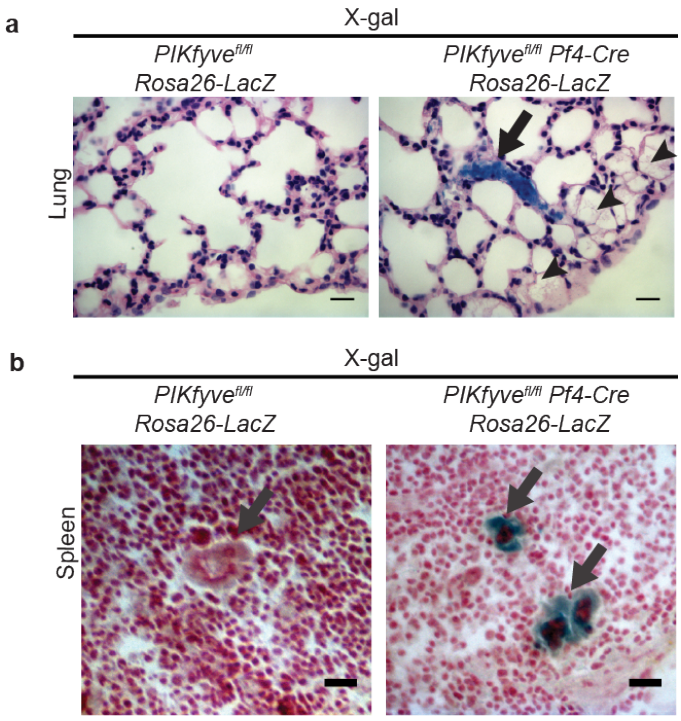

Figure 3.8. Pf4-Cre is specifically expressed in platelets and megakaryocytes. (a) X-gal staining of the lung tissues from *PIKfyve*$^{fl/fl}$ *Rosa26-LacZ* mice and *PIKfyve*$^{fl/fl}$ *Pf4-Cre Rosa26-LacZ* mice. Scale bar, 20 μm. The arrow indicates the platelet clumps in the lung vessels expressing LacZ (blue stain). Arrowheads indicate the vacuolated macrophages that did not stain blue. **(b)** Representative pictures of X-gal staining of the spleen sections from 10 week-old mice (n=5 per group). Arrows indicate megakaryocytes. Note the expression of LacZ (blue stain) only in the megakaryocytes of *PIKfyve*$^{fl/fl}$ *Pf4-Cre* / *Rosa-LacZ* mice. Scale bar, 20μm.

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 GP1bα antibodies(Bergmeier, Rackebrandt et al. 2000, Nieswandt, Bergmeier et al. 2000). 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 GP1bα antibodies twice weekly via intraperitoneal injections for 6 weeks. As reported previously, the injection of GP1bα antibodies efficiently induced acute thrombocytopenia in both sets of mice(Bergmeier, Rackebrandt et al. 2000, Nieswandt, Bergmeier et al. 2000). However, repetitive injections of GP1bα antibodies were less effective at inducing stable thrombocytopenia over time likely because of the reactive thrombocytopoiesis associated with complete platelet depletion with anti-platelet antibodies(Trowbridge and Martin 1984). Nevertheless, the *PIKfyve*$^{fl/fl}$ *Pf4-Cre* mice that were treated with GP1bα antibodies showed a significant delay in weight gain and attenuated hair loss as compared to the untreated *PIKfyve*$^{fl/fl}$ *Pf4-Cre* mice (Fig. 3.9a and 3.9b). 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.

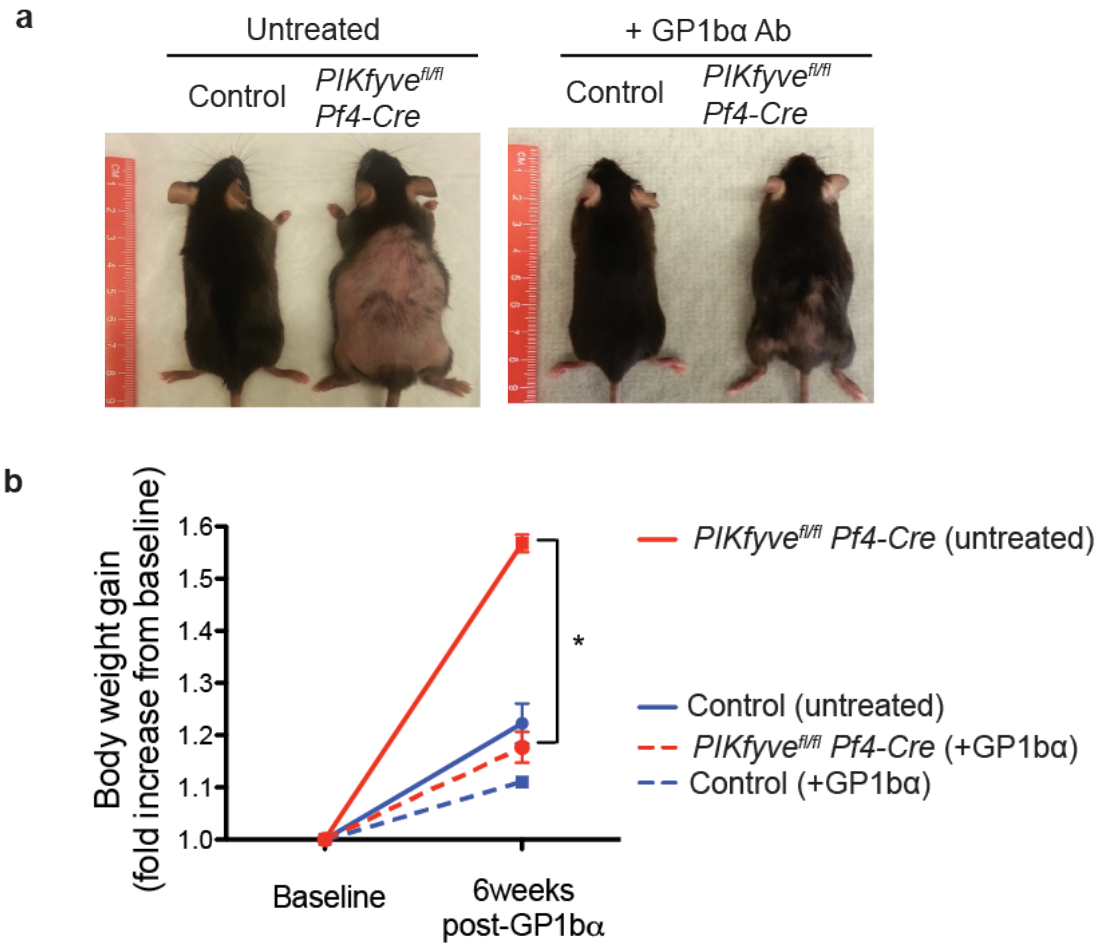

Figure 3.9. Platelets drive the multiorgan defects in the *PIKfyve*$^{fl/fl}$ *Pf4-Cre* mice. (**a**) Representative general morphology of 15-weeks-old control mice and *PIKfyve*$^{fl/fl}$ *Pf4-Cre* mice after 6 weeks of either no intervention (untreated) or biweekly injections of anti-GP1bα antibodies (+GP1bα Ab). (**b**) Body weight change of the control mice and *PIKfyve*$^{fl/fl}$ *Pf4-Cre* mice after 6 weeks of no intervention or treatment with anti-GP1bα antibodies (n=3 for each group). **P*<0.05. All error bars indicate mean ± S.D. Student's t-test.

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 (Figure 3.10b and 3.10e, 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. 3.10c and 3.10f). Together, these results further demonstrate that the phenotype of *PIKfyve*$^{fl/fl}$ *Pf4-Cre* mice can be either rescued or transferred by hematopoietic progenitor cells.

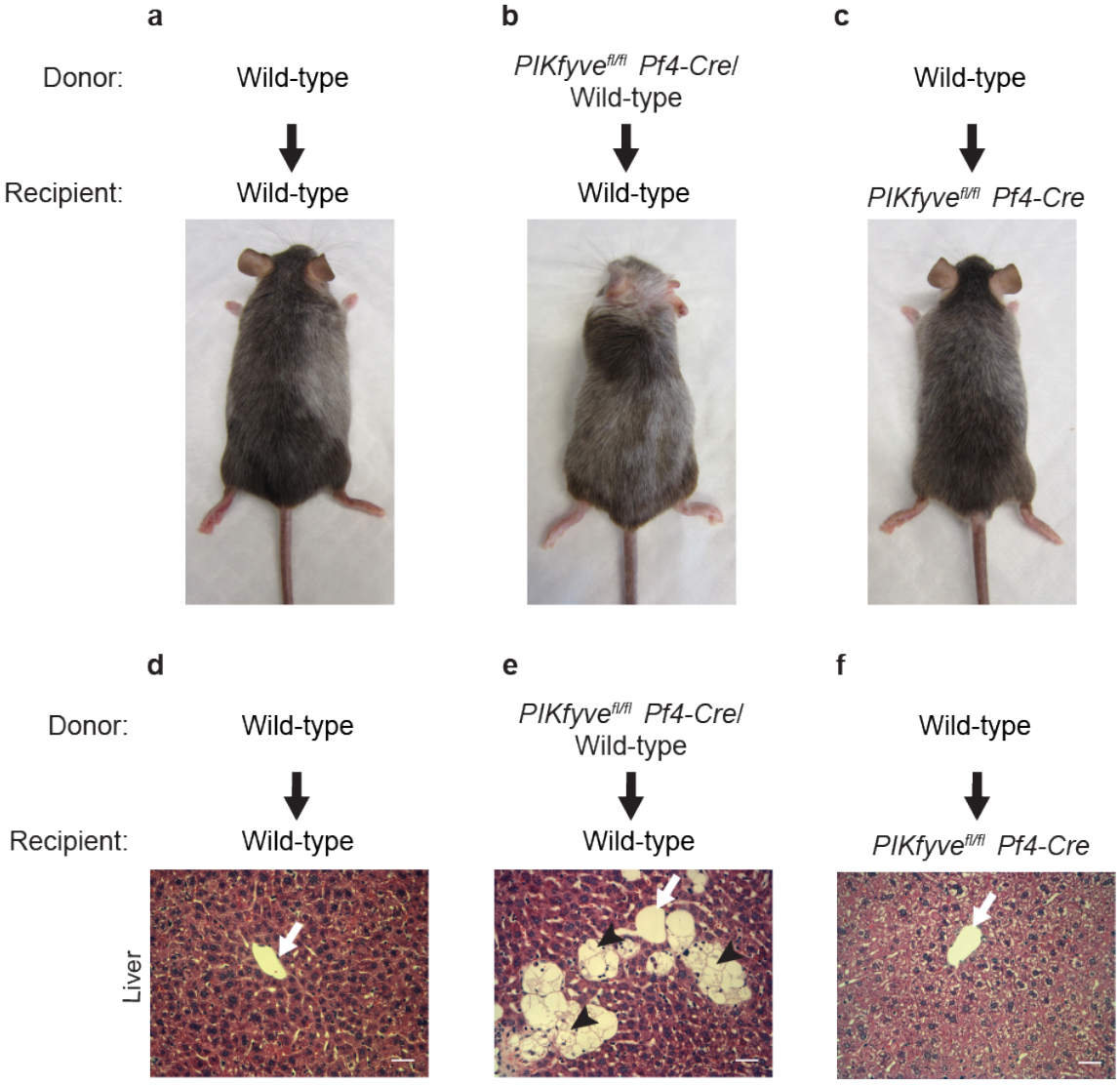

Figure 3.10. Transplanted bone marrow cells can recapitulate or revert the tissue infiltration of vacuolated macrophages in *PIKfyve*$^{fl/fl}$ *Pf4-Cre* mice. (a-c) General appearance of lethally irradiated recipient mice approximately 35 weeks after the transplantation of hematopoietic stem cells (HSC). **(a)** Wild-type HSC were transplanted into wild-type mice. **(b)** Mixture of HSC half derived from wild-type HSC and half derived from *PIKfyve*$^{fl/fl}$ *Pf4-Cre* HSC were transplanted into wild-type mice (n=5 per group). **(c)** Wild-type HSC were transplanted into *PIKfyve*$^{fl/fl}$ *Pf4-Cre* mice. **(d-f)** Liver sections from the respective recipient mice in panels (a-c). Black arrowheads indicate the vacuolated macrophages. White arrows indicate vessels. Scale bar, 20μm.

Megakaryopoiesis and thrombopoiesis do not require *PIKfyve*

We next studied the effects of PIKfyv*e* ablation in megakaryocytes of *PIKfyve*$^{fl/fl}$ *Pf4-Cre* mice. Fibroblasts and neurons of PtdIns(3,5)P2-deficient organisms typically develop large vacuoles(Takasuga and Sasaki 2013, McCartney, Zhang et al. 2014). Histological analysis by light microscopy of bone marrow sections showed that *PIKfyve-null* megakaryocytes were morphologically indistinguishable from control megakaryocytes (Fig. 3.11a). However, analysis by transmission electron microscopy of cultured megakaryocytes derived from *PIKfyve*$^{fl/fl}$ *Pf4-Cre* bone marrow revealed multiple enlarged cytoplasmic vacuoles (Fig. 3.11b). 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. 3.11c). Moreover, *PIKfyve-null* megakaryocytes had normal ploidy, implying that the loss of PIKfyve did not affect megakaryocyte maturation (Fig. 3.11d). Finally, counts of platelets and leukocytes as well as hemoglobin level were similar between control and *PIKfyve*$^{fl/fl}$ *Pf4-Cre* mice (Fig.3.11e-g). 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.

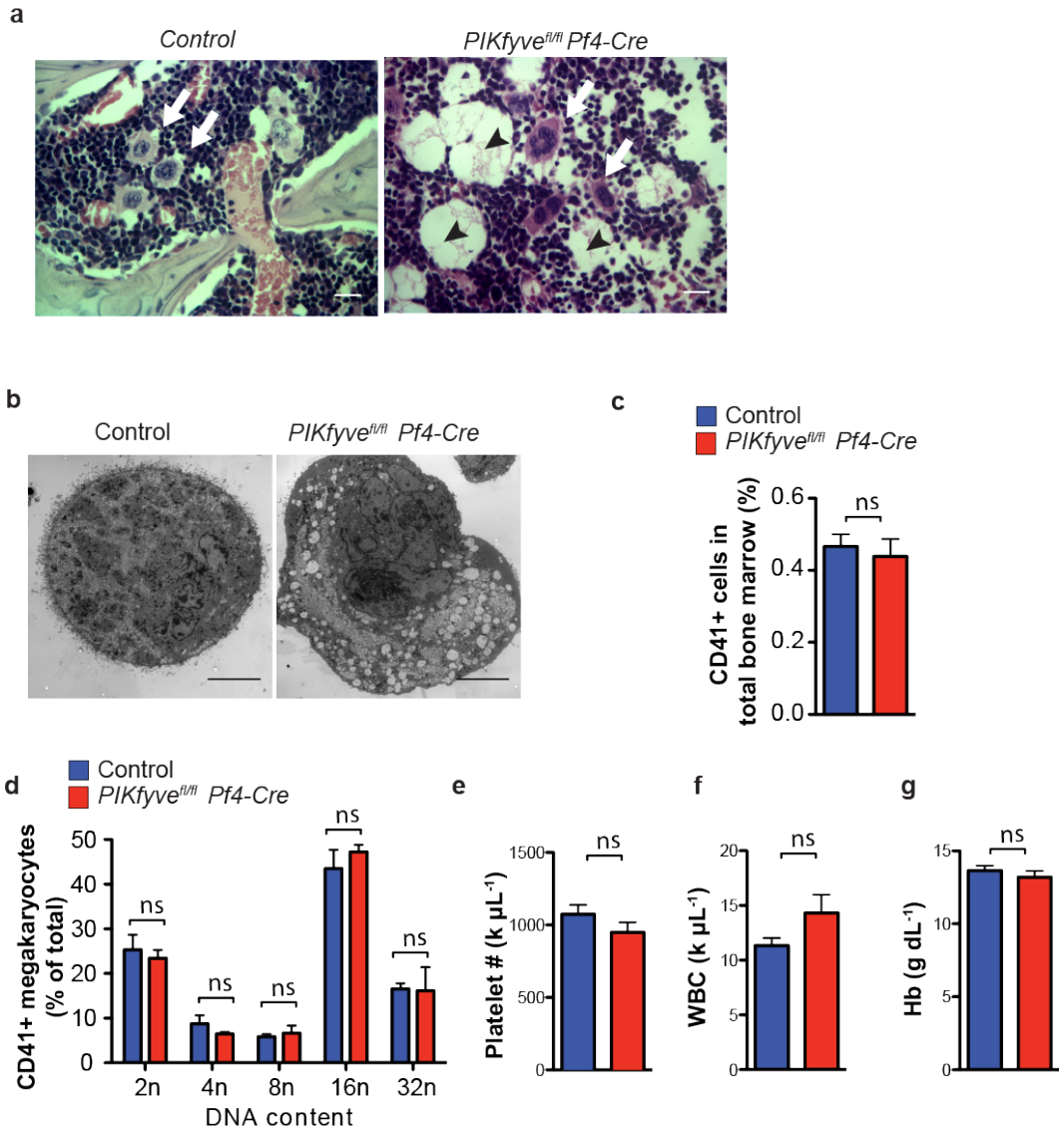

Figure 3.11. PIKfyve is not necessary for the development of megakaryocytes nor for platelet production. (**a**) Representative pictures of bone marrow sections from the control and *PIKfyve*$^{fl/fl}$ *Pf4-Cre* mice at 16 weeks of age. White arrows indicate megakaryocytes. Black arrowheads indicate the vacuolated macrophages. Scale bar 20µm. **(b)** 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 to the control mice. Scale bar, 10 µm. (**c**) CD41+ megakaryocyte counts of control and *PIKfyve*$^{fl/fl}$ *Pf4-Cre* mice (n=3 for each group). (**d**) Megakaryocyte ploidy (n=3 for each group). (**e-g**) Counts of platelets (**e**), WBC (**f**), and Hb (**g**) of control mice and *PIKfyve*$^{fl/fl}$ *Pf4-Cre* mice (n=3 for each group). All error bars indicate mean ± S.D. Student's t-test.

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 (Fig. 3.12a). To test whether the loss of PIKfyve impacted granule content, we assessed the abundance of representative components by immunoblotting. *PIKfyve-null* platelets expressed normal levels of the alpha granule components von Willebrand factor and platelet factor 4 (Fig. 3.12b-c). In contrast, *PIKfyve-null* platelets exhibited increased levels of the lysosomal protein LAMP-1 (Lysosomal-Associated Membrane Protein 1; Fig. 3.13a-b) and increased activity of the lysosomal enzyme β-hexosaminidase (Fig. 3.13c). These results suggest that *PIKfyve-null* platelets store normal levels of alpha granule components, but store excessive levels of lysosomal components.

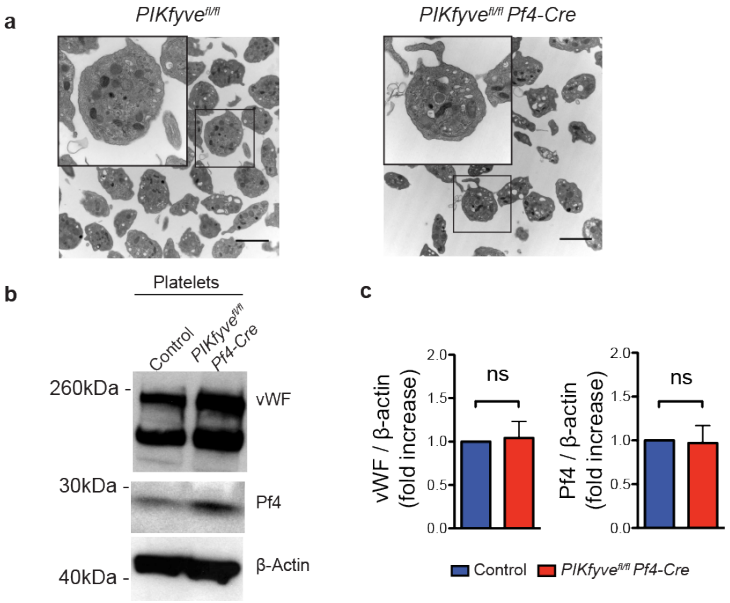

Figure 3.12. PIKfyve is not required for the storage of platelet alpha granules. **(a)** Representative electron micrographs of platelets extracted from *PIKfyve*$^{fl/fl}$ and *PIKfyve*$^{fl/fl}$ *Pf4-Cre* mice at 3 weeks of age. Scale bar 2μm. **(b)** Immunoblot of platelet lysates showing the expression of vWF and Pf4 in the control and *PIKfyve-null* platelets. β-actin was used as a loading control. **(c)** Quantification of panel (c) (n=3). All error bars

indicate mean ± S.D.

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 (Fig. 3.14a). Likewise, ATP secretion from dense granules was similar in control and *PIKfyve-null* platelets (Fig. 3.14b). However, the releasates from *PIKfyve-null* platelets showed increased activity of the lysosomal enzyme, β-hexosaminidase (Fig. 3.13c).

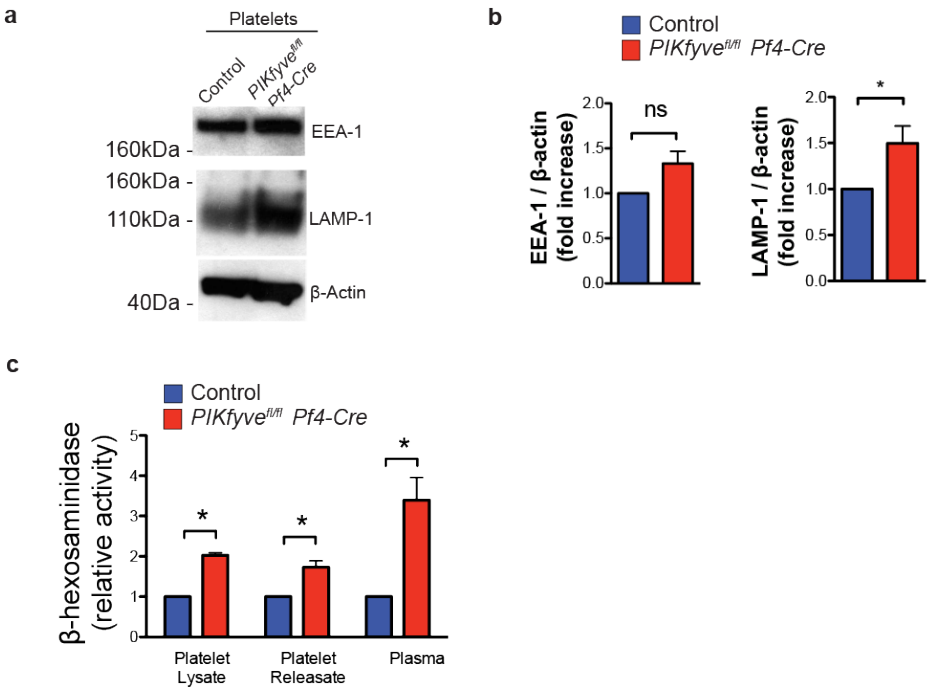

Figure 3.13. PIKfyve is critical for proper storage and release of lysosomal proteins in platelets. (**a**) Immunoblot of platelet lysates from the control mice and *PIKfyve*$^{fl/fl}$ *Pf4-Cre* mice showing the expression of EEA-1 and LAMP-1. β-actin was used as the loading control. (**b**) Quantification of protein bands shown in the panel a (n=3 for each group). (**c**) β-hexosaminidase activity in the platelet lysates of control mice (n=3) and *PIKfyve*$^{fl/fl}$ Pf4-Cre mice (n=3).

Further analysis showed that the *PIKfyve-null* platelets released a normal percentage of their total cellular β-hexosaminidase following agonist stimulation (Fig. 3.14c). 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. 3.13c). Together, these findings demonstrate that *PIKfyve-null* platelets store and release excessive levels of lysosomal cargo proteins.

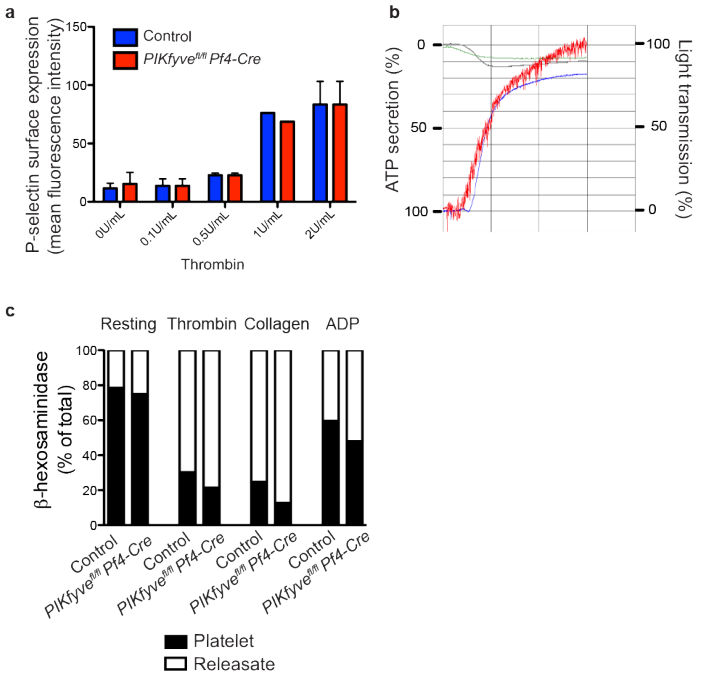

Figure 3.14. PIKfyve is not required for the efficient exocytosis of platelet granules. **(a)** Platelet surface translocation of P-selectin upon stimulation with thrombin at shown concentrations (n=3). **(b)** Representative graphs showing ATP secretion (green and black lines) and aggregation (red and blue lines) of control platelets (black and blue lines) and *PIKfyve-null* platelets (green and red lines). Platelets were stimulated with thrombin (1U ml^{-1}) or collagen (10µg ml^{-1}). **(c)** ß-hexosaminidase activity in the platelet releasates and lysates after stimulation with thrombin (1U ml^{-1}), collagen (10µg ml^{-1}), and ADP. White portions of the bar represent the enzymatic activity in the platelet releasates following platelet stimulation. Black portions of the bars represent the enzymatic activity in the residual platelet lysates after stimulation (n=3 per group).

To more comprehensively analyze 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 (Table 3.1 and 3.2). 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 (Table 3.1). Remarkably, levels of the cathepsin D were 20-fold higher in *PIKfyve-null* platelet releasates than in control releasates (Table 3.1).

Protein names	Gene names	Bayes Factor	Fold Change (KO/Cont)
Cathepsin D	Ctsd	4757.53	20.28
Serum amyloid A-1 protein	Saa1	266.90	15.48
Ig lambda-3 chain C region	Iglc3	263.45	14.34
Collagen alpha-1(I) chain	Col1a1	116.35	12.79
Proteasome subunit beta type-1	Psmb1	37.79	11.06
Proteasome subunit alpha type-6	Psma6	23.52	9.82
Fibulin-1	Fbln1	10000.00	8.87
Phosphatidylcholine-sterol acyltransferase	Lcat	8932.21	6.49
Complement C1q subcomponent subunit A	C1qa	18.07	3.46
Properdin	Cfp	749.23	3.21
Cathepsin B	Ctsb	14.55	3.21
Apolipoprotein B	Apob	10000.00	2.49
Kininogen 2	Kng2	200.88	2.42
Keratin, type I cytoskeletal 14	Krt14	14.80	2.33
Maltase-glucoamilase	Mgam	15.76	2.13
Inter-alpha-trypsin inhibitor heavy chain H3	Itih3	90.05	2.01
Complement C1s-A subcomponent	C1sa	14.43	1.99

Table 1. Upregulated proteins in the platelet releasates of *PIKfyve*$^{fl/fl}$ *Pf4-Cre* mice. The numbers of peptide spectral matches to each protein were used for quantitative comparison between the knockout (KO) and control (Cont) platelet samples. A Bayes Factor of at least 8 and a fold change of at least 2.0. were used for statistical analysis.

Protein names	Gene names	Bayes Factor	Fold Change
Tropomyosin alpha-4 chain	Tpm4	10000.00	-24.53
Myosin light polypeptide 6	Myl6	82.14	-12.77
Major urinary protein 20	Mup20	83.75	-12.57
Fermitin family homolog 3	Fermt3	248.02	-12.55
Zyxin	Zyx	254.52	-11.85
Filamin-A	Flna	10000.00	-11.07
Coagulation factor XIII A chain	F13a1	28.51	-10.91
Apolipoprotein C-III	Apoc3	139.36	-10.85
Beta-parvin	Parvb	28.66	-10.68
Protein disulfide-isomerase A3	Pdia3	80.52	-10.15
Glyceraldehyde-3-phosphate dehydrogenase	Gapdh	1515.81	-9.97
Arachidonate 12-lipoxygenase, 12S-type	Alox12	17.36	-9.42
von Willebrand factor	Vwf	10000.00	-7.77
Latent-transforming growth factor beta-binding	Ltbp1	1574.75	-6.72
Myosin regulatory light polypeptide 9	Myl9	404.59	-6.62
Interleukin-1 receptor accessory protein	Il1rap	1016.67	-6.18
Bridging integrator 2	Bin2	250.57	-6.09
Alpha-actinin-1	Actn1	10000.00	-5.84
14-3-3 protein eta	Ywhah	14.60	-5.34
Multimerin-1	Mmrn1	10000.00	-5.09
Vinculin	Vcl	10000.00	-4.69
L-lactate dehydrogenase A chain	Ldha	224.16	-4.63
Talin-1	Tln1	10000.00	-4.53
Galectin-3-binding protein	Lgals3bp	738.58	-4.36
Tubulin beta-4B chain;Tubulin beta-4A chain	Tubb4b	760.98	-4.14
WD repeat-containing protein 1	Wdr1	799.62	-4.13
Transforming growth factor beta-1	Tgfb1	37.64	-3.38
Tubulin alpha-1B chain	Tuba1b	596.11	-3.33
Heat shock cognate 71 kDa protein	Hspa8	33.49	-3.03
Myosin-9	Myh9	10000.00	-2.94
Tubulin beta-1 chain	Tubb1	133.34	-2.73
Glia-derived nexin	Serpine2	153.71	-2.68
78 kDa glucose-regulated protein	Hspa5	30.58	-2.68
Coagulation factor V	F5	10000.00	-2.61
Metalloproteinase inhibitor 3	Timp3	10.45	-2.59
Afamin	Afm	1394.27	-2.58
Cofilin-1	Cfl1	8.85	-2.54
14-3-3 protein zeta/delta	Ywhaz	10.93	-2.30

Table 2. Downregulated proteins in the platelet releasates of *PIKfyve*$^{fl/fl}$ *Pf4-Cre* mice.

The numbers of peptide spectral matches to each protein were used for quantitative comparison between the knockout (KO) and control (Cont) platelet samples. A Bayes Factor of at least 8 and a fold change of at least 2.0. were used for statistical analysis.

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 (Fig. 3.15a-c). To further validate these findings, we analyzed the expression of cathepsin D in platelet lysates by immunoblotting. Consistent with the proteomics data, both cathepsin D and procathepsin D were undedetectable in the lysates of wild-type platelets, and procathepsin D was highly elevated in *PIKfyve-null* platelets (Fig. 3.15d). 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 (Fig. 3.15e). Together, these data demonstrate that PIKfyve ablation in platelets results in a defective lysosomal pathway reflected by abnormal storage, maturation, and release of lysosomal enzymes.

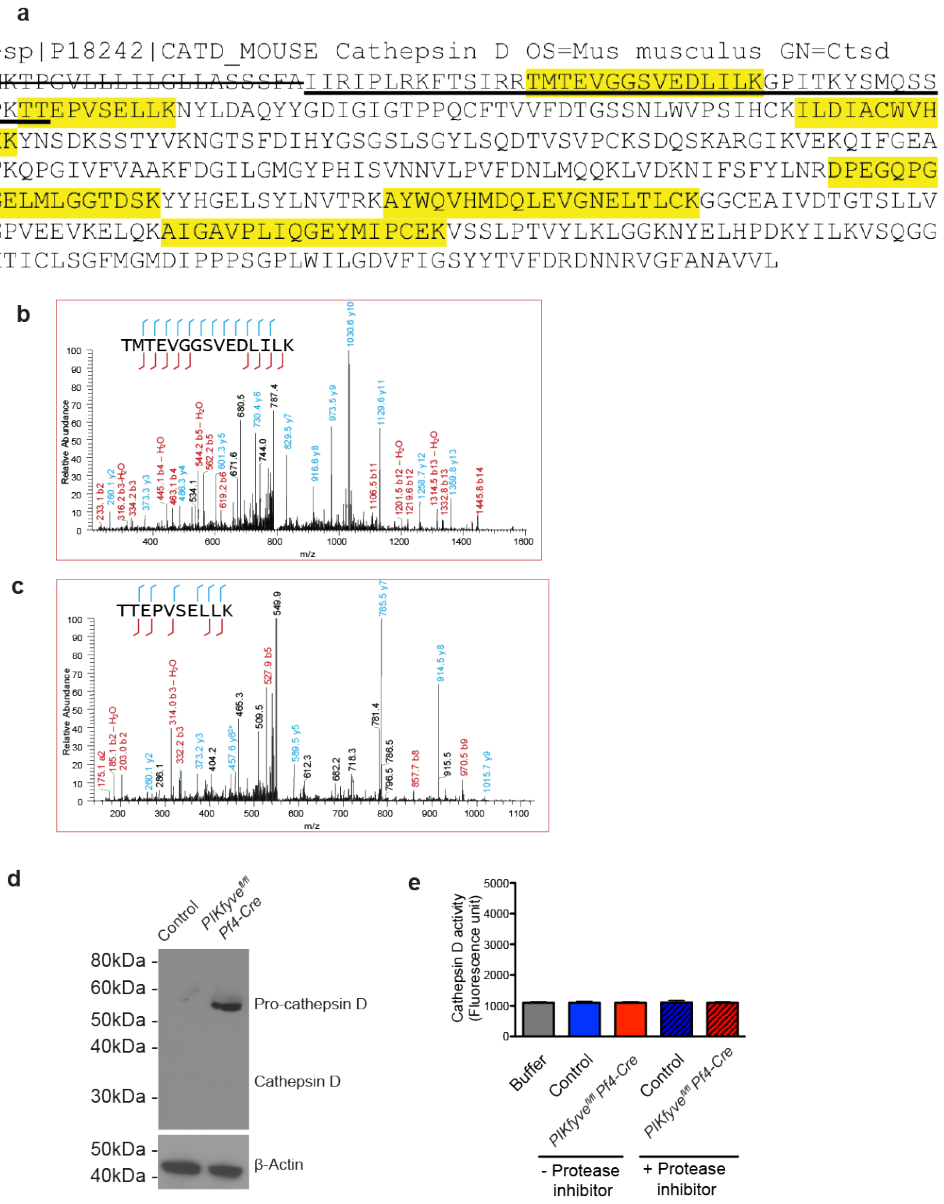

Figure 3.15. PIKfyve-null platelets release increased levels of procathepsin D. (a) Amino acid sequence of murine cathepsin D. The signal peptide sequence is crossed out and the propeptide sequence is underlined. The trypic peptides of cathepsin D are highlighted in yellow. **(b)** The assigned MS2 spectra of the tryptic peptides contained in the Cathepsin D propeptide are shown. **(c)** The peptide TTEPVSELLK spans the propeptide cleavage site. Note that the predicted N-terminal tryptic peptide of the active form of cathepsin D (EPVSELLK) was not observed in MS1 nor identified in MS2 with semi-tryptic enzyme specificity for the database search. **(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)** Cathepsin D activity of platelets from control and *PIKfyve-null* platelets in the absence or presence of protease inhibitor (n=3 per group).

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(Bentfeld-Barker and Bainton 1982, Rendu, Marche et al. 1987), 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 (Fig. 3.14b), suggesting that activation of the dominant platelet integrin, aIIbb3, is intact in the *PIKfyve-null* platelets. In addition, Akt phosphorylation was normally induced upon stimulation (Fig. 3.16b), suggesting that the intracellular signaling 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 analyzed 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 minutes vs. 6.1 ± 1.2 minutes, Fig. 3.16a). 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 inflammation(Lip and Blann 1997), were significantly elevated in the plasma of *PIKfyve*$^{fl/fl}$ *Pf4-Cre* mice when measured by immunoblotting (Fig. 3.16c) or ELISA (Fig. 3.16d). 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.

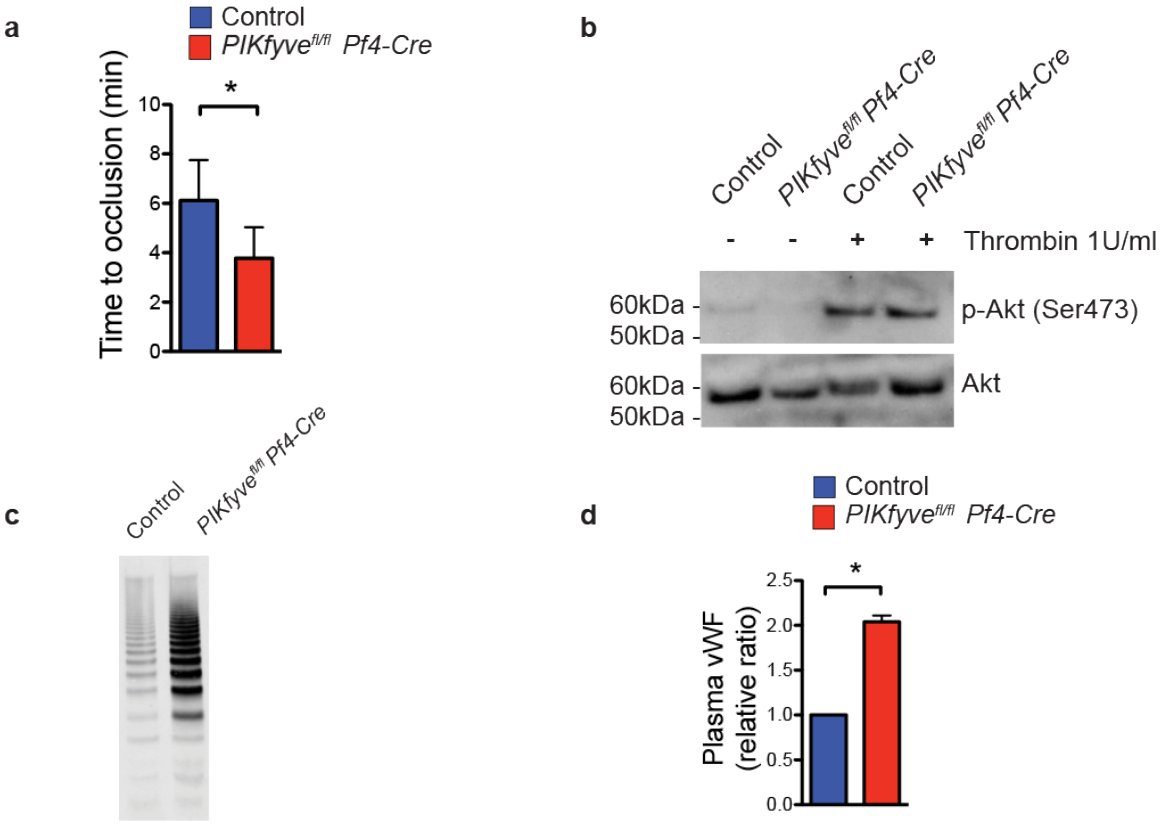

Figure 3.16. Loss of PIKfyve in platelets accelerates arterial thrombosis. (**a**) Time to occlusion of carotid arteries upon vascular injury with 7.5% of $FeCl_3$ in the control mice (n=9) and the *PIKfyve*$^{fl/fl}$ *Pf4-Cre* mice (n=8). (**b**) Immunoblot of platelet lysates from control platelets and *PIKfyve*$^{fl/fl}$ *Pf4-Cre* platelets showing the Akt phosphorylation in resting condition or stimulated with 1U/mL of thrombin for 10 minutes. (**c**) 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. (**d**) 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 ± S.D. Student's t-test.

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 (Cullinane, Curry et al. 2011). *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 (Marks, Heijnen et al. 2013, Wei and Li 2013). Importantly, stimulus-dependent secretion of lysosomal contents is impaired (but not ablated) in platelets from *pallid* mice (Novak, Hui et al. 1984). 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. 3.17a and 3.17b; note that because the *pallid* mutation also impairs melanosome biogenesis, *pallid* mice exhibit hypopigmented fur). As expected, platelet and plasma levels of b-hexosaminidase were lower in *PIKfyve*$^{fl/fl}$ *Pf4-Cre/pallid* mice than in *PIKfyve*$^{fl/fl}$ *Pf4-Cre* mice (Fig. 3.17c and 3.17d). Together, these data suggest that the release of platelet lysosomes contributes to the pathogenesis of multiorgan defects in *PIKfyve*$^{fl/fl}$ *Pf4-Cre* mice.

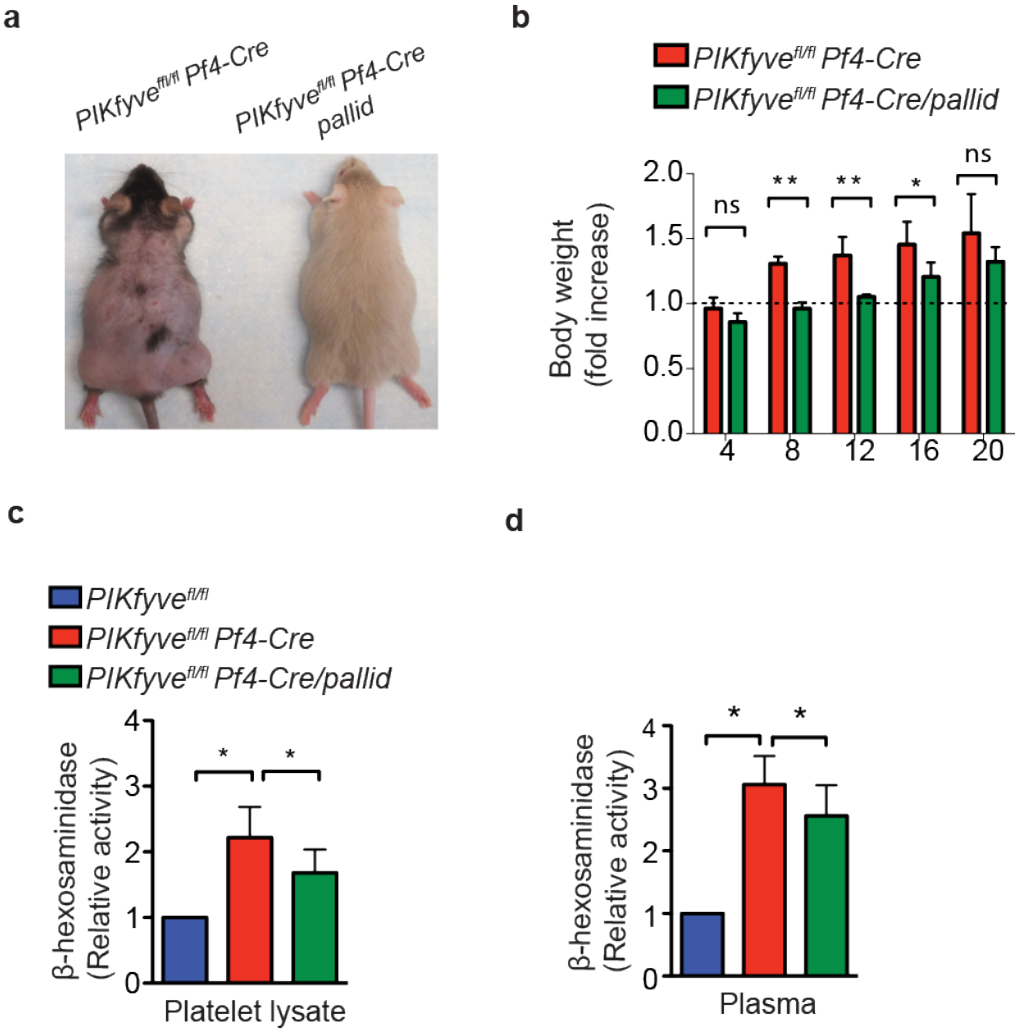

Figure 3.17. Impaired secretion of platelet lysosomes attenuates the phenotypes of *PIKfyve*$^{fl/fl}$ *Pf4-Cre* mice. (**a**) General appearance of *PIKfyve*$^{fl/fl}$ *Pf4-Cre* mice and *PIKfyve*$^{fl/fl}$ *Pf4-Cre/pallid* mice on C57BL/6 background at 17 weeks of age. In the presence of the *pallid* 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 5 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 ± S.D. Student's t-test.

CHAPTER 4: SUMMARY, DISCUSSION AND FUTURE DIRECTIONS

4.1) Summary

In this work, 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.

4.2) Discussion and future studies

PIKfyve regulation of platelet secretome

Our proteomic analysis by mass spectrometry identified 55 proteins that were differentially expressed in the releasates of *PIKfyve-null* platelets in comparison to the releasates of control platelets. These include 17 up-regulated proteins and 38 down-regulated proteins. Among all these proteins, 41 proteins (75% of total) have been previously reported as platelet-released proteins using a mass spectrometry-based approach (Coppinger, Cagney et al. 2004, Coppinger, O'Connor et al. 2007, Hernandez-Ruiz, Valverde et al. 2007, Maynard, Heijnen et al. 2007, Piersma, Broxterman et al. 2009).

Among those 17 proteins that were up-regulated in the releasates of *PIKfyve-null* platelets, three proteins are lysosomal enzymes (cathepsin D, cathepsin B and maltase-glucoamilase) and two proteins belong to the proteasome subunits (proteasome subunit alpha type 6 and proteasome subunit beta type 1). Because both the lysosome and the proteasome are the two main organelles for intracellular degradation, it is tempting to speculate that in the absence of PIKfyve, a defective lysosomal degradative function may feedback on the proteasome and activate the proteasomal degradative pathway.

Six of the up-regulated proteins in the releasates of *PIKfyve-null* platelets are acute phase reactants including proteins of the complement activation system (complement c1s subcomponent, complement c1q subcomponent and complement factor properdin), cholesterol metabolism (apolipoprotein B, phosphatidylcholine-sterol acyltransferase) and serum amyloid a-1 protein. Although all these proteins have been previously identified in the platelet releasates, they also belong to the plasma proteins. It is possible that these acute phase proteins may just reflect an active inflammatory response by *PIKfyve-null* platelets. However, we cannot exclude the possibility that some of these proteins represent plasma contaminants in the platelet releasate samples. Other proteins that were found also up-regulated in the platelet releasates of *PIKfyve-null* platelets include two extracellular matrix proteins normally absent in platelets (collagen and fibulin1), immunoglobulin light chains, kininogen 2, keratin and inter-alpha-trypsin inhibitor. However, the significance of the differential expression of these proteins in *PIKfyve-null* platelet releasates is currently unknown.

Cytoskeletal proteins have been consistently reported in the platelet releasates in the previous studies using a proteomic approach (Coppinger, Cagney et al. 2004, Hernandez-Ruiz, Valverde et al. 2007, Maynard, Heijnen et al. 2007), which suggest that they are authentic components of platelet releasates. Among those 38 down-regulated proteins in the releasates of *PIKfyve-null* platelets, 14 proteins are cytoskeletal proteins (tropomyosin, myosin light polypeptide 6, zyxin, filamin A, myosin regulatory light polypeptide 9, alpha-actinin 1, vinculin, talin 1, tubulin beta 4B chain, WD repeat-containing protein 1, tubulin alpha 1B chain, myosin 9, tubulin beta 1 chain and cofilin1). Interestingly, our immunoblotting analysis of platelet lysates for actin, tubulin and vinculin demonstrated no differences between the WT and *PIKfyve-null* platelets, which suggests that the total amount of cytoskeletal proteins is likely intact in *PIKfyve-null* platelets. The significance of a potential decrease of cytoskeletal proteins in the releasates of *PIKfyve-null* platelets remains to be explored.

Other proteins that are expressed lower in the *PIKfyve-null* platelet releasates include 13 proteins that have been previously detected in the releasates of platelet alpha granules or dense granules (Hernandez-Ruiz, Valverde et al. 2007, Maynard, Heijnen et al. 2007). These proteins include factor XIII A chain, beta parvin, protein disulfide-isomerase A3, vWF, latent-transforming growth factor beta binding protein, 14-3-3 protein eta, multimerin-1, transforming growth factor beta 1, heat shock cognate 71kDa protein, glia-derived nexin, 78kDa glucose-regulated protein, coagulation factor V, metalloproteinase inhibitor 3 and 14-3-3 protein zeta/delta. To validate these findings, we performed immunoblotting analysis of vWF and PF4 in the lysates from WT and *PIKfyve-null* platelets. Interestingly, similar to the cytoskeletal proteins, the expression of

vWF and PF4 was similar in the lysates from the WT and *PIKfyve-null* platelets. In addition, the secretion of P-selectin and ADP were also normal in thrombin-stimulated *PIKfyve-null* platelets, which suggests that the efficiency of exocytosis of alpha granules and dense granules are intact in *PIKfyve-null* platelets. Therefore, although our proteomic analysis provided a comprehensive list of potential candidate proteins that could be differentially expressed in the *PIKfyve-null* platelet releasates, it is essential that these findings need to be validated by a separate experimental approach before we draw any final conclusion.

Label-free mass spectrometry-based quantitative proteomics is a powerful technology for the analysis of protein abundance between samples. However, there still exist several limitations. 1) The use of spectral counts as a method of quantification only provides relative abundance of proteins in samples. In our study, the spectral counts were used to compare the abundance of a specific protein in the WT vs. *PIKfyve-null* platelet samples, but not for the analysis of relative abundance across different proteins. 2) The presence of highly abundant proteins may reduce the detection sensitivity of lower abundant proteins or peptides. This limitation may explain why the β-hexosaminidase level was found to be higher in the *PIKfyve-null* platelets using a chromogenic activity assay but not by mass spectrometry analysis. 3) Because mass spectrometry analysis is based on protein digests or peptides, information on the intact protein cannot be directly provided.

The role of PIKfyve in the maturation of lysosomal enzymes.

We show that the precursor lysosomal protease, procathepsin D, is excessively stored and released in *PIKfyve-null* platelets, which suggests that PIKfyve is essential in the maturation of procathepsin D to active cathepsin D. In normal physiological conditions, cathepsin D is synthesized as a pre-pro-enzyme in the endoplasmic reticulum. After removal of signal peptide, pre-pro-cathepsin D yields procathepsin D, which is modified by N-linked glycosylation and transported to Golgi. In Golgi, procathepsin D is further modified by the addition of mannose-6-phosphate residues (M6P), which bind to the mannose-6-phosphate receptors (M6PR) in the TGN for its delivery to endosomes. In the acidic endolysosomal compartments, procathepsin D is further processed by proteolytic cleavage to active cathepsin D (Kornfeld 1987). The M6PR must then recycle to the TGN for another round of transport. Therefore, procathepsin D maturation depends on the proper trafficking of procathepsin D from the TGN to endosomes for its proteolytic activation in acidic compartments. Hence, the defective maturation and accumulation of procathepsin D in *PIKfyve-null* platelets suggest that PIKfyve is critical for procathepsin D trafficking from the TGN to endosomes. This conclusion is consistent with a published study showing that PtdIns(3,5)P2 deficiency leads to the defective retrograde trafficking of M6PR from endosomes to the TGN (Rutherford, Traer et al. 2006), which would ultimately exhaust the M6PR from the TGN and result in a defective maturation and 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 D (Zhang, Zolov et al. 2007). Together, these studies

suggest that the PIKfyve synthesis of PtdIns(3,5)P2 regulates the trafficking from the TGN to the lysosome, essential for the maturation of procathepsin D.

However, the proteolytic maturation of procathepsin D also needs low pH in the lysosome. Consequently, a defective lysosomal acidification may also impair the proteolytic maturation of procathepsin D. In yeasts, PIKfyve is required for acidification of the lysosome-like vacuole (McCartney, Zhang et al. 2014). However, chemical inhibition and siRNA knockdown of PIKfyve in mammalian cells do not impair the low pH of endolysosomal comparments (Kim, Dayam et al. 2014). These studies suggest that PIKfyve might have a different role in the regulation of lysosomal pH in yeasts and mammalian cells. In future studies, it would be important to verify the lysosomal acidification as well as cellular functions that depend on the proper lysosomal acidification in *PIKfyve-null* mammalian cells.

The role of PIKfyve in the expression of lysosomal proteins

We also show that *PIKfyve-null* platelets have increased expression of lysosomal proteins, which include lysosomal membrane proteins such as LAMP-1 as well as lysosomal enzymes such as β-hexosaminidase and procathepsin D. These findings suggest that PtdIns(3,5)P2 deficiency might additionally feedback on lysosomal protein biosynthesis.

The gene network of lysosomal biogenesis and functions is tightly regulated by the master regulator transcription factor EB (TFEB) (Sardiello, Palmieri et al. 2009, Settembre, Fraldi et al. 2013). In basal conditions, TFEB is phosphorylated and localized

in the cytoplasm. However, in response to lysosomal dysfunction or starvation, TFEB is dephosphorylated and rapidly translocated into the nucleus. Phosphorylation of TFEB has been shown to be regulated by different kinases including mTORC1, ERK2 and PKCβ (Settembre, Fraldi et al. 2013). Interestingly, PIKfyve synthesis of PtdIns(3,5)P2 has been shown to be critical for the recruitment of mTORC1 to the plasma membrane and its activation in adipocytes (Bridges, Ma et al. 2012). In yeast, PtdIns(3,5)P2 recruits TORC1 to the lysosome-like vacuole and induces multiple TORC1-dependent pathways (Jin, Mao et al. 2014). However, whether PIKfyve synthesis of PtdIns(3,5)P2 and its activation of mTORC1 is necessary for the phosphorylation of TFEB to keep it inactive in the cytosol remains unknown. In our preliminary study, we found that *PIKfyve-deficient* cells have increased dephosphorylated forms of TFEB in their cell lysates (data not shown), which suggests that PIKfyve is required for the phosphorylation of TFEB. In future studies, one could test the hypothesis that PIKfyve deficiency impairs the activation of mTORC1 pathway and therefore the phosphorylation of TFEB, which results in the nuclear translocation of TFEB and activation of the lysosomal gene network. This hypothesis could be tested by comparing in the control and *PIKfyve-null* cells : 1) the activation of mTORC1 pathway by immunoblotting analysis, 2) the cellular localization of TFEB (cytosolic vs. nuclear) by immunofluorescence microscopy analysis, and 3) the expression of lysosomal genes by microarray analysis or quantitative RT-PCR. The impaired activation of mTORC1, nuclear translocation of TFEB, and increased transcripts of lysosomal genes in *PIKfyve-null* cells could explain the increased expression of lysosomal proteins in these cells.

The role of PIKfyve in the regulation of lysosomal functions

Although our study on the maturation of lysosomal enzymes was limited to procathepsin D in *PIKfyve-null* platelets, a defective protein trafficking from the TGN to endosomes could potentially impair the processing and functions of many lysosomal proteins and interfere with normal lysosomal functions. Most mammalian cells have lysosomes and their functions are mainly intracellular. For example, within the cell, lysosomes are critical for the degradation and recycling of extracellular materials, autophagy, nutrient sensing, and control of energy metabolism (Settembre, Fraldi et al. 2013). However, lysosomes can be exocytosed by professional secretory cells in physiological processes and the released lysosomal contents play essential roles at the extracellular space. These professional secretory cells include cytotoxic T lymphocytes that release granzyme and perforin for cytotoxicity, osteoclasts that release proteases for bone resorption, melanocytes that release and transfer melanin to keratinocytes for pigmentation, and platelets. However, the physiological role of the platelet secretion of lysosomes is poorly understood.

Megakaryocytes and platelets possess lysosomes that contain different hydrolases including proteases and glycosidases, which are released upon platelet activation (Bentfeld and Bainton 1975, Bentfeld-Barker and Bainton 1982) (Ciferri, Emiliani et al. 2000). It has been speculated that the release of platelet lysosomal enzymes may be required for the remodeling of extracellular matrix during wound healing (Rendu and Brohard-Bohn 2001). 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 arteries (Muir and Bowyer 1984). 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 an aberrant release of platelet lysosomal contents could be pathogenic and that the lysosomal homeostasis in platelets play a crucial role in limiting the proinflammatory and prothrombotic functions of platelets.

PtdIns(3,5)P2 deficiency is reminiscent of lysosomal storage disorders

Similar to PIKfyve-null platelets, excessive release of the lysosomal enzymes are also seen in the lysosomal storage disorders, Mucolipidosis II and Mucolipidosis III (Platt, Boland et al. 2012). 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 extracellularly (Vogel, Payne et al. 2009).

Like 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 diseases (Zhang, Chow et al. 2008).

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 compartments(Zhang, Zolov et al. 2007). 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.

PIKfyve deficiency induces secondary pathologies in other cells

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 (Boven, van Meurs et al. 2004, Vitner, Platt et al. 2010). Likewise, microglial activation is frequently associated with the neuropathology in Mucopolysaccaridoses (Parkinson-Lawrence, Shandala et al. 2010).

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 multiorgan failure 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. We further demonstrate that impairing the lysosomal secretion from platelets significantly attenuates the macrophage pathology leading to multiorgan abnormalities in *PIKfyve*$^{fl/fl}$ *Pf4-Cre* mice, which suggests that platelet release of lysosomes contribute to the macrophage

pathology. However, the exact mechanism by which the lysosomal contents of *PIKfyve-null* platelets induce the macrophage pathology remains unknown. Further studies are required to identify what components of platelet lysosomes contribute to the macrophage pathology. A strong candidate protein is the procathepsin D, which is the lysosomal protein with the highest relative abundance in the releasates of *PIKfyve-null* platelets in comparison to control platelets. One could test whether the releasates from *PIKfyve-null* platelets can directly contribute to the vacuolation and/or proliferation of macrophages by culturing WT macrophages in the presence of either the control or *PIKfyve-null* platelet releasates, and observing the morphological changes and cell proliferation by live cell imaging and biochemical assays. Similarly, WT macrophages could be treated either in the presence or in the absence of a recombinant procathepsin D, and examined for changes in their cell morphology or proliferation.

Once a specific *PIKfyve-null* platelet lysosomal proteins causing the macrophage pathology is identified, further studies will be necessary to determine how this protein induces cytoplasmic vacuolation and cell proliferation in macrophages. Similar to *PIKfyve-null* platelets, several cancer cells overexpress and hypersecrete procathepsin D. In some conditions, procathepsin D has a paracrine function in the tumor environment by increasing fibroblast outgrowth and tumor metastasis. One proposed model is that procathepsin D could function as a signaling molecule that activates MAPK signaling pathway and regulate cellular apoptosis and proliferation in cancer cells (Vetvicka, Vashishta et al. 2010). Thus, it would be intriguing to investigate whether the addition of *PIKfyve-null* platelet releasates could activate MAPK signaling pathway in WT macrophages. It has been also shown that the secreted procathepsin D by cancer cells can

be endocytosed by other cells in the tumor microenvironment and change the degradative functions of the lysosomes in the recipient cells. In future studies, it would be interesting to study whether the procathepsin D released by *PIKfyve-null* platelets could be endocytosed by WT macrophages and impair the lysosomal degradative functions leading to lysosomal vacuolation in 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 disorder (Chow, Zhang et al. 2007). 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 defects (Winters, Ferguson et al. 2011). 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.

4.3) Conclusion

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.

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