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Individual phosphatidylinositol transfer proteins have distinct functions that do not involve lipid transfer activity

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Abstract:
Platelets utilize signal transduction pathways facilitated by Class I phosphatidylinositol transfer proteins (PITPs). The two mammalian Class I PITPs, PITPα and PITPβ, are single PITP domain soluble proteins that are encoded by different genes and have 77% sequence identity, though their individual roles in mammalian biology remain uncharacterized. These proteins are believed to shuttle phosphatidylinositol and phosphatidylcholine between separate intracellular membrane compartments, thereby regulating phosphoinositide synthesis and second messenger formation. Previously, we observed that platelet-specific deletion of PITPα, the predominant expressed murine PITP isoform, had no effect on hemostasis, but had impaired tumor metastasis formation and disrupted phosphoinositide signaling. Here, we find that mice lacking the lesser expressed PITPβ in their platelets exhibit a similar phenotype. However, in contrast to PITPα-null platelet lysates that have impaired lipid transfer activity, PITPβ-null platelet lysates have essentially normal lipid transfer activity, although both isoforms contribute to phosphoinositide synthesis *in vitro*. Moreover, we found that platelet-specific deletion of both PITPs leads to *ex vivo* platelet aggregation/secretion and spreading defects, impaired tail bleeding, and profound tumor dissemination. Our studies also demonstrate that PITP isoforms are required for maintaining endogenous phosphoinositide PI(4,5)P2 levels and agonist stimulated second messenger formation. The data shown here demonstrate that both class I PITP isoforms contribute to phosphoinositide signaling in platelets, likely through distinct biochemical mechanisms or in different subcellular domains. They are functionally overlapping and either single isoform is able to maintain the homeostasis of platelets.

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Key Points

- Class I PITPs mediate *in vivo* platelet phosphoinositide signaling through both their transfer and co-factor activities.
- PITPα and PITPβ have both overlapping and distinct, but non-redundant functions.

Abstract

Platelets utilize signal transduction pathways facilitated by Class I phosphatidylinositol transfer proteins (PITPs). The two mammalian Class I PITPs, PITPα and PITPβ, are single PITP domain soluble proteins that are encoded by different genes and have 77% sequence identity, though their individual roles in mammalian biology remain uncharacterized. These proteins are believed to shuttle phosphatidylinositol and phosphatidylcholine between separate intracellular membrane compartments, thereby regulating phosphoinositide synthesis and second messenger formation. Previously, we observed that platelet-specific deletion of PITPα, the predominantly expressed murine PITP isoform, had no effect on hemostasis, but had impaired tumor metastasis formation and disrupted phosphoinositide signaling. Here, we find that mice lacking the less expressed PITPβ in their platelets exhibit a similar phenotype. However, in contrast to PITPα-null platelet lysates that have an impaired lipid transfer activity, PITPβ-null platelet lysates have essentially normal lipid transfer activity, although both isoforms contribute to phosphoinositide synthesis *in vitro*. Moreover, we found that platelet-specific deletion of both PITPs leads to *ex vivo* platelet aggregation/secretion and spreading defects, impaired tail bleeding, and profound tumor dissemination. Our studies also demonstrate that PITP isoforms are required for maintaining endogenous phosphoinositide PtdInsP2 levels and agonist stimulated second messenger formation. The data shown here demonstrate the two isoforms are functionally overlapping and either single isoform is able to maintain the homeostasis of platelets. However, both class I PITP isoforms contribute to phosphoinositide signaling in platelets through distinct biochemical mechanisms or in different subcellular domains.

Introduction

Phosphoinositides play important roles in both signal transduction and membrane trafficking.[1] The primary substrate of phosphoinositides, phosphatidylinositol (PtdIns), is produced in the endoplasmic reticulum and can be distributed by phosphatidylinositol transfer proteins (PITP) to different cellular organelles that participate in phosphoinositide synthesis. These proteins have a hydrophobic pocket which envelops a single phosphatidylinositol allowing transport through the cytoplasm to join in phosphoinositide synthesis.[2] The role of PITP in mediating phosphoinositide production has been demonstrated by *in vitro* studies in which mammalian PITP can substitute for *Sec14p*, the yeast lipid transfer protein, to support phosphoinositide synthesis in yeast.[3,4]

PITPα and PITPβ are ubiquitous proteins found in all mammals. In COS cell lines, they are expressed in different cellular compartments, with PITPα localizing in the cytosol and nucleus and PITPβ residing at the Golgi.[5,6] Early studies in permeabilized cells had indicated that PITP isoforms were able to support phospholipase C signaling and exocytosis by facilitating phosphoinositide synthesis.[7–9] Recent work has shown that PITP are essential for the PtdIns4P-dependent recruitment of GOLPH3 to Golgi membranes, an event that is required for Golgi biogenesis during neuronal development.[10,11] Together, the biochemical and genetic analyses performed to date indicate that PITP are essential for most phosphoinositide-dependent cellular events.

The relative expression levels of the individual isoforms vary in different cell types.[12] PITPα and PITPβ isoforms are expressed at similar levels in embryonic stem cells,[13] whereas their expression varies within adult mammalian tissues. With the exception of liver, lung, and kidney, the majority of tissues express PITPα as their most abundant isoform.[12] Likewise in

mouse platelets, PITPα expression is seven-fold more abundant than PITPβ.[14] PITPα knockout mice survived up to two weeks through birth, but had neurological defects, gut abnormalities, and hypoglycemia.[15] Mice with global deletion of PITPβ are viable and have no obvious pathological abnormality.[10] The contributions of each mammalian PITP remain elusive, but some recent studies found that PITPβ was significantly upregulated in peripheral blood of human atopic dermatitis patients[16], and PITPβ also was implicated in human epidermal growth factor signaling[17]. Together, these findings suggest that each specific PITP isoforms may have distinct functions as reflected in the manifestation of different human disease phenotypes.

PtdIns is an essential mediator of signal transduction pathways in all cells, including platelets. Given that PITPs were found to facilitate PtdIns pathways in yeast, we analyzed the role of the predominant platelet isoform, PITPα, in hemostasis, thrombosis, and tumor disseminations.[14] Surprisingly, platelet-specific deletion of PITPα did not disrupt *ex vivo* platelet function nor did it impact *in vivo* thrombosis and hemostasis, but it impaired signal transduction pathways that determine fibrin shroud formation around tumor cells, protecting them from immunological clearance.[14,18] In this study, we sought to investigate the specific contributions of platelet PITPβ-mediated phospholipid signaling to the same biological processes. We generated mice lacking either PITPβ, or both PITPα and PITPβ in their platelets and directly compared the biochemical and biologic functions of the discrete mammalian Class I PITP isoforms. Here, we show that two PITP isoforms function in an overlapping, yet not totally redundant way to support platelet homeostasis. Our results also provide the first *in vivo* evidence that PITPβ regulates platelet phosphoinositide synthesis through a mechanism that is at least in part independent of its lipid transfer activity.

Methods

Animals

Animal protocols were approved by the Institutional Animal Care and Use Committees of the University of Pennsylvania. Conditional knockout (*Pitpα*, *Pitpβ*, *Pitpα/β*) and transgenic (*Flp, Pf4-Cre)* mice with C57/BL6J genetic background were used in this study. The *Pitpα* conditional mouse was described previously.[14] To generate the *Pitpβ* conditional mouse, a 1.89kb region of the *Pitpβ* gene that includes exons 4-6 was flanked by insertions of loxP sites and *Frt/Neo* cassettes. The *Neo* cassette was then removed by crossing with *Flp* mice. Floxed mice were crossed with mice expressing CRE recombinase driven by the platelet factor-4 promoter (*Pf4-Cre*)[19] to generate $Pitp\beta^{fl/fl}Pf4\text{-}Cre^{+}$ mice which did not express PITPβ specifically in their platelets and megakaryocytes. These mice were further crossed with $Pitp\alpha^{fl/fl}Pf4\text{-}Cre^{+}$ to create platelet specific double-knockouts $Pitp\alpha^{fl/fl}/\beta^{fl/fl}$ $Pf4\text{-}Cre^{+}$. Mice were maintained on standard chow. Mice of both sexes between 8 and 20 weeks of age were used for experiments unless noted otherwise.

Additional methods are provided in the Supplemental Materials.

Results

PITPβ-null platelets are functionally normal, but deletion of both PITPs disrupts functions

To better understand the potential clinical significance of PITP isoforms, we analyzed the relative protein expression of the two Class I PITP isoforms in purified human platelets. As shown in **Figure 1A-B**, PITPβ was approximately six-fold more abundant than PITPα. These results contrast murine platelets where PITPα is more abundant than PITPβ. These data suggest that PITPβ may play more important functions than PITPα in human platelets.

In order to understand the role of PITPβ in platelet activation, we generated mice that specifically lacked PITPβ in their platelets and megakaryocytes by crossing *Pitpβ*$^{fl/fl}$ mice (**Figure 1C**) with *Pf4-Cre*$^{+}$ mice.[19] Mice lacking PITPβ only in their platelets and megakaryocytes were viable and produced platelets. Western blot analysis of platelet lysate probed with anti-PITPβ specific antibody indicated a successful deletion of the protein (**Figure 1D**). In the PITPβ-null platelets, we did not observe a compensatory increase of PITPα. Further, by pairing *Pitpα*$^{fl/fl}$*Pf4-Cre*$^{+}$ mice with *Pitpβ*$^{fl/fl}$*Pf4-Cre*$^{+}$ mice, we generated *Pitpα*$^{fl/fl}$/*β*$^{fl/fl}$*Pf4-Cre*$^{+}$ mice which lack both PITP isoforms in their platelets and megakaryocytes. Mice with deletion of both isoforms (*Pitpα*$^{fl/fl}$/*β*$^{fl/fl}$*Pf4-Cre*$^{+}$) were grossly normal with no apparent abnormalities in their body weight, organ morphology, and survival. We also did not observe any spontaneous thrombosis or hemorrhaging. However, *Pitpβ*$^{fl/fl}$*Pf4-Cre*$^{+}$ mice had minimal thrombocytopenia (85% of normal), while *Pitpα*$^{fl/fl}$/*β*$^{fl/fl}$*Pf4-Cre*$^{+}$ mice had an even more pronounced thrombocytopenia (60% of normal, **Figure 1E**). The complete blood cell count in our previous study demonstrated that *Pitpα*$^{fl/fl}$/*β*$^{fl/fl}$*Pf4-Cre*$^{+}$ mice have a slightly reduced WBC count (24%),[20] but the *Pitpβ*$^{fl/fl}$*Pf4-Cre*$^{+}$ mice have in general, normal blood cell counts (**Supplemental Table 1**).

Given that PITP is essential in phosphoinositide production in yeast and that phosphoinositide signaling is critical in platelet physiology, we examined if deleting PITPβ or both PITP isoforms in platelets affect *ex vivo* activation. Similar to PITPα-null platelets,[14] PITPβ-null platelets aggregated normally following stimulation with low doses of thrombin or collagen. However, PITPβ-null platelets had impaired ATP secretion when compared to their controls (**Figure 1F**). When both isoforms were deleted, platelets had severe *ex vivo* aggregation and secretion defects (**Figure 1G**). Interestingly, serotonin, which is stored in dense granules, in platelets lacking either PITPβ or both PITP were normal (**Supplemental Figure 3**) suggesting that these platelets may have secretion defects rather than biogenesis or loading defects. Finally, activated PITPβ-null platelets spread normally on fibrinogen, while PITPα/β-null platelets was impaired (**Figure 1H-J**). These findings demonstrate that both isoforms play roles in regulating platelet functions and either single isoform is able to maintain the normal platelet functions.

Mice lacking platelet PITPs have no defect in laser-induced *in vivo* thrombosis, but have prolonged tail bleeding times

We next assessed if the *ex vivo* platelet activation defects found in $Pitp\alpha^{fl/fl}/\beta^{fl/fl}Pf4\text{-}Cre^{+}$ mice (PITPα/β-null) disrupts *in vivo* thrombosis using two models: a laser injury-induced arteriolar thrombosis model and tail bleeding time assay. After initiating a laser-induced vascular injury of the cremaster arterioles, we visualized platelet accumulation (CD41) and α-granule secretion (P-selectin) in real-time. Mice with PITPα/β-null platelets had no discernable changes in platelet accumulation (**Figure 2A-B**) and exocytosis of alpha granules were normal (**Figure 2C**) despite the lack of granule contents as demonstrated by electron microscopy previously in MKs.[20] Next, we analyzed hemostasis using an independent *in vivo* model, the tail bleeding time assay. Bleeding times were normal in $Pitp\beta^{fl/fl}Pf4\text{-}Cre^{+}$ mice (**Figure 2D**), but there was a

statistically significant delay in *Pitpα*$^{f/f}$/*β*$^{fl/fl}$*Pf4-Cre*$^{+}$ mice when compared to their littermate controls (150s vs 35s, $p<0.05$) (**Figure 2E**) revealing a contribution of PITP-dependent signaling pathways on hemostasis that is different than shown by the laser injury model.

Loss of PITPs impairs tumor metastasis and platelet thrombin generation

In addition to hemostasis, platelets have been implicated in other processes, such as the dissemination of tumor metastases through effects of the coagulation system.[21, 22] We have previously demonstrated that PITPα-mediated phosphoinositide metabolism within platelets enables tumor cells to escape immune surveillance that requires platelet thrombin generation and fibrin formation.[14] To understand if PITPβ also contributes to this process, we intravenously injected B16F10 melanoma cells and analyzed the tumor metastases in the lungs of mice lacking either PITPβ or both PITPα and PITPβ in their platelets.

We found that mice lacking platelet PITPβ had impaired tumor metastasis formation (**Figure 3A**), similar to mice lacking platelet PITPα.[14] Additionally, mice lacking both platelet PITP had an even greater reduction in tumor metastasis as compared to the mice lacking a single isoform (**Figure 3B**). We previously demonstrated that the ability of platelets to support metastasis formation correlated with its ability to adhere to tumor cells.[14] Therefore, we analyzed the adhesion of PITPβ-null platelets to the tumor cells. We found that PITPβ deletion contributed to loss platelet-tumor adhesion, comparable to PITPα deletion (**Figure 3C**) and loss of both PITP isoforms did not further compound the adhesion defect (**Figure 3D**) revealing that adhesion is not the only mechanism driving metastasis formation.

Our previous study demonstrated that tumor cells can escape host immune surveillance by inducing thrombin generation on its surface, causing platelets to agglutinate, and shrouding the tumor cells from immune cells.[14] We observed that like PITPα, platelet PITPβ facilitates

tumor-induced thrombin generation and, in the absence of both PITP isoforms, thrombin generation in platelet rich plasma was essentially eliminated (**Figure 4A-B)**. Since the tissue factor (TF) on the tumor surface is likely to trigger thrombin production in platelets, we tested the ability of PITP-null platelets to generate thrombin when stimulated with recombinant TF alone. As predicted, TF-induced thrombin generation was markedly impaired in PITPβ-null platelets and essentially eliminated in platelets lacking both PITP isoforms (**Figure 4C-D**). In both cases, thrombin generation in platelet poor plasma was identical between PITP-null and wild-type following stimulation with either tumor cells (**Figure 4E**) or TF (**Figure 4F**), confirming that PITP-mediated thrombin generation is platelet-dependent. Together, these results demonstrate that both PITP isoforms contributes to tumor dissemination by a pathway dependent on platelet PITP-mediated thrombin generation.

Thrombin generation depends on flipping of phosphatidylserine (PS) from the inner to the outer leaflet of platelet cell membrane. To determine if platelets lacking PITPβ or both PITP isoforms have impaired PS exposure, we analyzed Annexin V-binding to detect PS on the surface of platelets. In resting platelets, PS exposure is minimal and unaffected by the absence of either PITP isoform (**Figure 4G**). However, PS exposure was significantly reduced in activated platelets lacking PITPβ, similar to our previous study with PITPα[14], and further reduced when both isoforms were deleted. The ability of PITPs to support the flipping of PS on the cell membrane likely accounts for the mechanism by which PITP enable tumor-mediated thrombin generation.

PITPβ is required to maintain endogenous phospholipid homeostasis

PITPs are named for their ability to transport PtdIns from endoplasmic reticulum to different subcellular compartments to facilitate intracellular phospholipid signaling. We analyzed

the relative contributions of either single isoform to the synthesis of platelet phosphoinositide PtdInsP and PtdInsP2 by mass spectrometry analysis. The most abundant phosphoinositide was distributed in the fraction of C38:4, and the least abundant phosphoinositide was in the fraction of C36:2 (**Supplemental Figure 4A**). In both resting and thrombin activated conditions, platelets lacking either PITPα or PITPβ have no significant reduction of total endogenous PtdInsP which includes PtdIns(3)P, PtdIns(4)P, and PtdIns(5)P. However, platelets lacking both isoforms have markedly reduced amounts of endogenous PtdInsP, and thrombin activation failed to induce additional PtdInsP production (**Figure 5A, Supplemental Figure 4A**). The data shown here suggest that either isoform alone is able to maintain sufficient PtdInsP required for platelet normal function. Surprisingly, PITPβ is able to compensate for the loss of PITPα to maintain the PtdInsP levels even though there is relatively little PITPβ in platelets.

We also analyzed total PtdInsP2 levels [PtdIns(3,4)P2, PtdIns(3,5)P2, and PtdIns(4,5)P2] in platelets derived from all fractions (C38:4, C38:3 and C36:2). In contrast to PtdInsP production, we found that platelets lacking either single isoform or both PITPs had only minor and PI3 kinase independent reduction of endogenous PtdInsP2 analyzed at resting conditions and after thrombin stimulation (**Figure 5B, Supplemental Figure 4A-B**).

In our studies of platelets lacking PIP5KI, we observed that phospholipase C generates the second messenger IP_3, which derive from pools of PtdIns(4,5)P2 within specific microdomains.[37] We hypothesized that the loss of individual PITP isoforms could impair the synthesis of discrete pools of PtdIns(4,5)P2 required for second messenger formation without markedly affecting the levels of total cellular PtdIns(4,5)P2. To test this hypothesis, we analyzed IP_3 production following thrombin activation in platelets lacking either PITPβ or both isoforms.

While activation caused a rapid increase of IP_3 formation in wild-type platelets, IP_3 production in PITPβ-null platelets was reduced by up to 50% following activation (**Figure 5C**), similar to the previously reported result found in PITPα-null platelets.[14] Further, platelets lacking both isoforms had PLC activity independent, essentially no IP_3 production following stimulation (**Figure 5D, Supplemental Figure 4C**). These data indicate that although PITP isoforms are not critical for the synthesis of total cellular PtdInsP2, they are completely essential for the synthesis of discrete pools of the PtdIns(4,5)P2. Since PITPα is more abundant than PITPβ, these data also demonstrate the unexpectedly large contribution by the PITPβ on discrete pools of PtdIns(4,5)P2 required for IP_3 production.

PITPβ is a co-factor for phosphoinositide synthesis

Since the primary function of PITPs has been discovered by their ability to transfer PtdIns, we compared the *in vitro* transfer activity of tritiated phosphatidylinositol from microsomes to liposomes in the presence of lysates of PITPα-null and PITPβ-null platelets.[23] As predicted, when both PITP isoforms were deleted in platelets, the PtdIns transfer activity was completely ablated (**Figure 6A**). Interestingly, PITPα-null platelets lysates had more than 90% reduction in transfer activity in comparison to the lysates from the control platelets. However, PITPβ-null platelet lysates had almost no reduction of PtdIns transfer (**Figure 6A**). The data demonstrate that PITPα is responsible for essentially all the PtdIns transfer activity within platelets. The difference of the transfer activity mediated by PITPα and PITPβ could be due to the difference in the levels of PITP protein expression within platelet.[14] Importantly, this data indicate that PITPβ does not support PtdIns transfer activity in platelets in a significant way and thus, the effect it has on PtdInsP2 production is mediated through another mechanism.

In addition to phospholipid transfer, recent studies have demonstrated that PITPs also directly interact with lipid kinases enhancing their kinetics.[1,24,25] Thus, we analyzed the potential impact of each individual isoform on platelet lipid kinase activity in mediating PtdInsP synthesis *in vitro*. We utilized a cell-free system, with excess lipid substrate, to assess the co-factor capability of PITP to enhance the lipid kinase function. Since lipid availability was not rate limiting and cellular compartments were eliminated, this assay analyzed each individual PITP isoform for their so-called "nanoreactor" ability to assist phosphoinositide kinases through a lipid transfer independent mechanism.[24] The data show that PITPα-null platelet lysate have reduced PtdInsP production after thrombin activation. Augmentation with either recombinant PITPα (rPITPα) or recombinant PITPβ (rPITPβ) fully restored PtdInsP production (**Figure 6B**). Conversely, lysates from PITPβ-null platelets also had reduced PtdInsP production and similar to PITPα-null platelet lysate, augmentation with rPITPα or rPITPβ restored the ability of PITPβ-null platelet lysates to generate PtdInsP (**Figure 6C)** showing that each isoform can compensate for the loss of deleted isoform in mediating PtdInsP synthesis *in vitro*. Together, these results show the first evidence that both platelet PITP isoforms have co-factor function in phosphoinositide synthesis.

PITP isoforms distributed differentially within platelets

Given that PITPα is the predominant isoform involved in lipid transfer in platelets yet both isoforms are interchangeable in their ability to provide co-factor activity to lipid kinases, we hypothesized that the isoforms may be differentially distributed within platelets and contribute to phosphoinositide synthesis in different subcellular compartments. To address this hypothesis, we first fractionated wild-type platelets into cytosolic, membrane, and cytoskeletal fractions, and immunoblotted for PITPα and PITPβ. We found that PITPα was overwhelmingly localized in the

cytosol (89.5%), with very little found in the membrane (4.8%) and cytoskeletal (5.7%) fractions. In contrast, PITPβ localization was more dispersed amongst the cytosolic (62.8%), membrane (13.2%), and cytoskeletal fractions (24%). Finally, thrombin activation did not change the respective distribution of each isoform (**Figure 7A-B**). Next, we investigated the cellular distributions of PITPα in PITPβ-null platelets and, conversely, the PITPβ distributions in PITPα-null platelets. PITPα distribution in either resting and activated PITPβ-null platelets were shifted away from the cytosol to the membrane (14.7%) and cytoskeleton (25.3%) fractions as compared to wild-type (**Figure 7C-D**). Furthermore, PITPβ distribution in PITPα-null platelets shifted away from the membrane and cytoskeleton, to the point it was undetectable, towards the cytosol (**Figure 7E)**. Thus, in both cases, the distribution of the compensating isoform shifted toward the distribution profile of the missing isoform suggesting a spatial compensation of the missing isoform. We further examined human platelets, and the spatial distribution of the isoforms was quite different from mouse platelets, as the majority of PITPα was found in the membrane (up to 60%) while the cytoskeleton contained the least amount of the protein (**Figure 7F-G**). However, PITPβ was distributed predominantly in cytosol and cytoskeleton and was undetectable in membrane fraction (**Figure 7F-G**). These data demonstrate that the distinct PITP isoforms diverge in their spatial localization within human platelets, which could lend to their differing roles in platelet signal transduction.

Discussion

In this study, we sought to determine how PITPβ contributes to platelet intracellular signaling, platelet activation, hemostasis, and non-hemostatic tumor metastasis formation. We were also interested in comparing the results with the previous PITPα study to determine any redundancies or differences in function. Our major findings are: (1) PITPβ is the more abundant

isoform in human platelets. (2) Mice lacking PITPβ or lacking both PITP isoforms in their megakaryocytes and platelets can survive normally, but developed thrombocytopenia. (3) Platelets have a severe aggregation and dense granule defect when both PITP isoforms are deleted. (4) Deletion of the PITPβ isoform had comparable effects with PITPα on the levels of endogenous PtdInsP, PtdInsP2, and agonist induced second messenger production, showing that PITPβ is as important as PITPα to platelet signal transduction. (5) Like PITPα, PITPβ contributes to a process that enables tumors to escape innate immunity. (6) PITPα contributes to the majority of PtdIns transfer activity in murine platelets. (7) Both PITP isoforms serve as co-factors for lipid kinases, and either isoform can functionally compensate for the loss of another (**Table 1**). (8) PITP isoforms likely exert their distinct contributions to platelet biology by regulating spatially distinct pools or pathways of phosphoinositide demonstrated by their differential intracellular compartmentalization.

Unlike deletion in other tissues, such as PITPα in neurons or PITPβ in embryonic stem cells,[26] deletion of PITP in murine platelets did not cause a severe phenotype. Dense granule secretion and platelet spreading in PITPβ-null platelets were only partially reduced. However, when both isoforms were deleted, platelet aggregation, secretion, and spreading were impaired significantly with a concomitant marked reduction in phosphoinositide production. We found that each PITP isoform can compensate for the loss of the other isoform demonstrating that these isoforms have overlapping, but complementary roles when facilitating phospholipid signaling, which drives platelet shape change and granule secretion. Strikingly, significant *in vivo* impairment was absent in the laser injury-induced thrombosis model even when both isoforms were deleted in platelets. This suggests that enough platelet phosphoinositide was produced despite the PITP deletion to recruit platelets for normal hemostasis, perhaps facilitated by other

PITP classes or mechanisms independent of PITP. Studies have found that PITPα associated *in vivo* pathogenesis are largely independent on the levels of the protein expression and even only 10% of normal expression can support the minimum homeostasis requirements[38]. However, in other *in vivo* assays, such as tail bleeding time and tumor metastasis models showed that PITPs are required for both hemostasis and non-hemostatic functions. The discrepancy among the *in vivo* models suggests that an essential role for PITP-mediated phosphoinositide signaling is not universal, and perhaps speak to how different biological processes are specialized via compartmentalization of local PITP protein function within platelets. Additionally, our previous study[14] together with the data shown here demonstrate that PITP can regulate platelet thrombin generation by supporting the externalization of PS to the platelet surface. This suggests that platelet PITP may play large roles in processes that are highly dependent on thrombin activation such as tail bleeding and tumor metastasis.

It is notable that while murine platelet PITPβ levels are expressed at lower levels than PITPα, PITPβ is just as important as PITPα to phosphoinositide production. Previous studies with N-ethyl maleimide (which blocks PITP opening its hydrophobic pocket) have shown that the rate of lipid exchange by PITPβ is about five times faster than PITPα [27]. Experiments with FRET also demonstrated that PITPβ has a higher transfer rate than PITPα thus PITPβ may have higher specific activity than the alpha isoform.[28,29] This illustrates that PITPβ could potentially be more efficient than PITPα requiring less expression. While PITPs were initially identified for their lipid transfer function, there is growing evidence that they present PtdIns directly to phosphoinositide kinases and therefore have co-factor activity.[24, 25] For example, Kular et al. demonstrated that formylmethionyl-leucylphenylalanine - sensitive PtdIns(3,4,5)P3 production by PtdIns 3-kinase γ in HL60 cells requires PITP to function as a co-factor rather than as a lipid

transfer protein.[30] Fensome et al. found that PITP can restore secretory function in HL60 cells, and that PITP promoted PtdIns(4,5)P2 synthesis to restore exocytosis.[31] Furthermore, Panaretou et al. demonstrated that PITP physically associates with the p150-PtdIns 3-kinase complex *in vitro* to activate lipid kinase activity.[32] Finally, Phillips et al. showed that a yeast mutant PITP sec14 that lacked lipid transfer activity could rescue the secretion defects of lethal *sec14* null yeast mutations.[33]

Cockcroft has described PtdIns presentation (co-factor) supplementing the lipid transfer function of PITP.[1] Recently, Cockcroft and Atkinson have demonstrated that the PITPβ binds to membrane lipids with higher affinity than PITPα using dual polarization interferometry.[36] It has been proposed that PITP functions as a lipid kinase co-factor by increasing the accessibility of the kinases to the head groups of phospholipids that are normally buried within the lipid bilayer of membranes. Since PITPs prefer highly curved membranes to bind lipids, PITP once bound to a membrane may serve as an adaptor protein for lipid kinases.[17,34,35] In this way, PITP isoforms would interact within a larger protein complex regulating the lipid kinase machinery.

While further inquiry is needed to refine these models, our evidence demonstrates that *in vivo,* both PITP isoforms must regulate phosphoinositide production beyond their traditional transfer ability role. Since PITPα and PITPβ differ in their intracellular localization in various tissue cell lines,[5,6] this indicates that they may play unique intracellular roles.[25] Our data here supports this model demonstrating that PITPβ is more evenly distributed in the membrane and cytoskeleton as opposed PITPα which is almost entirely restricted to the cytosol. We hypothesize that PITPβ functions as the co-factor in local phosphoinositide synthesis at the cell membrane or perhaps the cytoskeleton. Furthermore, the data shown here suggest that the loss of either isoform causes spatial redistribution of the other extant isoform, perhaps to maintain the

basic functional requirements such as phosphoinositide synthesis. We hypothesize that in platelet phospholipid signaling, PITPα serves the conventional lipid transport function role while both PITPβ and PITPα serve as co-factors in lipid kinase, possibly, in different cellular compartments. We further propose that in certain pathways PITP may operate serially (**Figure 7H**).

Our work demonstrates that the PITP isoforms have overlapping, but non-redundant roles in supporting platelet functions. Both single isoforms contribute to platelet phospholipid signaling, possibly compartmentalized to impart differential functionality. Despite being significantly less abundant than PITPα, PITPβ contributes to normal platelet physiology in both hemostatic and non-hemostatic functions.

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Authorship

Contributions: L.Z., C.L.T., A.S., T.J.S., S.H.M., S.K., S.C., and C.S.A. designed the experiments. L.Z., C.L.T., A.S., T.J.S., S.C. performed the experiments. L.Z., C.L.T., A.S., T.J.S., S.H.M., S.K., S.C., and C.S.A. analyzed the data. L.Z., C.L.T., A.S., T.J.S., and C.S.A. wrote the manuscript. L.Z., C.L.T., A.S., T.J.S., S.H.M., S.K., S.C., and C.S.A. reviewed and approved the manuscript.

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Data and Materials Availability: All data needed to evaluate the conclusions in this paper are present in the paper or the Supplementary Materials. All relevant data are available from the corresponding author upon reasonable request.

References

1. Cockcroft S. The diverse functions of phosphatidylinositol transfer proteins. *Curr. Top. Microbiol. Immunol.* 2012;362:185–208.
2. Yoder MD, Thomas LM, Tremblay JM, et al. Structure of a multifunctional protein. Mammalian phosphatidylinositol transfer protein complexed with phosphatidylcholine. *J. Biol. Chem.* 2001;276(12):9246–9252.
3. Cockcroft S, Carvou N. Biochemical and biological functions of class I phosphatidylinositol transfer proteins. *Biochim. Biophys. Acta BBA - Mol. Cell Biol. Lipids.* 2007;1771(6):677–691.

4. Ile KE, Kassen S, Cao C, et al. Zebrafish class 1 phosphatidylinositol transfer proteins: PITPbeta and double cone cell outer segment integrity in retina. *Traffic Cph. Den*. 2010;11(9):1151–1167.
5. Morgan CP, Allen-Baume V, Radulovic M, et al. Differential expression of a C-terminal splice variant of phosphatidylinositol transfer protein beta lacking the constitutive-phosphorylated Ser262 that localizes to the Golgi compartment. *Biochem. J.* 2006;398(3):411–421.
6. Phillips SE, Ile KE, Boukhelifa M, Huijbregts RPH, Bankaitis VA. Specific and nonspecific membrane-binding determinants cooperate in targeting phosphatidylinositol transfer protein beta-isoform to the mammalian trans-Golgi network. *Mol. Biol. Cell*. 2006;17(6):2498–2512.
7. Hay JC, Fisette PL, Jenkins GH, et al. ATP-dependent inositide phosphorylation required for Ca(2+)-activated secretion. *Nature*. 1995;374(6518):173–177.
8. Hay JC, Martin TF. Phosphatidylinositol transfer protein required for ATP-dependent priming of Ca(2+)-activated secretion. *Nature*. 1993;366(6455):572–575.
9. Thomas GM, Cunningham E, Fensome A, et al. An essential role for phosphatidylinositol transfer protein in phospholipase C-mediated inositol lipid signaling. *Cell*. 1993;74(5):919–928.
10. Xie Z, Hur SK, Zhao L, Abrams CS, Bankaitis VA. A Golgi Lipid Signaling Pathway Controls Apical Golgi Distribution and Cell Polarity during Neurogenesis. *Dev. Cell.* 2018;44(6):725-740.e4.
11. Gatt MK, Glover DM. The Drosophila phosphatidylinositol transfer protein encoded by vibrator is essential to maintain cleavage-furrow ingression in cytokinesis. *J. Cell Sci*. 2006;119(Pt 11):2225–2235.

12. Cosker KE, Shadan S, van Diepen M, et al. Regulation of PI3K signalling by the phosphatidylinositol transfer protein PITPalpha during axonal extension in hippocampal neurons. *J. Cell Sci*. 2008;121(Pt 6):796–803.
13. Alb JG, Phillips SE, Rostand K, et al. Genetic ablation of phosphatidylinositol transfer protein function in murine embryonic stem cells. *Mol. Biol. Cell*. 2002;13(3):739–754.
14. Zhao L, Thorsheim CL, Suzuki A, et al. Phosphatidylinositol transfer protein-α in platelets is inconsequential for thrombosis yet is utilized for tumor metastasis. *Nat. Commun*. 2017;8(1):1216.
15. Alb JG, Cortese JD, Phillips SE, et al. Mice lacking phosphatidylinositol transfer protein-alpha exhibit spinocerebellar degeneration, intestinal and hepatic steatosis, and hypoglycemia. *J. Biol. Chem*. 2003;278(35):33501–33518.
16. Won Kon Kim, Hyun Ju Cho, Sang Chul Lee et al. Comparative proteomic analysis of peripheral blood mononuclear cells from atopic dermatitis patients and healthy donors. *BMB Rep.* 2008 Aug 31;41(8):597-603.
17. Kauffmann-Zeh A, Thomas GM, Ball A, et al. Requirement for phosphatidylinositol transfer protein in epidermal growth factor signaling. *Science*. 1995;268(5214):1188–1190.
18. Palumbo JS, Talmage KE, Massari JV, et al. Platelets and fibrin(ogen) increase metastatic potential by impeding natural killer cell-mediated elimination of tumor cells. *Blood*. 2005;105(1):178–185.
19. Tiedt R, Schomber T, Hao-Shen H, Skoda RC. Pf4-Cre transgenic mice allow the generation of lineage-restricted gene knockouts for studying megakaryocyte and platelet function in vivo. *Blood*. 2007;109(4):1503–1506.

20. Capitano M, Zhao L, Cooper S, et al. Phosphatidylinositol transfer proteins regulate megakaryocyte TGF-β1 secretion and hematopoiesis in mice. *Blood*. 2018;132(10):1027–1038.
21. Gay LJ, Felding-Habermann B. Contribution of platelets to tumour metastasis. *Nat. Rev. Cancer*. 2011;11(2):123–134.
22. Xu XR, Yousef GM, Ni H. Cancer and platelet crosstalk: opportunities and challenges for aspirin and other antiplatelet agents. *Blood*. 2018;131(16):1777–1789.
23. Cockcroft S. Measurement of phosphatidylinositol and phosphatidylcholine binding and transfer activity of the lipid transport protein PITP. *Methods Mol. Biol. Clifton NJ*. 2009;462:363–377.
24. Bankaitis VA, Ile KE, Nile AH, et al. Thoughts on Sec14-like nanoreactors and phosphoinositide signaling. *Adv. Biol. Regul*. 2012;52(1):115–121.
25. Cunningham E, Thomas GM, Ball A, Hiles I, Cockcroft S. Phosphatidylinositol transfer protein dictates the rate of inositol trisphosphate production by promoting the synthesis of PIP2. *Curr. Biol. CB*. 1995;5(7):775–783.
26. Alb JG, Phillips SE, Rostand K, et al. Genetic ablation of phosphatidylinositol transfer protein function in murine embryonic stem cells. *Mol. Biol. Cell*. 2002;13(3):739–754.
27. Shadan S, Holic R, Carvou N, et al. Dynamics of lipid transfer by phosphatidylinositol transfer proteins in cells. *Traffic Cph. Den*. 2008;9(10):1743–1756.
28. Baptist M, Panagabko C, Cockcroft S, Atkinson J. Ligand and membrane-binding behavior of the phosphatidylinositol transfer proteins PITPα and PITPβ. Biochem. *Cell Biol. Biochim. Biol. Cell*. 2016;94(6):528–533.

29. Krauss M, Haucke V. Phosphoinositide-metabolizing enzymes at the interface between membrane traffic and cell signalling. *EMBO Rep*. 2007;8(3):241–246.
30. Kular G, Loubtchenkov M, Swigart P, et al. Co-operation of phosphatidylinositol transfer protein with phosphoinositide 3-kinase γ in the formylmethionyl-leucylphenylalanine-dependent production of phosphatidylinositol 3,4,5-trisphosphate in human neutrophils. *Biochem. J*. 1997;325(2):299–301.
31. Fensome A, Cunningham E, Prosser S, et al. ARF and PITP restore GTPγS-stimulated protein secretion from cytosol-depleted HL60 cells by promoting PIP2 synthesis. *Curr. Biol*. 1996;6(6):730–738.
32. Panaretou C, Domin J, Cockcroft S, Waterfield MD. Characterization of p150, an adaptor protein for the human phosphatidylinositol (PtdIns) 3-kinase. Substrate presentation by phosphatidylinositol transfer protein to the p150.Ptdins 3-kinase complex. *J. Biol. Chem*. 1997;272(4):2477–2485.
33. Phillips SE, Sha B, Topalof L, et al. Yeast Sec14p deficient in phosphatidylinositol transfer activity is functional in vivo. *Mol. Cell*. 1999;4(2):187–197.
34. Lomize AL, Pogozheva ID, Lomize MA, Mosberg HI. The role of hydrophobic interactions in positioning of peripheral proteins in membranes. *BMC Struct. Biol*. 2007;7:44.
35. Tilley SJ, Skippen A, Murray-Rust J, et al. Structure-function analysis of human [corrected] phosphatidylinositol transfer protein alpha bound to phosphatidylinositol. *Struct. Lond. Engl*. 1993. 2004;12(2):317–326.
36. Baptist M, Panagabko C, Cockcroft S, Atkinson J. Ligand and membrane-binding behavior of the phosphatidylinositol transfer proteins PITPα and PITPβ. Biochem. *Cell Biol. Biochim. Biol. Cell*. 2016;94(6):528–533.

37. Wang Y, Litvinov RI, Chen X, Bach TL, Lian L, Petrich BG, Monkley SJ, Kanaho Y, Critchley DR, Sasaki T, Birnbaum MJ, Weisel JW, Hartwig J, Abrams CS. *J Clin Invest*. 2008;118(2):812-9.

38. James G.AlbJr. Scott E.Phillips, Lindsey R.Wilfley Benjamin D.Philpot, Vytas A.Bankaitis. The pathologies associated with functional titration of phosphatidylinositol transfer protein α activity in mice. *J Lipid Res*. 2007;48(8):1857-72.

Tables

Table 1. Phenotype differences due to platelet specific deletion of murine PITPα, PITPβ, or both isoforms compared to wild-type controls. N/A indicates that experiments were not performed.

	PITPα-null	**PITPβ-null**	**PITPα/β-null**
Platelet count	Reduced by 15%	Reduced by 15%	Reduced by 40%
Aggregation	Normal	Normal	Impaired
Dense granule secretion (ATP)	Normal	Impaired	More severe
Platelet spreading	Normal	Normal	Impaired
***In vivo* thrombosis**	Normal	N/A	Normal
***In vivo* α-granule secretion (P-selectin)**	Normal	N/A	Normal
Hemostasis: Tail bleeding time	Normal	Normal	150s versus 35s
Tumor metastases	Fewer	Fewer	Markedly fewer
Platelet adhesion to tumor cells	Decreased	Decreased	Decreased

***In vitro* thrombin generation**	Impaired	Impaired	Completely eliminated
PIP response	Normal	Normal	Decreased
IP_3 formation	Decreased	Decreased	More severe

Figure Legends

Figure 1. Platelet-specific loss of both PITPs impairs *ex vivo* platelet aggregation /secretion and spreading. (A) Western blot analysis of PITP expression in human platelet as compared to their mouse counterparts. (B) Western blot based densitometry quantification for individual PITP isoform in human platelet. (C) Schematic representation of the conditional targeting strategy for *Pitpβ*. A 1.89 kb genomic DNA of PITPβ (which includes exon 4-6) was targeted by the insertion of loxP recombination sites. The Neo cassette was removed by crossing with FRT mice before further crossing with *PF4-Cre* transgenic mice. (D) Western blot of platelet lysates demonstrating the specific deletion of the PITPβ isoform in $Pitp\beta^{fl/fl}Pf4\text{-}Cre^{+}$ mice. (E) Complete blood count (CBC) analyses show mild thrombocytopenia in $Pitp\beta^{fl/fl}\ Pf4\text{-}Cre^{+}$ mice and more severe thrombocytopenia in $Pitp\alpha^{fl/fl}/\beta^{fl/fl}Pf4\text{-}Cre^{+}$ mice compared to their respective littermate controls (n = 6 for $Pitp\beta^{fl/fl}Pf4\text{-}Cre^{-}$ mice, n = 8 for $Pitp\beta^{fl/fl}Pf4\text{-}Cre^{+}$ mice, n = 13 for $Pitp\alpha^{fl/fl}/\beta^{fl/fl}Pf4\text{-}Cre^{-}$ mice, n = 9 for $Pitp\alpha^{fl/fl}/\beta^{fl/fl}Pf4\text{-}Cre^{+}$ mice; error bars are standard deviation (s.d.); p-values are shown obtained from unpaired Student's t-test). (F and G) *Ex vivo* analysis of platelet aggregation and dense granule secretion. PITPβ-null platelets aggregate normally, but dense granule secretion was impaired in response to low dose thrombin (0.05 U/mL) and collagen (10 μg/mL) as measured by ATP release (F). Deleting both PITP isoforms increased the severity of aggregation and secretion defects (G). ATP secretion traces start at 100% and trend

down, and aggregation traces start at 0% and trend up. Traces are representative of five separate experiments per condition. (H) Spreading of PITPβ-null and PITPα/β-null platelets on fibrinogen after stimulation with thrombin (0.025 U/mL) revealed that PITPα/β-null platelets had a spreading defect, but PITPβ-null platelets spread normally. (I) PITPα/β-null platelet spreading was quantified as total cumulative area of platelets per field (n = 3 per group). (J) The adherent number of PITPα/β-null platelet was quantified as average number per field under 100x microscope. (n = 5 per group; error bars are s.d.; p-values are shown, unpaired Student's t-test).

Figure 2. Mice lacking platelet PITPs have prolonged tail bleeding time, but have no defects in laser-induced *in vivo* thrombosis.

Laser-induced injury model demonstrates normal *in vivo* thrombosis and platelet secretion in *Pitpα*$^{fl/fl}$/*β*$^{fl/fl}$*Pf4-Cre*$^{+}$ mice. (A) Representative images show platelet accumulation (CD41, red) and P-selectin exposure (green, overlay of red/green is yellow) three minutes post laser-induced injury to cremaster arterioles. Images are binary representations of 2D confocal fluorescence images overlaid on the brightfield. White arrows indicate the direction of flow; scale bar = 10 μm. Shown in (B) are graphs of the CD41-positive area over time (left, mean ± standard error of the mean [SEM]), the median CD41-positive area over time (middle), and CD41 peak area (right, lines are median ± interquartile range). Shown in (C) are graphs of the P-selectin-positive area over time (left, mean ± standard error of the mean [SEM]), the median P-selectin-positive area over time (middle), and P-selectin peak area (right, lines are median ± interquartile range). n = 20 thrombi in four wild-type mice and n = 29 thrombi in four *Pitpα*$^{fl/fl}$/*β*$^{fl/fl}$*Pf4-Cre*+ mice. Statistics were performed using a two-tailed Mann-Whitney test. (D and E) Tail bleeding times in mice lacking PITPβ (D) or both PITP isoforms as compared to their littermate controls (E). Tail bleeding was normal in mice with platelets lacking PITPβ (NS, Mann Whitney test; n = 52

for *Pitpβ*$^{fl/fl}$*Pf4-Cre*$^{-}$ mice, *n* = 58 for *Pitpβ*$^{fl/fl}$*Pf4-Cre*$^{+}$ mice). When both PITP isoforms were deleted in platelets, there was a mild increase in bleeding time (p = 0.0013, two-tailed Mann-Whitney test; *n* = 57 for *Pitpα*$^{fl/fl}$/*β*$^{fl/fl}$*Pf4-Cre*$^{-}$ mice, *n* = 54 for *Pitpα*$^{fl/fl}$/*β*$^{fl/fl}$*Pf4-Cre*$^{+}$ mice).

Figure 3. Platelets lacking PITPβ or both PITP isoforms were less susceptible to tumor metastasis. (A and B) Lungs harvested from *Pitpβ*$^{fl/fl}$*Pf4-Cre*$^{-}$ mice and *Pitpβ*$^{fl/fl}$*Pf4-Cre*$^{+}$ mice (A) or from *Pitpα*$^{fl/fl}$/*β*$^{fl/fl}$*Pf4-Cre*$^{-}$ mice and *Pitpα*$^{fl/fl}$/*β*$^{fl/fl}$*Pf4-Cre*$^{+}$ mice (B) two weeks after tail vein injection with B16F10 melanoma cells demonstrated that loss of PITP impairs metastasis. Shown are representative lungs two weeks after tumor cell injection (top); the number of tumor nodules on the lung surface two weeks after tumor cell injection (center); the wet lung weights three weeks after tumor injection (bottom). For tumor nodule counting, *n* = 21 lungs for *Pitpβ*$^{fl/fl}$*Pf4-Cre*$^{-}$ mice, *n* = 19 for *Pitpβ*$^{fl/fl}$*Pf4-Cre*$^{+}$ mice, *n* = 13 for both *Pitpα*$^{fl/fl}$/*β*$^{fl/fl}$*Pf4-Cre*$^{-}$ mice and *Pitpα*$^{fl/fl}$/*β*$^{fl/fl}$*Pf4-Cre*$^{+}$ mice. For lung weights, *n* = 21 lungs for *Pitpβ*$^{fl/fl}$*Pf4-Cre*$^{-}$ mice, *n* = 20 for *Pitpβ*$^{fl/fl}$*Pf4-Cre*$^{+}$ mice, *n* = 17 for *Pitpα*$^{fl/fl}$/*β*$^{fl/fl}$*Pf4-Cre*$^{-}$ mice, and *n* = 18 for *Pitpα*$^{fl/fl}$/*β*$^{fl/fl}$*Pf4-Cre*$^{+}$ mice. Statistical analysis was performed using an unpaired Student's t-test. Black scale bars are 10 mm. (C and D) *Ex vivo* adhesion of PITPβ-null (C) or PITPα/β-null (D**)** platelets to tissue cultured tumor cell monolayer was impaired compared to wild-type controls. Error bars are s.d., *n* = 3 for each genotype.

Figure 4. Loss of PITP in platelets impairs thrombin generation and Annexin V binding. (**A**-**D**) Representative kinetics of thrombin generation induced by BF610 tumor cells (A and B) or by TF (C and D) in platelet rich plasma (PRP) from *Pitpβ*$^{fl/fl}$*Pf4-Cre*$^{-}$ mice (wild-type control, navy trace), *Pitpβ*$^{fl/fl}$*Pf4-Cre*$^{+}$ mice (PITPβ-null, red trace), and *Pitpα*$^{fl/fl}$/*β*$^{fl/fl}$*Pf4-Cre*$^{+}$ mice (PITPα/β-null, teal trace). The endogenous thrombin potential (ETP) shown is the mean value of total thrombin induced by B16F10 tumor cells (B) or by TF (D) over 90 minutes of reaction time

in PRP contains PITPβ-null platelets (red bars), PITPα/β-null platelets (teal bars) and their wild-type controls (navy bars). (E and F) Platelet poor plasma (PPP) was used as a control to demonstrate the platelet-intrinsic nature of thrombin generation upon stimulation with B16F10 tumor cells (E) or TF (F). n = 3 mice per group. Statistical analysis was performed using an unpaired Student's t-test. Error bars are s.d. (G) Platelets lacking PITPβ and both PITP isoforms have an impaired ability to bind Annexin V after activation by the combination of 5μg/mL collagen and 0.05U/mL thrombin. The mean values are averaged from four independent experiments. Data were analyzed by unpaired Student's t-test. Error bars are s.d.

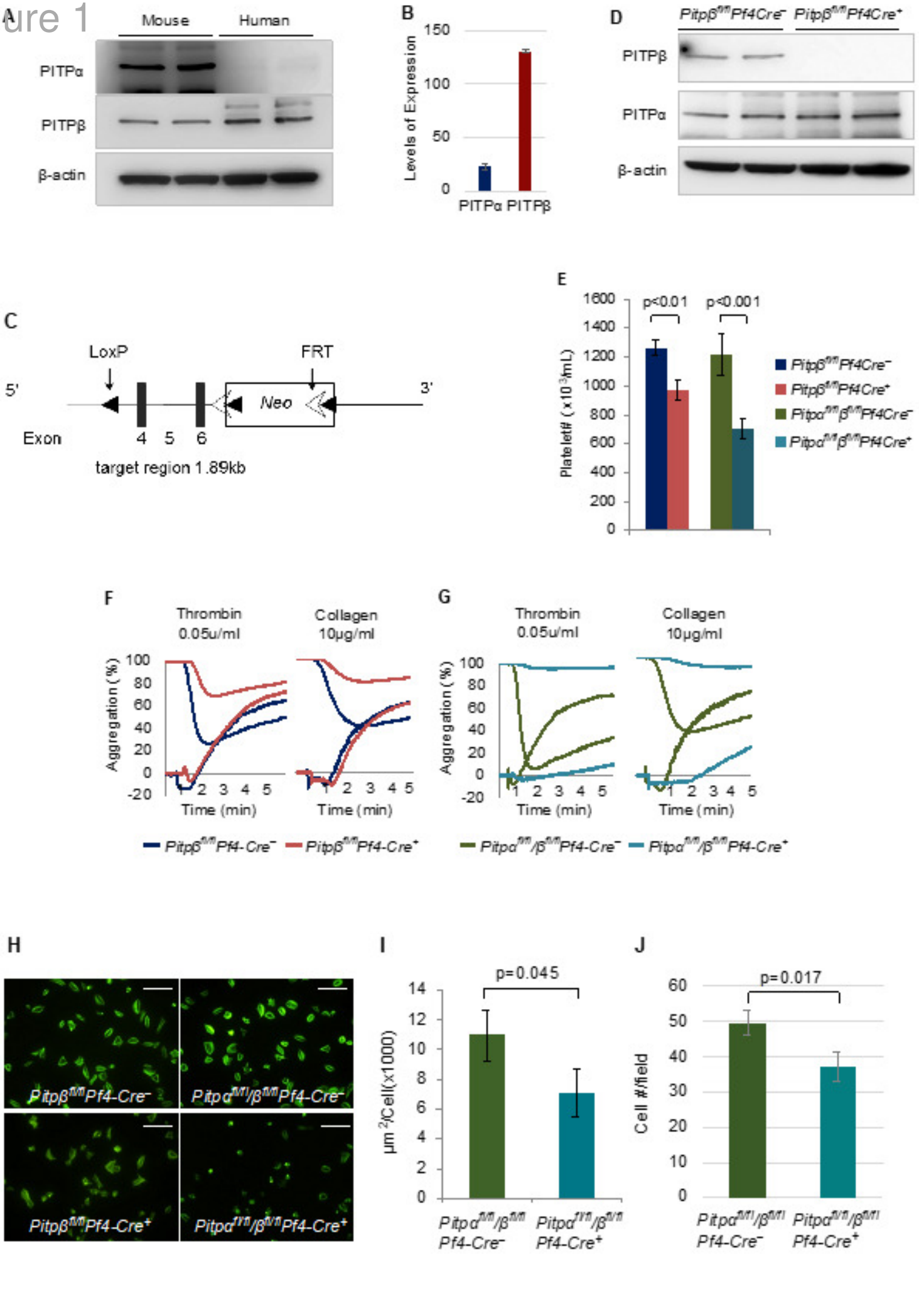
Figure 5. Mass spectrometry analysis on endogenous phosphoinositide and IP_3 production in murine platelets. (A) Endogenous levels of PtdInsP(PIP) in platelets lacking a single isoform of PITPα, PITPβ, and both PITP isoforms. Total PIP response include PtdIns(3)P, PtdIns(4)P and PtdIns(5)P in all fraction species including C38:4, C38:3 and C36:2. (B) PtdInsP2 (PIP2) production in platelets with deletion of either single isoform or deletion of both PITPs. Total PtdInsP2 response include PtdIns(3,4)P2, PtdIns(3,5)P2 and PtdIns(4,5)P2 in all fractions. The assay was repeated three times for each group. The data represented endogenous phosphoinositide levels in 5×10^6 cells and normalized by added known amounts of phosphoinositide as an internal control (mean ± standard deviation). IP_3 production was impaired in thrombin-stimulated (1U/mL for 1 minute) PITPβ-null (C, red trace) platelets and PITPα/β-null (D, teal trace) platelets compared to wild-type littermate controls.

Figure 6. PITPβ does not have transfer activity but has co-factor activity. (A) *In vitro* [^{3}H]-labeled PtdIns transfer activity from microsomes (permeabilized HL60 cells) to liposomes (PC:PI=98:2) is mediated by platelet PITPα, but not PITPβ. (B and C) Lipid kinase assays were

performed to determine the effects of platelet PITPα (left) and platelet PITPβ (right) on PtdInsP synthesis *in vitro* before and after thrombin stimulation (3 min: time of thrombin stimulation [1U/mL]). This assay, which does not require transfer activity, demonstrated that both PITP isoforms were required for phospholipid kinases to generate phosphoinositides. Phosphoinositide production was restored in PITP-null platelets by the addition of recombinant PITP (rPITPα: recombinant human PITPα; rPITPβ: recombinant human PITPβ). (**$p < 0.01$).

Figure 7. Fractioned distribution of PITPα and PITPβ within platelets. (A) Representative immunoblot and (B) densitometry quantification of wild-type platelets indicates that PITPα is mostly distributed in the cytosol, while PITPβ has a disproportionate amount of its total protein in the membrane and cytoskeleton (n = 3 separate experiments) in both resting and thrombin activated platelet (1U/mL for 1 minute). (C) Representative immunoblot of fractioned distribution of PITPα in platelet lacking PITPβ. (D) Densitometry quantification of PITPα fractioned distribution in platelet lacking PITPβ. (E) The distribution of PITPβ in platelet lacking PITPα (bottom). (F) Fractioned distribution of PITPα and PITPβ in resting (left) and thrombin activated (right) human platelet. (G) Densitometry quantification of PITPα and PITPβ in different cellular fractions of resting human platelets. All densitometry data was summed from three separate experiments and the percentage relative to total single PITP isoform was graphed for each fraction. (H) In this model, PITPα serves in its traditional role of transferring PtdIns (PI) from one subcellular compartment to another such as plasma membrane, and PITPβ in turn, serves as a co-factor for PI kinase-mediated PtdInsP (PIP) synthesis.

Figure 1


A
Mouse
Human
PITPα
PITPβ
β-actin
B
Levels of Expression
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100
50
0
PITPα PITPβ
D
Pitpβ^fl/fl Pf4Cre^-
Pitpβ^fl/fl Pf4Cre^+
PITPβ
PITPα
β-actin
C
LoxP
FRT
5'
3'
Neo
Exon
4 5 6
target region 1.89kb
E
p<0.01
p<0.001
Platelet# (x10^3/mL)
1600
1400
1200
1000
800
600
400
200
0
Pitpβ^fl/fl Pf4Cre^-
Pitpβ^fl/fl Pf4Cre^+
Pitpα^fl/fl β^fl/fl Pf4Cre^-
Pitpα^fl/fl β^fl/fl Pf4Cre^+
F
Thrombin 0.05u/ml
Collagen 10μg/ml
Aggregation (%)
Time (min)
Pitpβ^fl/fl Pf4-Cre^-
Pitpβ^fl/fl Pf4-Cre^+
G
Thrombin 0.05u/ml
Collagen 10μg/ml
Aggregation (%)
Time (min)
Pitpα^fl/fl β^fl/fl Pf4-Cre^-
Pitpα^fl/fl β^fl/fl Pf4-Cre^+
H
Pitpβ^fl/fl Pf4-Cre^-
Pitpα^fl/fl β^fl/fl Pf4-Cre^-
Pitpβ^fl/fl Pf4-Cre^+
Pitpα^fl/fl β^fl/fl Pf4-Cre^+
I
p=0.045
μm^2/Cell(x1000)
Pitpα^fl/fl β^fl/fl Pf4-Cre^-
Pitpα^fl/fl β^fl/fl Pf4-Cre^+
J
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Cell #/field
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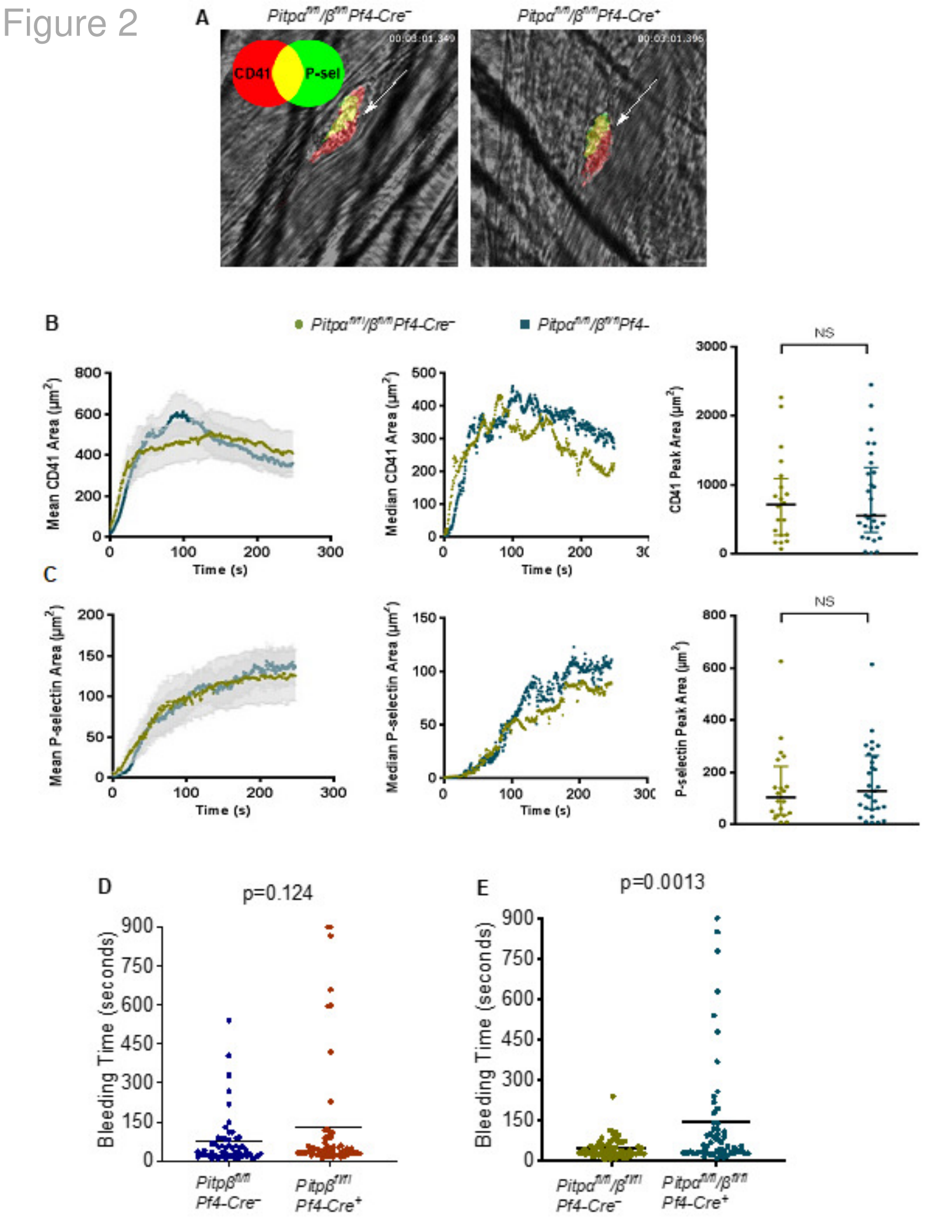
Pitpα^fl/fl β^fl/fl Pf4-Cre^+

Figure 2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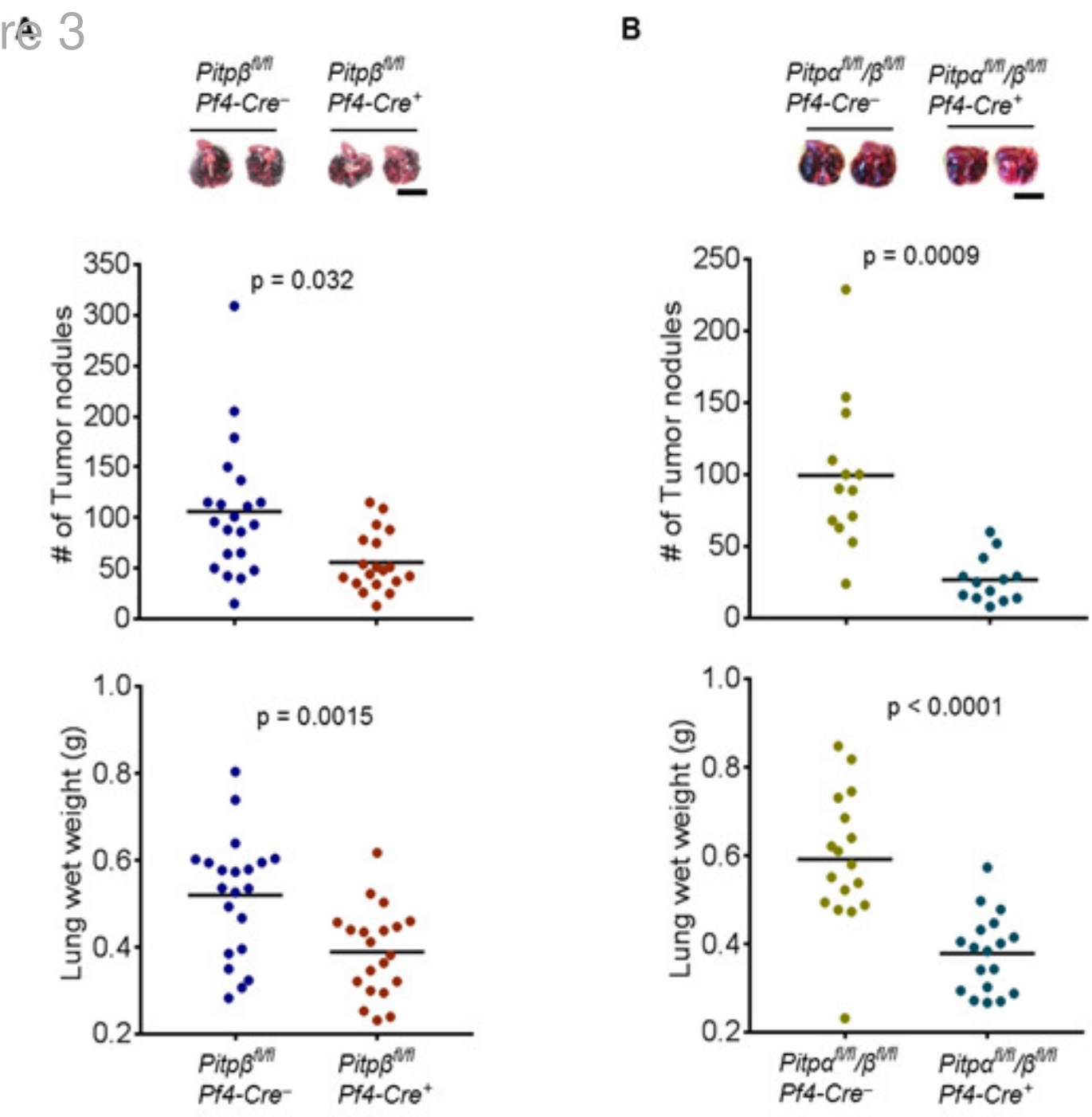
Pitpα fl/fl/β fl/fl Pf4-Cre−
Pitpα fl/fl/β fl/fl Pf4-Cre+
CD41
P-sel
B
Pitpα fl/fl/β fl/fl Pf4-Cre−
Mean CD41 Area (µm²)
Median CD41 Area (µm²)
Time (s)
CD41 Peak Area (µm²)
NS
C
Mean P-selectin Area (µm²)
Median P-selectin Area (µm²)
P-selectin Peak Area (µm²)
D
p=0.124
Bleeding Time (seconds)
Pitpβ fl/fl Pf4-Cre−
Pitpβ fl/fl Pf4-Cre+
E
p=0.0013
Pitpα fl/fl/β fl/fl Pf4-Cre−
Pitpα fl/fl/β fl/fl Pf4-Cre+

Figure 3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Pitpβ fl/fl Pf4-Cre+
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p = 0.0015
B
Pitpα fl/fl/β fl/fl Pf4-Cre−
Pitpα fl/fl/β fl/fl Pf4-Cre+
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Lung wet weight (g)
p < 0.0001

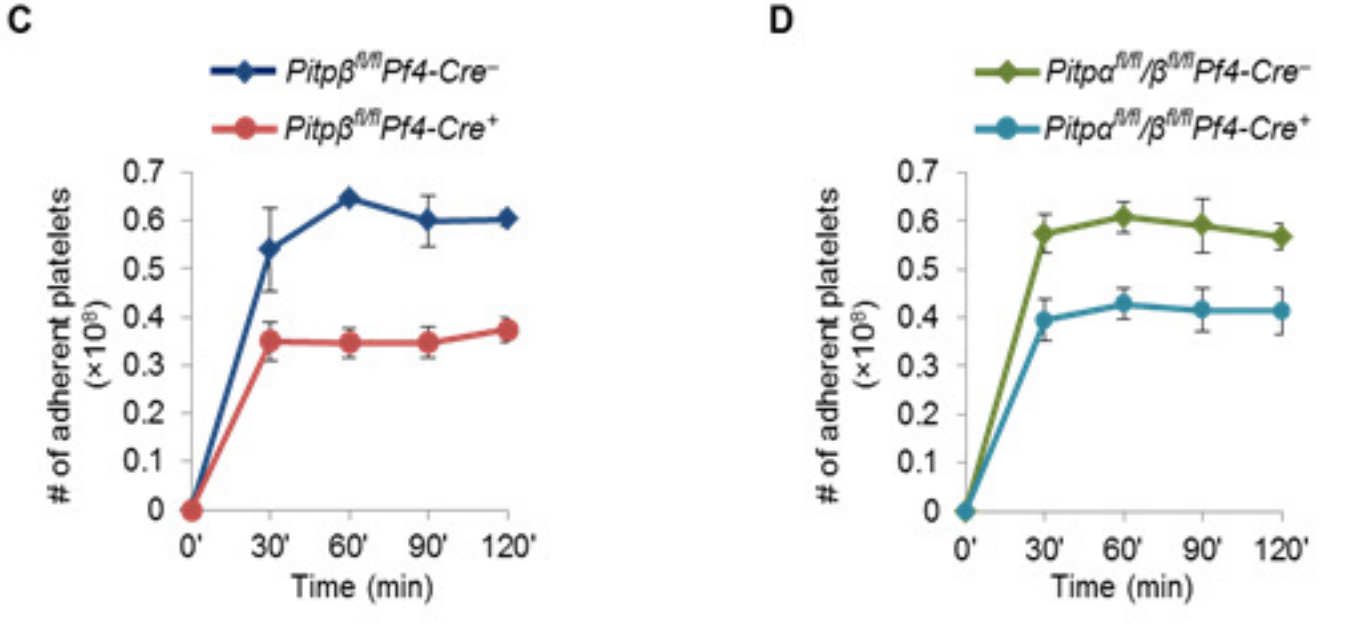

C
Pitpβ fl/fl Pf4-Cre−
Pitpβ fl/fl Pf4-Cre+
# of adherent platelets (×10⁸)
Time (min)
D
Pitpα fl/fl/β fl/fl Pf4-Cre−
Pitpα fl/fl/β fl/fl Pf4-Cre+
# of adherent platelets (×10⁸)
Time (min)

Figure 4

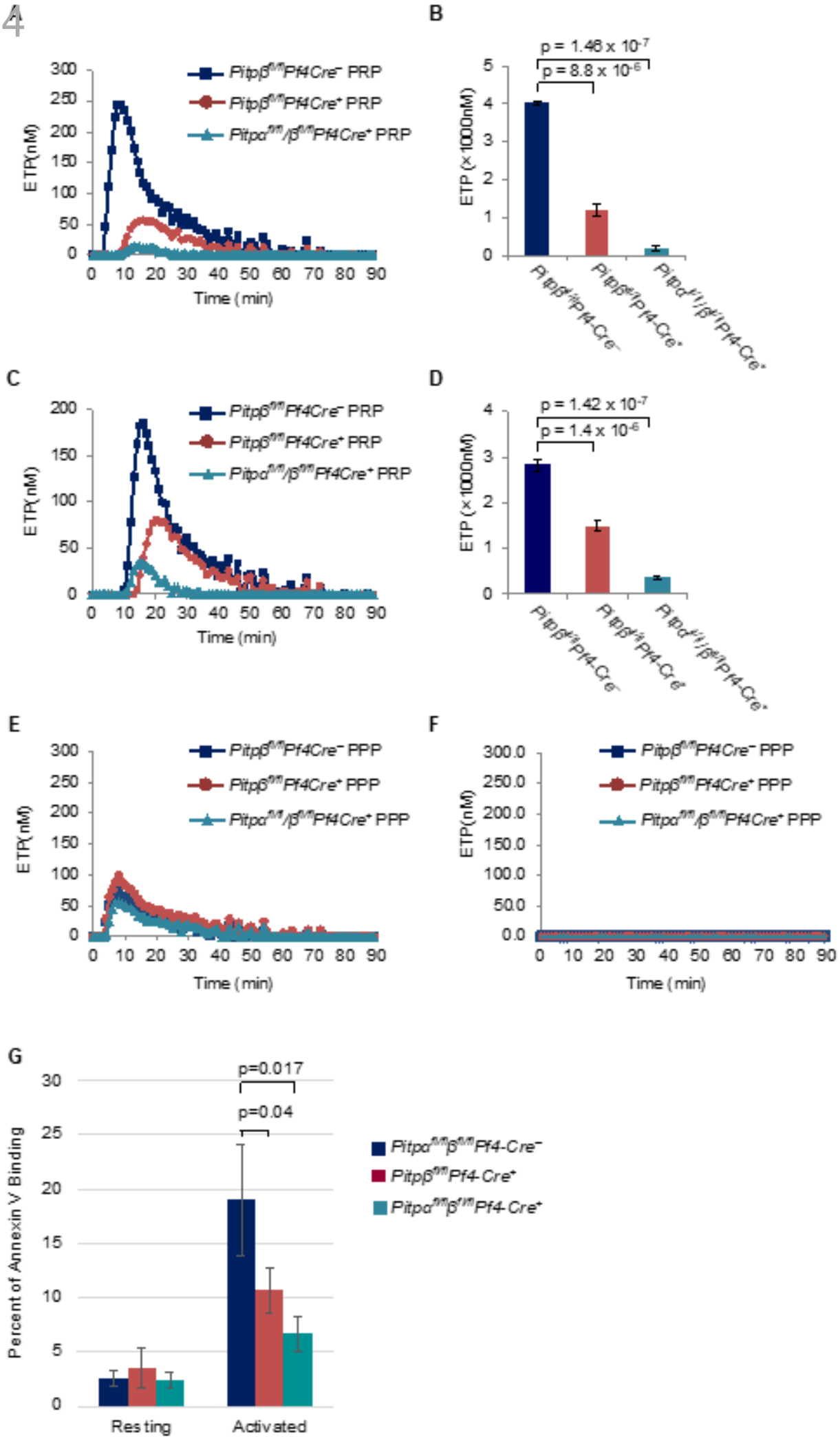

A
B
C
D
E
F
G
ETP(nM)
Time (min)
ETP (×1000nM)
p = 1.46 x 10-7
p = 8.8 x 10-6
p = 1.42 x 10-7
p = 1.4 x 10-6
PRP
PPP
Percent of Annexin V Binding
p=0.017
p=0.04
Resting
Activated

Figure 5

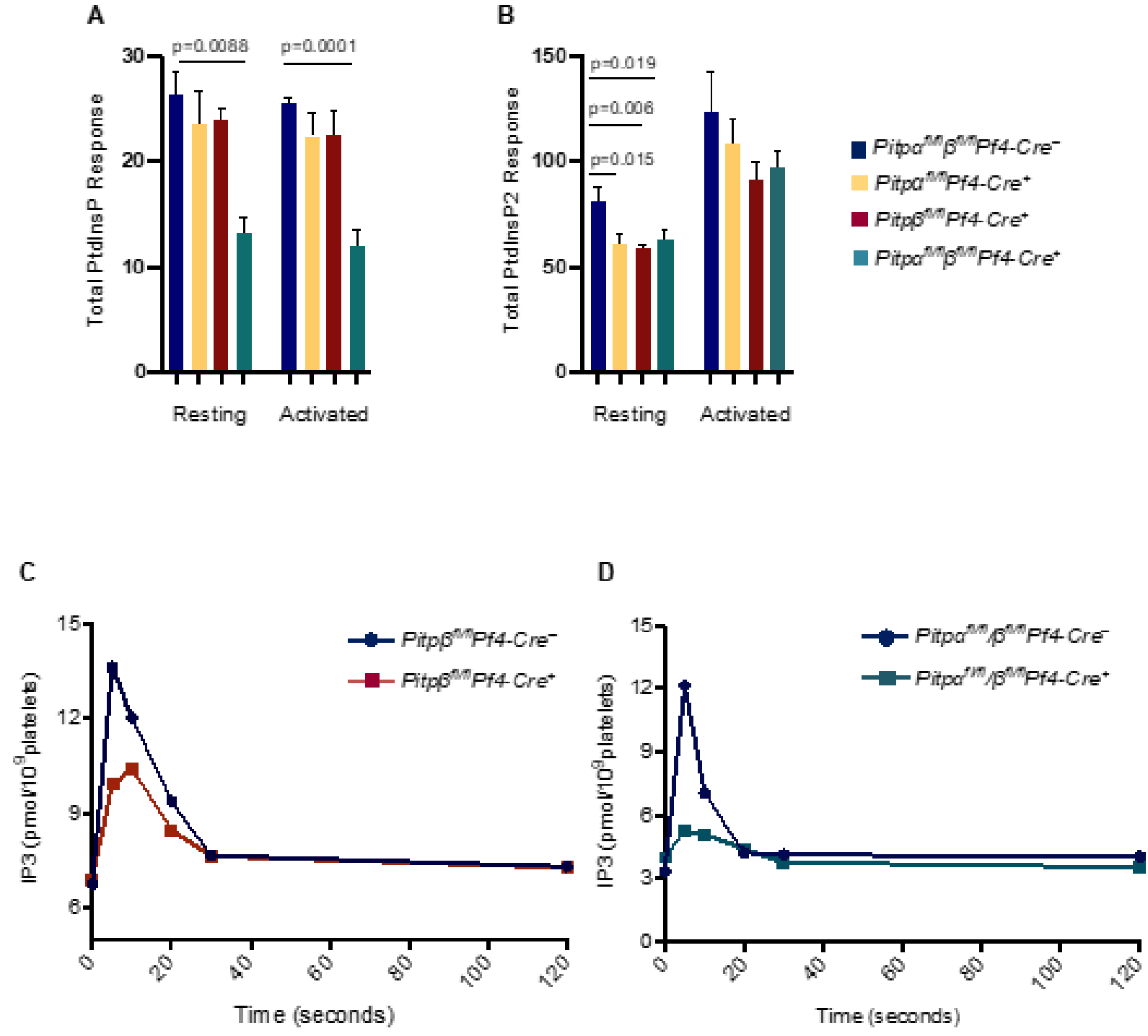
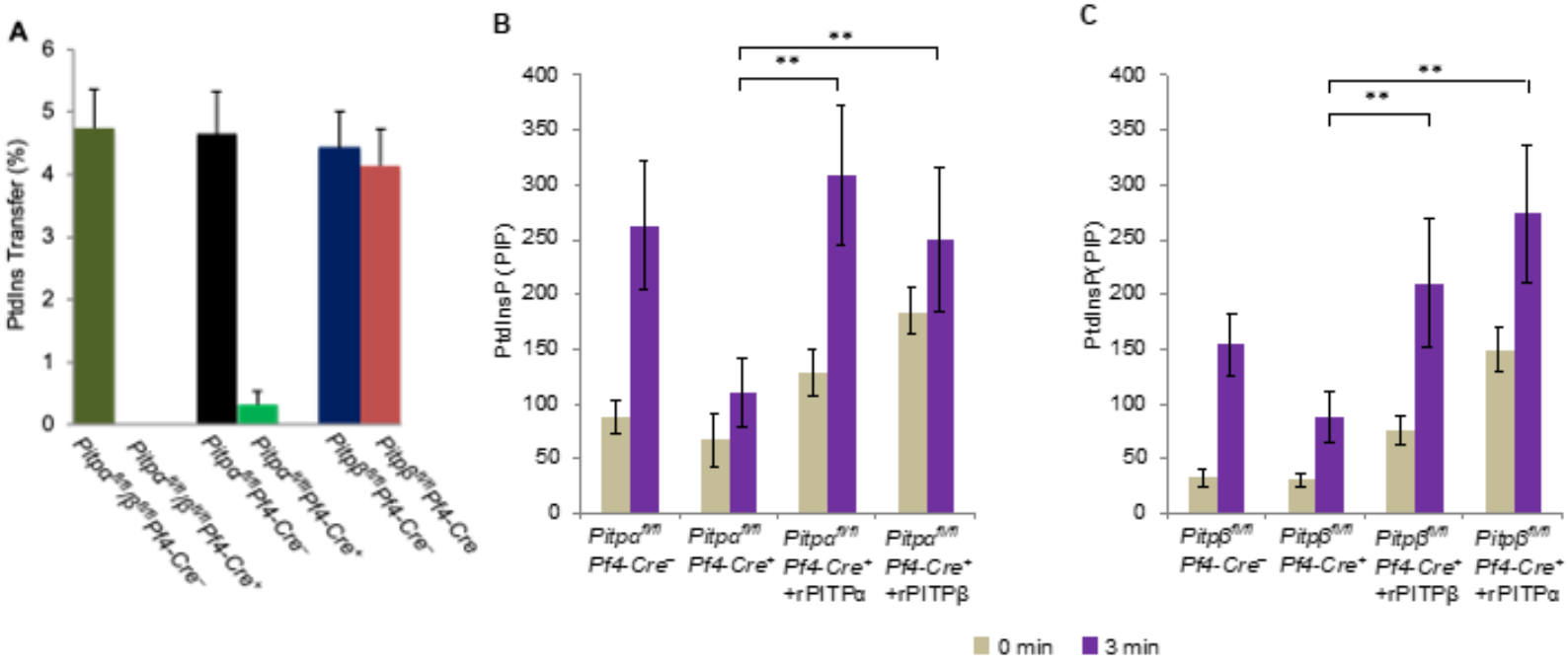
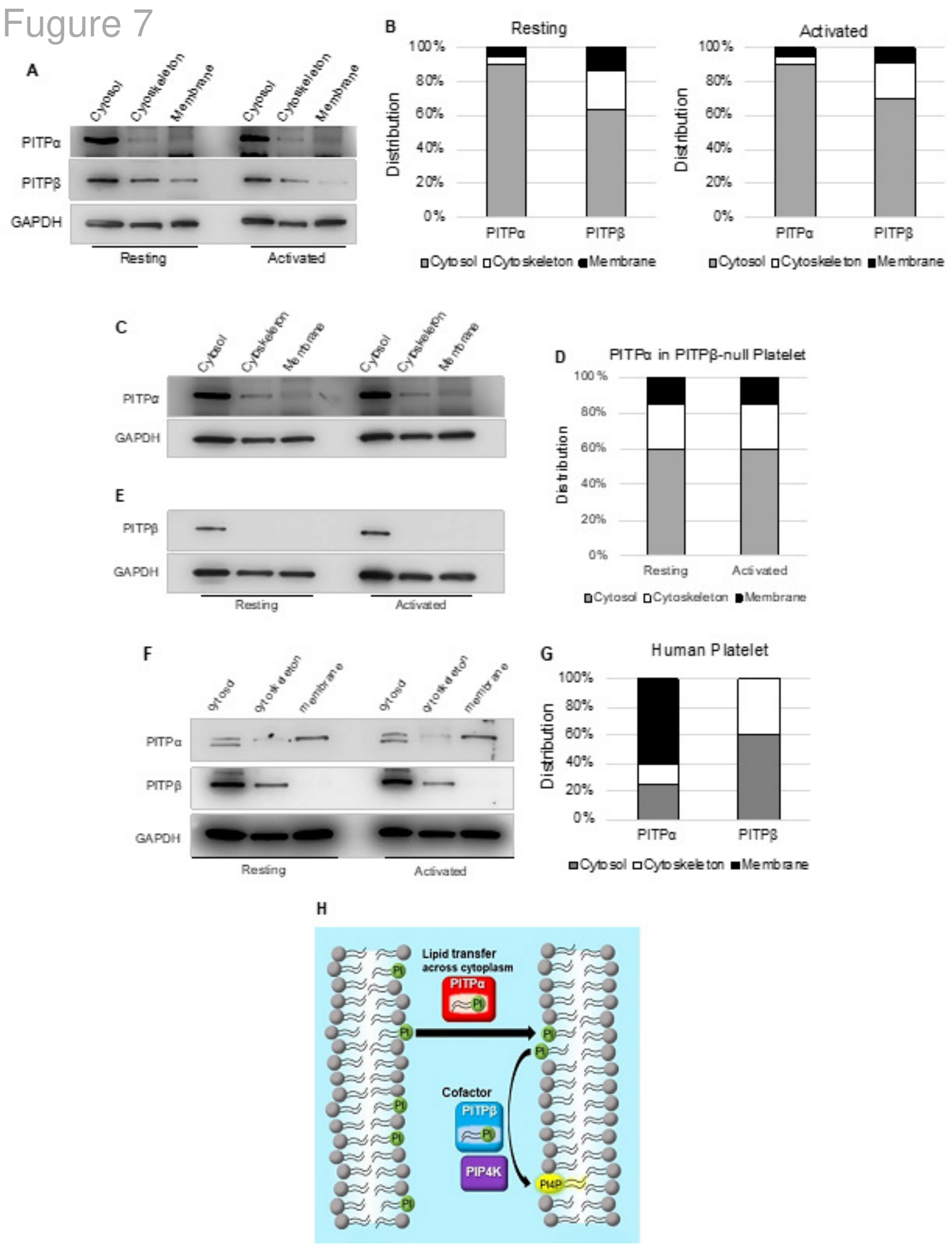

A
p=0.0088
p=0.0001
Total PtdInsP Response
30
20
10
0
Resting
Activated
B
p=0.019
p=0.006
p=0.015
Total PtdInsP2 Response
150
100
50
0
Resting
Activated
Pitpα^fl/fl β^fl/fl Pf4-Cre^-
Pitpα^fl/fl Pf4-Cre^+
Pitpβ^fl/fl Pf4-Cre^+
Pitpα^fl/fl β^fl/fl Pf4-Cre^+
C
IP3 (pmol/10^9 platelets)
15
12
9
6
0
20
40
60
80
100
120
Time (seconds)
Pitpβ^fl/fl Pf4-Cre^-
Pitpβ^fl/fl Pf4-Cre^+
D
IP3 (pmol/10^9 platelets)
15
12
9
6
3
0
20
40
60
80
100
120
Time (seconds)
Pitpα^fl/fl β^fl/fl Pf4-Cre^-
Pitpα^fl/fl β^fl/fl Pf4-Cre^+

Figure 6


A
PtdIns Transfer (%)
0
1
2
3
4
5
6
Pitpα fl/fl β fl/fl Pf4-Cre-
Pitpα fl/fl β fl/fl Pf4-Cre+
Pitpα fl/fl Pf4-Cre-
Pitpα fl/fl Pf4-Cre+
Pitpβ fl/fl Pf4-Cre-
Pitpβ fl/fl Pf4-Cre+
B
PtdInsP (PIP)
0
50
100
150
200
250
300
350
400
**
**
Pitpα fl/fl Pf4-Cre-
Pitpα fl/fl Pf4-Cre+
Pitpα fl/fl Pf4-Cre+ +rPITPα
Pitpα fl/fl Pf4-Cre+ +rPITPβ
C
PtdInsP(PIP)
0
50
100
150
200
250
300
350
400
**
**
Pitpβ fl/fl Pf4-Cre-
Pitpβ fl/fl Pf4-Cre+
Pitpβ fl/fl Pf4-Cre+ +rPITPβ
Pitpβ fl/fl Pf4-Cre+ +rPITPα
0 min
3 min

Fugure 7


A
Cytosol
Cytoskeleton
Membrane
Cytosol
Cytoskeleton
Membrane
PITPα
PITPβ
GAPDH
Resting
Activated
B
Resting
Activated
100%
80%
60%
40%
20%
0%
Distribution
PITPα
PITPβ
Cytosol
Cytoskeleton
Membrane
C
Cytosol
Cytoskeleton
Membrane
Cytosol
Cytoskeleton
Membrane
PITPα
GAPDH
D
PITPα in PITPβ-null Platelet
100%
80%
60%
40%
20%
0%
Distribution
Resting
Activated
Cytosol
Cytoskeleton
Membrane
E
PITPβ
GAPDH
Resting
Activated
F
cytosol
cytoskeleton
membrane
cytosol
cytoskeleton
membrane
PITPα
PITPβ
GAPDH
Resting
Activated
G
Human Platelet
100%
80%
60%
40%
20%
0%
Distribution
PITPα
PITPβ
Cytosol
Cytoskeleton
Membrane
H
Lipid transfer
across cytoplasm
PITPα
PI
PI
PI
PI
PI
PI
PI
PI
Cofactor
PITPβ
PI
PIP4K
PI4P