Signaling Mechanisms of the Glycoprotein Ib-IX-V Complex and Role of LIM kinase 1 in Platelet Activation

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THESIS

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This thesis is dedicated to my parents, Annette Rodriguez and Rafael Antonio Estevez, who provided me with endless encouragement, support, and love that inspired me to complete this work. This thesis is also dedicated to my grandparents, Sixto Luis Estevez, Maria Mercedes Checo-Estevez, Francisco Rodriguez, and Guadalupe Sanabria Rodriguez, for their incredible sacrifices, kindness, and generosity, and for instilling our family with these same values.

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CONTRIBUTION OF AUTHORS

Chapter 1 is a literature review that gives the background for my research. Included are portions of a review written in collaboration with my mentor Dr. Xiaoping Du and colleague Dr. Bo Shen (the figures on pages 36 and 43, and portions of the text on pages 40-41 was originally published in Estevez B, Shen B, Du X. Targeting Integrin and Integrin Signaling in Treating Thrombosis. *Arteriosclerosis, thrombosis, and vascular biology*. 2015;35(1):24-29. doi:10.1161/ATVBAHA.114.303411. Wolters Kluwer Health Lippincott Williams & Wilkins©. No permission letter is needed for reuse (see APPENDIX). Chapter 2 is my rationale and own writing regarding the purpose of my research. Chapter 3 are methods taken from publications described in Chapters 4 and 5 (see below). Chapter 4 represents a published original research manuscript that was written, edited, and conducted in collaboration with my mentor Dr. Xiaoping Du (Figures 7 through 14; text verbatim from pages 89 to 110), which was published in Blood. Estevez B, Stojanovic-Terpo, A, Delaney MK, O'Brien KA, Berndt MC, Ruan C, Du X. LIM kinase-1 selectively promotes glycoprotein Ib-IX–mediated TXA_2 synthesis, platelet activation, and thrombosis. Blood. 2013 May 30; 121(22): 4586–4594. © The American Society of Hematology (See APPENDIX). Chapter 5 also represents previously published work done in collaboration with my mentor Dr. Xiaoping Du who helped plan the research, and write the manuscript (Figures 15-23; text verbatim from pages 111-139), which was originally published in Blood. Estevez B, Kim K., Delaney MK, Stojanovic-Terpo A, Shen B, Ruan C, Cho J, Ruggeri ZM, Du, X. Signaling-mediated cooperativity between glycoprotein Ib-IX and protease-activated receptors in thrombin-induced platelet activation. Blood. 2016 Feb 4;127(5):626-36. doi: 10.1182/blood-2015-04-638387. © The American Society of Hematology. (See APPENDIX).

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LIST OF ABBREVIATIONS

AA	arachidonic acid
ABP	actin-binding protein
AC	adenylyl cyclase
ACD	acid citrate dextrose
ADF	actin depolymerizing factor
Asn	asparagine
Asp	aspartic acid
ATP	adenosine trisphosphate
ATPase	adenosine triphosphatase
Btk	bruton's tyrosine kinase
Ca^{2+}	calcium ion
CalDAG-GEFI	calcium and diacyglycerol-regulated guanine nucleotide exchange factor I
cAMP	cyclic adenosine monophosphate
CHO	Chinese hamster ovary
CIN	chronophin
COX	cyclooxygenase
Cys	cysteine
DAG	diacylglycerol
DMSO	dimethyl sulfoxide
DTS	dense tubular system
EDTA	ethylenediaminetetraacetic acid
ERK	extracellular signal-regulated kinase
FcRγ	Fc receptor gamma
FcγRIIA	fc gamma receptor II A
Fg	fibrinogen
Fn	fibronectin
G protein	guanosine nucleotide-binding protein

LIST OF ABBREVIATIONS (continued)

GAP	GTPase activating protein
GDP	guanosine diphosphate
GEF	guanine nucleotide exchange factor
GP	glycoprotein
GPCR	G protein-coupled receptor
GPIb-IX-V	glycoprotein Ib-IX-V
GPVI	glycoprotein VI
GTP	guanosine triphosphate
HEPES	4-(2-hydroxyethyl) -1-piperazineethanesulfonic acid
His	Histidine
Ig	immunoglobulin
IP_3	inositol 1,4,5-trisphosphate
ITAM	immunoreceptor tyrosine-based activation motif
JNK	jun N-terminal kinase
kDa	kilodaltons
KO	knockout
LAT	linker for activated T cells
LIM	lin-11, isl-1, and mec-3
LIMK	LIM kinase
LRR	leucine-rich repeat
Lys	Lysine
MAPK	mitogen-activated protein kinase
MLC	myosin light chain
MLCK	myosin light chain kinase
NSF	N-ethylmaleimide sensitive fusion protein
OCS	open canalicular system
PAGE	polyacrylamide gel electrophoresis
PAK	p21-activated kinase

LIST OF ABBREVIATIONS (continued)

PAR	protease-activated receptor
PAR4-AP	PAR4 activating peptide
PBS	phosphate-buffered saline
PC	phosphatidylcholine
PCR	polymerase chain reaction
PDZ	post synaptic density protein, drosophila disc large tumor suppressor protein, and zona occludens-1 protein
PF4	platelet factor 4
PG	prostaglandin
PGE_1	prostaglandin E1
PGE_2	prostaglandin E2
PGH_2	prostaglandin H2
PH	pleckstrin homology
PI3K	phosphatidylinositol-3 kinase
PIP_2	phosphatidylinositol 4,5-bisphosphate
PIP_3	phosphatidylinositol 3,4,5-bisphosphate
PKA	cyclic AMP-dependent protein kinase
PKC	cGMP-dependent protein kinase
PKC	protein kinase C
PLA_2	phospholipase A2
PLCβ	phospholipase C beta
$PLC\gamma_2$	phospholipase C gamma2
PMSF	phenylmethylsulfonyl fluoride
PPACK	D-phenylalanyl-prolyl-arginine chloromethyl ketone
PRP	platelet-rich plasma
PS	phosphatidylserine
RGDS	arginine-glycine-aspartic acid-serine
RIAM	rap1-GTP-interacting adaptor molecule

LIST OF ABBREVIATIONS (continued)

ROCK	Rho-associated kinase
SDS	sodium dodecyl sulfate
Ser	serine
SFK	src family kinase
SLP-76	src homology 2 domain containing leukocyte phosphoprotein of 76 kDa
SNAP	soluble NSF-attachment protein
SNARE	soluble NSF-attachment protein receptor
SOCE	store-operated calcium entry
SSH	slingshot
STIM1	stromal interaction molecule 1
Thr	threonine
TP	thromboxane prostanoid
tSNARE	target soluble NSF-attachment protein receptor
TXA_2	thromboxane A2
TXB_2	thromboxane B2
Tyr	tyrosine
VAMP	vesicle-associated membrane protein
vSNARE	vesicle soluble NSF-attachment protein receptor
VWF	von willebrand factor
WT	wildtype

SUMMARY

Upon blood vessel wall damage circulating platelets rapidly adhere and secrete their granule contents, which elicits activation and recruitment of other platelets that together will form aggregates to seal the lesion. This process is known as primary hemostasis and it is critical in preventing life-threatening bleeding. Platelets are essential for hemostasis as demonstrated by the excessive bleeding observed in patients with any of the various acquired or inherited thrombocytopenia. A critical receptor for initial platelet adhesion to the damaged endothelial wall, wherein adhesive matrix proteins are exposed, is the platelet glycoprotein Ib-IX-V receptor complex (GPIb-IX-V) that binds to the adhesive matrix protein von willebrand factor (VWF). VWF binding to GPIb-IX-V elicits an intracellular signaling cascade that leads to platelet activation. Currently, the most proximal step in VWF/GPIb-IX-V signaling is the activation of Src family kinase (SFK) Lyn. Lyn then activates the small RhoGTPase Rac1 via phosphorylation of guanine nucleotide exchange factor (GEF) Vav. Rac1 activates the PI3K/Akt pathway which leads to activation of the cGMP/PKG and MAPK pathways. The Rac1 and MAPK pathways have been shown to play an important role in promoting thromboxane A2 (TXA_2) production, an event which is particularly important for amplification of platelet activation. However, the mechanism by which VWF/GPIb-IX-V signaling regulates TXA_2 production is unclear.

SUMMARY (continued)

We tested the hypothesis that LIMK1, a serine/threonine kinase, is involved in GPIb-IX-V-mediated signaling leading to platelet activation. We found that LIMK1 plays a stimulatory role in promoting platelet response via VWF/GPIb-IX-V signaling. Interestingly, we also found that LIMK1 negatively regulates other GPIb-IX independent platelet activation pathways. In addition, we discovered that LIMK1 null platelets displayed an absence of TXA_2 production in response to VWF stimulation, suggesting that LIMK1 promotes VWF-induced TXA_2 production. In contrast, we determined that LIMK1 does not play a role in other TXA_2 generating pathways. Thus, the stimulatory role of LIMK1 is selective for VWF/GPIb-IX-V-mediated TXA_2 production. LIMK1 null platelets displayed partially defective stable adhesion to immobilized VWF under shear conditions, similar to aspirin-treated controls, which could be rescued by the addition of the TXA_2 analogue U46619. Stimulation of isolated human or mouse platelets with VWF induced LIMK1 activation and phosphorylation of its substrate cofilin. Deletion of LIMK1 in mouse platelets diminished cofilin and $cPLA_2$ phosphorylation, a rate limiting enzyme in TXA_2 production. Remarkably, the phenotype of LIMK1 null mice on in vivo hemostasis and thrombosis did not mimic previous reports of the effect of aspirin-treatment on these assays in mice. Previously others have shown that aspirin-treated or TP knockout mice display defective thrombosis and hemostasis in vivo, in contrast, LIMK1 null mice were only defective in thrombosis. Taken together, our data show that LIMK1 selectively promotes VWF/GPIb-IX-V mediated TXA_2 production leading to platelet activation and thrombosis.

Another major ligand for the GPIb-IX-V complex is the coagulation protease thrombin. Thrombin binding to GPIb-IX-V has been shown to promote platelet response to low concentrations of thrombin. Early investigators demonstrated the contribution of

GPIb-IX-V to thrombin-dependent platelet response using antibodies directed against GPIbα or by pretreating with a trypsin-generated fragment of the extracellular domain of GPIbα, which would inhibit thrombin binding and platelet response to thrombin. The lack of response to low concentrations of thrombin was also observed in Bernard-Soulier syndrome patients' platelets, which lack or have abnormal expression of the GPIb-IX-V complex. Later others would localize the thrombin binding site to an anionic region on extracellular domain of GPIbα that contained three sulfated tyrosines. Mutations, antibodies, or peptides designed to target this site abolished the binding of thrombin to GPIbα. In addition, the absence of any aggregation or calcium mobilization response to thrombin stimulation in platelets isolated from protease-activated receptor 4 (PAR4) knockout mice suggested that PARs were required for platelet response to thrombin. Nonetheless, other researchers reported that platelets could respond to catalytically inactive thrombin, suggesting the existence of PAR-independent platelet activation mechanisms. Thus, how the GPIb-IX-V complex promotes platelet response to thrombin remains unclear.

The current model is that GPIb-IX-V functions as a dock for thrombin to cleave and activate PARs. However, this model does not take into account evidence for the ability of catalytically inactive thrombin to activate platelets. Our hypothesis was that GPIb-IX functions as a thrombin receptor and transduces intracellular signals independent from PARs. To clearly dissect the contribution of GPIb-IX, we reconstituted platelet thrombin receptor signaling in Chinese hamster ovary (CHO) cells that express endogenous PAR1. Utilizing CHO cells stably expressing wildtype human GPIb-IX

receptor complex, thrombin binding mutant GPIb-IX, and GPIb-IX with a cytoplasmic C-terminal deletion of a critical 14-3-3 binding site, we investigated the role of GPIb-IX in thrombin-induced calcium mobilization response. We found that cells expressing WT GPIb-IX complex (i.e., in the absence of GPV) displayed enhanced response to thrombin stimulation, which could be abolished by mutation of a critical thrombin binding site. Surprisingly, CHO cells expressing the 14-3-3 binding mutant GPIb-IX complex also displayed abolished response to thrombin stimulation. To further investigate the role of 14-3-3-GPIb-IX interaction in platelets, we tested the effect of cell-permeable peptide inhibitor, MPαC, on several parameters of thrombin-induced platelet activation. We discovered that only at low concentrations of thrombin were the platelet activation responses inhibited. By contrast to a previous study by our lab, wherein MPαC inhibited VWF binding to platelets, pretreatment with MPαC did not affect thrombin binding to platelets, indicating that GPIb-IX-14-3-3 was not required for ligand binding to GPIb-IX. We hypothesized that thrombin signaling was similar to VWF signaling wherein Rac1 and LIMK1 play important roles. With that premise, we determined that Rac1 and LIMK1 were also important for transducing thrombin-induced GPIb-IX dependent signaling at low concentrations of thrombin. We then investigated the contribution of PAR signaling under conditions where we observed GPIb-IX-dependent responses to thrombin (i.e., at low concentrations of thrombin). We found that PAR activation is required for GPIb-IX-dependent response to thrombin using GPIb-IX-expressing cells or

MPαC-pretreated platelets. Antagonism of PAR1 with the PAR1-selective antagonist, SCH79797, abolished the stimulatory effect of GPIb-IX expression in cells. We also investigated whether we could detect GPIb-IX-dependent response to catalytically inactive thrombin.

Surprisingly, we found that a widely used tool, S195A mutant thrombin, displayed residual catalytic activity. To further inactivate this reagent we used PPACK. After PPACK-treatment of S195A thrombin we abolished all activity of S195A mutant thrombin on platelets and cells, and this inactivation was verified using a fluorescent thrombin substrate assay. To determine how GPIb-IX cooperated with PARs to promote thrombin response we pretreated cells or platelets with PAR-antagonists and measured activation of LIMK1. We found that in the presence of PAR-antagonists LIMK1 activation was abolished. However, at low concentrations GPIb-IX was also required for LIMK1 activation, and we found that inhibition of 14-3-3-GPIb interaction also abolished LIMK1 activation. These data indicate that at low concentrations of thrombin both GPIb-IX and PAR are required to transduce optimal levels of signaling leading to platelet activation. We then investigated whether low concentrations of thrombin were generated in vivo using a mouse cremaster laser injury model. Using an anti-thrombin monoclonal antibody conjugated to Alexa Fluor 488 we detected in vivo thrombin generation after laser injury. To approximate the concentration of thrombin in vivo, we performed a standard curve using known concentrations of thrombin and compared these data to those obtained in vivo. We discovered that approximately 4nM of thrombin was generated around 260 seconds after laser injury.

SUMMARY (continued)

Taken together, our results show that GPIb-IX cooperates with PARs via a GPIb-IX-dependent 14-3-3/Rac1/LIMK1 signaling pathway at low concentrations of thrombin, which are important in vivo.

1. LITERATURE REVIEW

1.1 Platelets

Platelets are small anucleate blood cells that patrol the bloodstream and use their highly specialized adhesive properties to sense and respond to vascular abnormalities; upon blood vessel wall damage, they rapidly adhere to exposed matrix proteins, and this initial adhesion elicits intracellular signaling that recruits additional platelets which then leads to the formation of hemostatic thrombi that seal the wound. In mammals, platelets originate from fragmented cytoplasmic pseudopod extensions released from large polyploid precursor hematopoietic cells called megakaryocytes which reside primarily in the bone marrow. The hemostatic function of platelets is evolutionarily conserved in other vertebrates, such as fish, amphibians, and birds, whose functional analog of the mammalian platelet contains a nucleus and is called a thrombocyte. Human platelets circulate at concentrations which vary between individuals from $150x10^9$ to $400x10^9$ per liter(Giles 1981), and they circulate in the blood for 7-10 days (Aas and Gardner 1958). Human platelets range in size from 1.5-3 microns in diameter and their mean volume varies from 7-10.5 femtoliters (Weiss 1975, Frojmovic and Milton 1982).

1.1.1 Platelet Morphology and Structure

The morphological features of platelets, such as their small size, resting discoid shape, and dramatic shape change upon blood clotting, assisted early investigators, which used light microscopy on living and excised blood vessels, in distinguishing them from red and white

blood cells. Based on these morphological characteristics they were able to identify platelets as normal constituents of the bloodstream. In 1865, Max Schultze described numerous colorless little ‘granular masses’ or ‘spherules’ and many clumps thereof in normal human blood; moreover, he found these ‘granules’ displayed membrane protrusions and were associated with fibrous material after the blood had clotted (Schultze 1865). In 1881, platelets and their role in thrombosis were more definitively identified and characterized by Giulio Bizzozero (Bizzozero 1881). Bizzozero noted the disc-like initial appearance of platelets prior to vessel injury and, using microscopy on arterial blood vessels damaged by a needle, he observed platelet adhesion to the damaged vessel wall, platelet aggregates, and the formation of large platelet-rich thrombi. Bizzozero called these unique blood cells *petite plaques* or small plates, and this term was later modified to the English word platelets (Brewer 2006).

Based on investigations using electron microscopy (EM), the platelet anatomy can be divided into three distinct regions: the ‘peripheral zone’, composed of the glycocalyx, surface membranes and surface-associated membrane structures; the ‘sol-gel zone’, composed primarily of the cytoskeleton and its associated proteins; and the ‘organelle zone’ which contains granules, mitochondria, a dense tubular channel system and glycogen (White and Gerrard 1976).

1.1.1.1 <u>Surface Membrane Systems and Glycocalyx</u>

The plasma membrane lipid bilayer functions to regulate the passage of molecules into the cytosol as well as to sense extracellular signals and elicit intracellular signaling in response to changes in the environment. The plasma membrane of platelets consists of a bilayer of phospholipids, transmembrane and membrane-associated proteins, and carbohydrates similar

to plasma membranes in all other cells (Edidin 2003, Barber and Jamieson 1970, Robertson 1981). Also similar to other cells, the platelet membrane displays asymmetry in the composition of its plasma membrane phospholipids(Bevers, Comfurius, and Zwaal 1983). The outer leaflet contains primarily phosphatidylcholine and sphingomyelin whereas the inner leaflet contains primarily phosphatidylserine, phosphatidylethanolamine, and phosphoinositides (Lhermusier, Chap, and Payrastre 2011). In platelets, phosphatidylserine (PS) is exposed on the outer leaflet upon platelet activation. The exposure of PS promotes the binding and activity of coagulation factors which facilitates blood coagulation (Bevers et al. 1982).

When visualized by transmission electron microscopy (EM), a 15-20 nanometer thick amorphous fuzzy coating, called the glycocalyx, is observed on the outer surface of the platelet plasma membrane. The glycocalx is composed of mucopolysaccharides, glycolipids and glycoproteins (Behnke 1968b, a, 1969). Glycocalyx coated plasma membranes are also found on many other cell types, such as endothelial cells, fibroblasts, leukocytes, and erythrocytes (Reitsma et al. 2007, Rambourg and Leblond 1967) . A major constituent of the platelet glycocalyx is the platelet adhesion receptor glycoprotein Ibα (discussed further below, see section 1.2.1) which contains sialic acid groups that account for 10% of platelet sialic acid (Okumura, Lombart, and Jamieson 1976a, Okumura and Jamieson 1976b). Moreover, several other acidic polysaccharides and glycoproteins exist on the platelet surface resulting in a net negative charge on the outer platelet surface membrane (Gröttum and Solum 1969, Jamieson and Pepper 1971).

Freeze-fracture and scanning electron microscopy techniques show numerous invaginations of the platelet membrane towards the platelet interior that appear as “pits” on the

resting platelet surface (Allen et al. 1979, Zucker-Franklin 1981, White and Gerrard 1976). These “pits” are actually channels continuous with, cytochemically and morphologically identical to, the plasma membrane and are known as the surface-connected canalicular system or open canalicular system. Therefore, the open canalicular system is an inward extension of the platelet plasma membrane as shown by their shared coating of glycocalyx which is not found on other intracellular platelet membranes (White and Clawson 1980, Behnke 1968b, Behnke 1967, White and Gerrard 1976); moreover, these tunnels of platelet surface membrane form labyrinth-like networks throughout the interior of resting platelets (White and Clawson 1980). The open canalicular system (OCS) provides a common pathway for two-way transport of intra- and extra-platelet substances: extracellular substances can enter into platelets and, at the same time, intracellular granules can fuse with the OCS during secretion (Escolar and White 1991). In addition, the OCS provides a reserve of surface membrane during platelet spreading (Escolar, Leistikow, and White 1989).

The platelet cytoplasm also contains several elongated membrane enclosed channels known as the dense tubular system (DTS). The platelet DTS is suggested to be the residual smooth endoplasmic reticulum left over from megakaryocytes (Daimon and Gotoh 1982, Ebbeling et al. 1992, White and Gerrard 1976). The DTS is the major calcium storage compartment responsible for sequestering and maintaining homeostatic intracellular calcium levels in platelets, and is similar to the sarcoplasmic reticulum of muscle cells. Upon platelet activation the DTS releases the stored Ca^{2+} into the cytosol. The DTS membranes are also believed to be the site of prostaglandin biosynthesis (Gerrard et al. 1976).

1.1.1.2 Cytoskeleton and Membrane Skeleton

Primarily based on morphological studies of cell motility and mitosis in living cells, early investigators hypothesized the existence of a 'viscoelastic' cytoplasmic apparatus that appeared to impart motile and dividing cells with rigidity, contractile function, and highly regulated organelle localization; they called the supposed cytoplasmic network controlling these transformations in cellular morphology the 'cytoskeleton' (Zampieri, Coen, and Gabbiani 2014). The filamentous structure of the cytoskeleton and its components were later directly observed and characterized in numerous cell types, including platelets, using biochemical methods in combination with polarized light microscopy, fluorescence microscopy and EM (Brøndsted and Carlsen 1951, Lazarides and Weber 1974, Inoue 2008). It is now widely accepted that a cytoskeleton is present in all eukaryotes and prokaryotes (Wickstead and Gull 2011, Jekely 2014). In addition, the localization and activity of cytoskeletal filaments is not homogenous within cells; these properties are tightly regulated by specific cytoskeletal accessory proteins which crosslink and/or modulate their polymerization both in basal conditions and in response to chemical and mechanical stimuli (Fletcher and Mullins 2010, Bezanilla et al. 2015, Pollard and Cooper 2009). Consistent with other cells the platelet cytoskeleton is also dynamic and tightly regulated.

The platelet cytoskeleton is composed of polymers of actin and tubulin that form three distinct structures: a membrane-associated actin filamental structure called the membrane skeleton; a network of cytoplasmic actin filaments; and a microtubule coil that wraps several times around the periphery of the platelet. The platelet cytoskeleton regulates platelet shape, surface membrane stability, granule secretion, and spatially organizes and integrates intracellular signaling events. Importantly, cytoskeleton dependent processes, such as cell

shape changes and maintenance of membrane stability, are critical for platelets to carry out their normal functions. Maintenance of the discoid shape of circulating platelets and stability of the platelet membrane in the face of high shear forces, due to the blood flow on platelets near the endothelial wall, are properties regulated by the cytoskeleton. Similarly, transformations in membrane morphology, during agonist-induced platelet activation and when spreading on immobilized ligands, are products of the underlying structure and activity of the intracellular cytoskeleton and its associated proteins. Under normal conditions circulating platelets maintain a biconvex disc-shaped appearance and have a relatively smooth surface. In contrast, when exposed to agonist(s) and/or to subendothelial matrix proteins platelets will transition through a sequence of predictable and stereotypical morphological changes. They first change shape from discoid to spherical, and then project several spiny membrane protrusions, called filopodia. Platelets allowed to adhere to matrix proteins or foreign substances, such as glass, will first project several filopodia and then spread out into thin, often ellipsoid or circular, films displaying characteristic surface membrane ruffles called lamellipodia, and later they will retract these membrane extensions (Allen et al. 1979).

The membrane skeleton coats the cytoplasmic face of the inner leaflet of the plasma membrane; using EM on detergent permeabilized rapidly frozen and dried metal coated platelet membranes, it appears as a fishnet-like structure that is composed of actin filaments and actin filament crosslinking proteins (Hartwig and DeSisto 1991). The thread-like appearance of the membrane skeleton lining the cytoplasmic side of the bilayer is primarily due to the presence of a 240 kilodalton protein called spectrin that forms anti-parallel $\alpha\beta$ dimers that then associate to form heterotetramers (Hartwig et al. 1999, Bearer 2005, Fox 2001). There are several spectrin isoforms expressed in platelets, but the non-erythroid α2-

and β2-spectrins appear to be predominantly expressed (Patel-Hett et al. 2011, Fox et al. 1987, Fox 2001). Spectrin tetramers, which exist in the same plane as the plasma membrane, bind to the barbed ends of actin filaments that are extending from the inner cytosol towards membrane. Thus, spectrin tetramers interconnect via intervening actin filaments to form repeating units (i.e., spectrin-actin filament-spectrin-actin filament) throughout the inner face of the plasma membrane. The functional importance of this assembly of membrane-associated filaments was shown using lentiviral vector mediated expression of an α2 spectrin chain peptide in megakaryocytes, designed to prevent the formation of spectrin αβ tetramers, which blocked the development of internal membrane systems, proplatelet production, and fission of proplatelets, suggesting a role for spectrin in thrombopoiesis (Patel-Hett et al. 2011). Various other signaling, cytoskeletal and transmembrane proteins are also present in the membrane skeleton such as myosin, adducin, talin, moesin, GPIb-IX-V, and αIIbβ3 (Bearer 2005, Fox 2001).

A major membrane skeleton protein important for maintaining normal platelet morphology and membrane stability is an intracellular F-actin binding protein filamin. Filamin crosslinks actin filaments, but it also binds to membrane proteins and thereby links the actin cytoskeleton to the lipid bilayer. Filamin is a 280 kilodalton (kDa) cytoplasmic protein(also known as actin binding protein 280, ABP-280) which functions as a homodimer. In platelets, filamin binds to the cytoplasmic domain on the α-chain of glycoprotein Ib of the membrane receptor complex GPIb-IX-V (Okita et al. 1985, Hartwig 2013, Fox 1985). Filamin A is the dominant isoform expressed in platelets, but Filamin B is also present at low levels (Takafuta et al. 1998); however, Filamin B is not sufficient to compensate for loss of Filamin A function in megakaryocytes or platelets (Falet et al. 2010, Jurak Begonja et al. 2011). The importance of

the membrane skeleton in platelet morphology, membrane stability and function is exemplified by studies disrupting the linkage between GPIbα and filamin. Deletion of Filamin A in the megakaryocyte lineage results in the formation of large and fragile platelets that appear to be rapidly cleared from the circulation. Filamin A knockout (Filamin $A^{-/-}$) platelets display defective GPIb-IX-V complex surface expression, enhanced GPIbα proteolysis, and loss of GPIbα association with the cytoskeleton (Jurak Begonja et al. 2011). Filamin $A^{-/-}$ mice phenocopy the macrothrombocytopenia observed in GPIb$\alpha^{-/-}$ mice (Ware, Russell, and Ruggeri 2000), and expression of the transmembrane and cytoplasmic domains of human GPIbα is sufficient to partially rescue the platelets size and platelet count defects in GPIbα null mice (Kanaji, Russell, and Ware 2002), which supports an important role for the GPIbα-membrane skeleton interaction in regulating platelet morphology and platelet production (Hartwig 2013). Platelets from Filamin $A^{-/-}$ mice also spontaneously form microvesicles after prolonged incubation at 37°C, which is consistent with evidence from transgenic mice expressing a filamin-binding defective mutant form of human GPIbα whose platelets display abnormal membrane tether formation, defective adhesion, and that disintegrate into microvesicles when exposed to high shear conditions (Cranmer et al. 2011).

Severing of preexisting actin filaments, and subsequent reorganization and polymerization of actin, the core building block of actin filaments, are critical for changes in platelet shape. Actin is an abundantly expressed protein in platelets. Actin is approximately 42 kDa. Actin exists in two forms: monomeric globular actin (G-actin) and filamentous actin (F-actin), which is made up of non-covalently bound ATP- and ADP-actin monomers. Approximately, 40 percent of the actin in platelets is in the filamentous form and upon thrombin stimulation this increases to about 70-80 percent. The change in the amount of resting versus

activated platelet F-actin is highly regulated, in a spatial, temporal and agonist-dependent manner, by several types of actin binding proteins. Moreover, actin binding proteins interact with distinct sites on F-actin: some bind to the sides of F-actin, others bind the barbed end, and still others bind the pointed end of F-actin. Depending on the availability of free actin monomers and the type of actin binding proteins, these interactions have distinct outcomes such as to stabilize, to promote or to inhibit actin polymerization. In addition, actin binding proteins may promote a combination of outcomes such as reorganization and polymerization (Hartwig 2013, Fox 2001, Bearer 2005).

Encircling the long axis of resting platelets is a microtubule coil, which is responsible for the discoid shape of circulating platelets. This microtubule coil is found just below the inner surface of the platelet plasma membrane and it winds around the periphery of the cytoplasm about 8-12 times. The platelet microtubule coil appears as a thick rope-like structure when observed by EM (Hartwig 2013). Microtubules are polymers composed of α and β-tubulin dimers. Each monomeric α or β-tubulin subunit is ~50 kDa. The $\alpha\beta$-heterodimers polymerize into filaments that then assemble laterally into long tubular hollow structures composed of 13 $\alpha\beta$-tubulin protofilaments, and thus are known as a microtubules. About 50 percent of the tubulin is polymerized in resting platelets. Similar to actin filaments, microtubules (MT) are polar structures with plus and minus ends, wherein the former growing end binds tubulin subunits during MT polymerization. In addition, MT polymerization is regulated by tubulin binding proteins and is a dynamic process (Hartwig 2013). Platelets express several β tubulin isotypes including β1, β2, and β5, however, β1 appears to be the dominant β tubulin in platelets (Italiano et al. 2003, Schwer et al. 2001). β1 tubulin is specifically expressed in mature megakaryocytes and platelets (Schwer et al. 2001, Lecine et al. 2000). β1 tubulin

protein can also be detected in spleen and lung, but these signals appear to be due to megakaryocytes and platelets residing or entrapped in those tissues. β1 tubulin knockout mice display thrombocytopenia, and their platelets lack the normal resting discoid shape (Schwer et al. 2001). These defects are due to the importance of microtubule coils that form looped peripheral bundles in the pseudopods of megakaryocytes during formation of intermediate platelet structures known as proplatelets (Italiano et al. 1999). β1 tubulin appears to play an important role in proplatelet formation from megakaryocytes. In addition, β1 tubulin null platelets show reduced microtubule coils, which contains only 1-3 microtubule coils and appears disorganized upon platelet activation, as determined by fluorescence microscopy, immunostaining, and EM approaches (Italiano et al. 2003, Schwer et al. 2001). Although, β1 tubulin does appear to be important in proplatelet formation and thrombopoiesis in megakaryocytes, the importance of these structural MT abnormalities in platelet function is unclear. Remarkably, β1 tubulin null mice also display prolong tail bleeding, however, with only what appears to be a minor defect in p-selectin exposure; it is likely that this hemostatic defect is due to reduced megakaryocyte production of platelets in β1 null mice rather than defective platelet function (Schwer et al. 2001).

Platelet activation results in a morphological shape change, from disc-like to spherical, which involves reorganization of preexisting actin filaments at the periphery of the platelet. This reorganization consists of severing or breaking actin filaments into short fragments. At the center of this process is gelsolin, an approximately 82 kDa cytosolic protein (Hartwig, Chambers, and Stossel 1989, Lind, Yin, and Stossel 1982), which mediates F-actin severing that is important for platelet shape change. Gelsolin is not associated with F-actin in resting platelets, but upon stimulation of platelets and subsequent intracellular calcium release the F-

actin binding function of gelsolin is activated (Barkalow et al. 1996, Hartwig et al. 1999). Once bound to F-actin gelsolin remains on the newly formed barbed end. In addition, gelsolin appears to preferentially localize at the plasma membrane during early stages of platelet activation (Hartwig, Chambers, and Stossel 1989). Thus, upon platelet activation at the periphery of the platelet cytoplasm gelsolin creates numerous new barbed ends and simultaneously caps them preventing actin polymerization and reannealing of severed F-actin. Gelsolin is released from F-actin barbed ends by the binding of membrane phosphoinositides which are synthesized during platelet activation and this 'uncapping' allows actin polymerization to occur (Janmey and Stossel 1987, Hartwig et al. 1995). The importance of gelsolin in platelet shape change is supported by gelsolin null mice whose platelets have defects in spreading, abnormal basal F-actin levels, and reduced ability to nucleate actin assembly (Witke et al. 1995). Importantly, gelsolin knockout mice display prolonged tail bleeding time suggesting a role for gelsolin in hemostasis.

Also known to be important in actin reorganization are the ADF/cofilin family of actin binding proteins: actin depolymerizing factor (ADF/destrin), non-muscle cofilin (n-cofilin/cofilin-1), and muscle cofilin (m-cofilin/cofilin 2). ADF/cofilin proteins have been shown to be critical for chemotaxis (Tang et al. 2011, Mouneimne et al. 2006), cytokinesis (Hotulainen et al. 2005, Gohla, Birkenfeld, and Bokoch 2005), and cell motility (Mseka and Cramer 2011, Hotulainen et al. 2005, Dawe et al. 2003, Ghosh et al. 2004). Cofilin (~19kDa) binds both G-actin and F-actin (i.e., monomers and filaments) and it has higher affinity for ADP-bound actin than ATP-bound actin. Importantly, cofilin severs F-actin; and this severing activity, which is the process by which non-covalent interactions between adjacent actin molecules are disrupted, exposes new barbed ends that can initiate actin polymerization. Each cofilin molecule bridges two

longitudinally associated actin molecules within an actin filament, and thereby cofilin stabilizes the portion of the actin filament to which it binds; however, these interactions also disrupt the conformation of cofilin-bound actin molecules, particularly subdomain 2 of actin monomers, which leads to rotations or twisting of the actin filament (McGough et al. 1997, Galkin et al. 2011). This distortion of F-actin conformation is transmitted along the filament and renders the adjacent area not decorated by cofilin molecules unstable (McGough et al. 1997, Galkin et al. 2011, Ngo et al. 2015), which results in a break (or severing) between cofilin bound and unbound regions (Suarez et al. 2011). The key determinant of whether depolymerization or polymerization occurs after cofilin-mediated F-actin severing is the relative concentrations of ATP-G-actin and active cofilin. Based on in vitro experiments, the outcome of cofilin activity on actin filament reorganization appears to be dependent on the concentration of active cofilin (Andrianantoandro and Pollard 2006): at low concentrations of cofilin actin filament severing is promoted, at intermediate concentrations filaments are stabilized, and at high concentrations it directly nucleates actin polymerization. Whether and how cells maintain the supposed gradient of cofilin activity, for example during lamellopodia formation, is unclear. Moreover, actin filaments are polar structures: ADP-G-actin subunits dissociate at the pointed end and ATP-G-actin subunits associate at the faster growing barbed end. The critical concentration for actin polymerization at the barbed end is much lower (~12-15 times) than the pointed end, which is dependent on actin monomer binding to ATP, and results in growth preferentially at the barbed end (Dos Remedios et al. 2003, Lodish et al. 2000). Thus, since actin is the most abundant platelet protein (0.55mM) it would be expected that nearly all actin would be in the polymerized form. However, only approximately 40 percent of the resting platelet actin is polymerized. 60 percent is bound to the actin monomer sequestering protein β4-thymosin (Hartwig 2013). β4-

thymosin is also abundant in platelets (~0.55mM), and it has high affinity for ATP-actin. Importantly, F-actin barbed ends have higher affinity for ATP-actin monomers than β4-thymosin, thus when barbed ends are exposed ATP-actin is released from β4-thymosin, allowing for actin polymerization to occur. In addition, 98 percent of the barbed-ends in resting platelets are capped. Therefore, a critical step in platelet actin polymerization is the uncapping of barbed ends, which are inactivated by binding membrane phosphoinositides, such as phosphatidylinositol 4,5-bisphosphate (PIP_2) (Hartwig 2013). On the other hand, cofilin activity is inhibited by PIP_2-binding at the plasma membrane. A subtle point here is that unphosphorylated cofilin is still inactive upon binding to PIP_2, and therefore, cofilin dephosphosphorylation itself may not be sufficient for cofilin to bind F-actin (Song et al. 2006). This inhibitory interaction is then released upon PIP_2 hydrolysis, for example by PLC, which is thought to lead to local F-actin severing and formation of membrane protrusions (Bravo-Cordero et al. 2013). Indeed, it has been shown that at least some proportion of active cofilin (unphosphorylated at Ser3) is found at the plasma membrane, whereas inactive cofilin (phosphorylated at Ser3) is more distant from the plasma membrane and that the membrane-bound pool is released by PLC (Van Rheenen et al. 2007). Thus, these findings indicate that active cofilin, released from PIP_2, generates new barbed ends which should displace β4-thymosin from ATP-G-actin and lead to actin polymerization, and also that cofilin activation is an early step in actin polymerization, which is required for membrane protrusion activity in cells. Interestingly, it was also shown that actin depolymerization promoted retraction at the rear of the cell, in polarizing and migrating cells, which was correlated with the activity of cofilin (Mseka and Cramer 2011). Taken together, it would be of interest to simultaneously measure F-actin, active and inactive cofilin concentrations in the rear of cells and compare these to the

local levels found in lamellipodia. These data would enable better understanding of how cells achieve localized shape changes. Although cofilin is a critical component of actin dynamics, in cells actin reorganization is complex with many other factors contributing to the outcome of cofilin activation: localization and activities of cofilin regulatory proteins (e.g., LIMK, SSH), competition between other actin-binding proteins, and activity of barbed/pointed-end capping proteins all of which influence active cofilin concentrations.

1.1.1.3 Granules

Platelets contain three major secretory organelles: dense (δ) granules that contain small molecules (e.g., divalent cations, ADP, ATP, serotonin, and pyrophosphate); alpha (α) granules that contain adhesion proteins (e.g., VWF, fibrinogen), growth factors (e.g., platelet-derived growth factor, vascular endothelial growth factors), coagulation factors(e.g., factor V, factor XI) and chemokines (e.g., platelet factor 4 (PF4), neutrophil-activating peptide-2); and lysosomes that contain degradative enzymes (e.g., cathepsin D, β-hexosaminidase). There are about 50-80 α granules per platelet, and they are 10-fold more abundant than the dense granules (3-6/platelet); moreover, at 200-500 nanometers each α-granules are also larger than dense granules (150 nanometers) (Fitch-Tewfik and Flaumenhaft 2013, Koseoglu and Flaumenhaft 2013, Blair and Flaumenhaft 2009). Remarkably, α-granules were determined by proteomic methods to contain 284 different proteins many with opposing functions (Maynard et al. 2007). Platelet granule exocytosis or secretion amplifies platelet activation signaling, recruits circulating platelets, and stablizes platelet aggregation, which is important for primary hemostasis. In addition, granule secretion contributes to other physiological responses to vessel injury (e.g, vascular remodeling, inflammation), and it also plays an important role in

disease progression (e.g., atherosclerosis, cancer metastasis) (Lievens and von Hundelshausen 2011, Labelle, Begum, and Hynes 2011, Schumacher et al. 2013, Massberg et al. 2006, Duerschmied et al. 2013, Ghasemzadeh et al. 2013, Golebiewska and Poole 2015).

Nearly all platelet agonists cause granule release. A critical event for release of granules is fusion of intracellular vesicle membranes with target membranes. Platelet granule membranes are thought to fuse with surface plasma membranes and invaginations of the open canalicular system, enabling pore formation that allows granule cargo to then diffuse outward into the extracellular milieu. Granule or vesicle fusion requires highly regulated and coordinated interactions between N-ethylmaleimide sensitive fusion protein (NSF), soluble NSF-attachment protein (SNAP), and soluble NSF-attachment protein receptors (SNAREs). SNAREs are grouped into three subfamilies: synaptosomal-associated protein (SNAP) type, vesicle-associated membrane proteins (VAMPs), and syntaxins. Upon platelet activation, vesicle SNAREs (vSNAREs) interact with target SNAREs (tSNARES) on surface membranes to form a transbilayer complex that is required for membrane fusion. A critical vSNARE in platelets appears to be VAMP8, whereas the essential tSNAREs appear to be syntaxin-11 and SNAP-23. VAMP8 promotes agonist-induced secretion of α- and lysosomal granules, but it may play a less critical role in δ-granules release and secretion at high doses of agonist; thus other VAMPs are likely to play a role in secretion. The essential tSNAREs appears to be syntaxin-11 and SNAP-23. Patients lacking syntaxin-11 display severe reductions in agonist-induced α- and δ- granule release. At present, the evidence for SNAP-23 is suggestive of a role in promoting secretion, but these are largely based on pharmacological methods and other less direct approaches, which would benefit from platelet-specific deletion of this and other members of the SNARE complex. There are several regulatory molecules and second

messengers that promote SNARE complex assembly : elevations in intracellular Ca^{2+} levels which, in addition to regulating the activity of several signaling molecules, may induce changes in SNARE complex conformation (Südhof and Rothman 2009); PKC, and more recently IKK, -dependent SNARE phosphorylation; and SNARE chaperones Munc13d, and Munc18b. Munc13d, unlike Munc18b, appears to be required for agonist-induced δ granule release and plays a critical role in both α-granule and lysosome release (Ren et al. 2010). Given the numerous regulators of the secretion complex, it is not surprising that many signaling molecules have been shown to promote secretion: SFK activation, mainly Lyn, an early step proximal to activation of adhesion receptors and GPCRs that is upstream of PI3K/AKT and NO/cGMP/PKG pathways (Senis, Mazharian, and Mori 2014); adhesion receptor or GPCR-induced TXA2 synthesis that provides positive feedback leading to secretion; MAPK family member signaling; CalDAG-GEFI, Rap1b, and Rac1 signaling; and integrin outside-in signaling (Su et al. 2008, Law et al. 1999, Shen, Delaney, and Du 2012, Flevaris et al. 2009, Flevaris et al. 2007, Gong et al. 2010). Whether these signaling pathways or others utilize the same SNARE core complex (vamp-8/SNAP-23/syntaxin-11) to induce secretion of various granule cargoes is unclear. Future research may determine the mechanism for regulated release of α-granules that contain diverse cargo, many with alternative and antagonistic functions, which ultimately must mediate productive platelet-dependent physiological responses.

1.2 Platelet adhesion receptors and signaling

Upon blood vessel injury platelets rapidly respond to form a hemostatic plug. The ability of platelets to rapidly respond to vascular damage is due to an extraordinarily intricate tapestry of surface receptors and finely regulated intracellular signaling networks that together coordinate adhesion, shape change, granule secretion, and platelet aggregation leading to

thrombus formation and cessation of bleeding. On the other hand, inappropriate activation of platelets under pathological conditions induces occlusive thrombosis that often leads to heart attack and stroke, which are leading global causes of death. En route to formation of hemostatic thrombi platelet activation signaling proceeds through several characteristic stages: (1) Exposure of adhesion receptors and agonist receptors to their respective ligands, which induces early platelet activation signaling; (2) convergence of early signaling events on common intermediates; (3) inside-out signaling; and (4) outside-in signaling. In addition, platelet activation signaling utilizes granule secretion and other second messengers to rapidly amplify initial receptor-induced activation signals. Understanding the molecular mechanisms that regulate platelet activation signaling is essential for developing novel antithrombotics with minimal side effects. In this review we discuss recent advances in the understanding of platelet activation signaling.

GPIb-IX-V, GPVI, and αIIbβ3, are the major adhesion receptors present on the platelet surface; and they are critical not only for adhesion of platelets to damaged vessel walls, but also for transduction of platelet activation signals leading to shape change, granule secretion, and thrombus formation.

1.2.1 GPIb-IX-V complex

The GPIb-IX-V complex is the platelet receptor for von Willebrand factor (VWF). Upon blood vessel injury, the platelet GPIb-IX-V complex binds to VWF to mediate the initial adhesion of platelets. The GPIb-IX-V receptor complex not only mediates the initial adhesion of platelets to VWF, but also elicits intracellular signaling that leads to platelet shape change

and granule release that induces integrin αIIbβ3 activation; and thereby, GPIb-IX signaling promotes stable adhesion, platelet aggregation, and thrombus formation.

1.2.1.1 GPIb-IX-V organization and structure

The hetero-oligomeric GPIb-IX-V complex was originally characterized by the contribution of its largest subunit, GPIbα, to the carbohydrate-rich and negatively-charged platelet surface glycocalyx (Behnke 1968b, Okumura, Lombart, and Jamieson 1976b, Nurden 1974, Nurden and Caen 1975). The name Glycoprotein I (GPI), and later GPIbα, is derived from the initial grouping of the major platelet membrane glycoproteins into three subgroups based on their molecular weight and carbohydrate staining on one- and two-dimensional polyacrylamide gels (Takahashi et al. 2002, Phillips and Agin 1977a, Phillips 1972) . The GPIb-IX-V receptor complex is composed of four different single pass transmembrane subunits, which are encoded by four different genes. In humans the chromosomal locations of the GPIb-IX-V complex genes are as follows: GPIbα is on chromosome 17, GPIb β on chromosome 22, and GPIX and GPV are on different regions of chromosome 3 (Wenger et al. 1989, Lopez et al. 1988, Lopez et al. 1987, Yagi et al. 1995, 1994). In addition, each polypeptide of the platelet GPIb-IX-V complex is encoded by a single exon, and upstream of the coding sequence all four genes contain GATA-1 and ETS family member, Fli-1, transcription factor binding sites, which are thought to be important for promoting megakaryocyte/platelet-specific gene expression (Bastian et al. 1999, Deveaux et al. 1996, Wang et al. 2002). Interestingly, there are reports that the GPIb-IX-V complex is also expressed in endothelial cells, and except for a longer GPIbβ chain, the endothelial polypeptides are similar, in size and surface expression ratios, to the platelet GPIb-IX-V complex (Wu et al. 1997, Kelly et al. 1994). In addition, the same group

reported that endothelial GPIb-IX-V expression is increased by shear force and cytokine stimulation (Beacham et al. 1999, Beacham, Tran, and Shapiro 1997).

Each subunit of the GPIb-IX-V complex is a member of the leucine-rich repeat superfamily of proteins. The extracellular leucine-rich repeat (LRR) motifs found in the subunits of the GPIb-IX-V complex consist of an approximately 24 amino acid sequence that repeats 8 times in GPIbα, 15 times in GPV and once in both GPIbβ and GPIX. In addition, the tandem and single LRR motifs are flanked by disulfide loop structures in each of the GPIb-IX-V subunits (Shen et al. 2002, Hickey et al. 1993, Hickey, Williams, and Roth 1989, Uff et al. 2002, Lopez et al. 1988, Lopez et al. 1987). The GPIbα and GPIbβ subunits are linked by disulfide bonds (Phillips and Agin 1977a) while GPIX and GPV are associated with the GPIb complex noncovalently (Berndt et al. 1985, Modderman et al. 1992, Luo et al. 2007). Additional evidence suggests that GPIbα, GPIbβ, GPIX and GPV may be expressed with a stoichiometry of 2:4:2:1 on the platelet surface (Luo et al. 2007).

The α-chain of the GPIb-IX-V complex, GPIbα, is approximately 135 kDa and owes approximately 50% of its molecular weight to glycosylation (Okumura, Lombart, and Jamieson 1976b, Berndt et al. 1985, Judson, Anstee, and Clamp 1982, KORREL et al. 1984, Fox, Aggerbeck, and Berndt 1988). The extracellular region of GPIbα, beginning from the N-terminus of the mature polypeptide, contains: an N-terminal β-hairpin loop (His^{2} - Asp^{18}) (Uff et al. 2002), which is formed by a disulfide bond between cysteines 4 and 17; eight tandem leucine-rich repeats (Lys^{19}-Gly^{204}), that together impart a concave structure on this region (Uff et al. 2002, Lopez et al. 1988, Lopez et al. 1987); two N-linked glycosylation sites (Asn^{21} and Asn^{159}); a C-terminal flanking region (Asn^{205}-Gly^{268}) that contains two disulfide bonds (Cys^{209}-Cys^{248} and Cys^{211}- Cys^{264}) which forms a ‘disulfide knot’ structure, and also a loop called the

β-switch (Val^{227}-Val^{243}) that plays a role in VWF binding to GPIb (Huizinga et al. 2002, Lou and Zhu 2008, Shen et al. 2002, Uff et al. 2002); an anionic region (Gly^{271}-Glu^{285}), which contains three sulfated tyrosines known to be important for thrombin binding to GPIb (De Marco et al. 1994, Marchese et al. 1995, Zarpellon et al. 2011); a macroglycopeptide region (Thr^{292}- Ser^{428}) that is heavily O-glycosylated on serine and threonine residues (KORREL et al. 1984, Judson, Anstee, and Clamp 1982); and just before the transmembrane region (Leu^{486}-Gly^{514}), are two disulfide bonds at cysteines 484 and 485 that covalently link one GPIbα to two different GPIbβ molecules (Luo et al. 2007). In humans, differences in the length of the macroglycopeptide region can occur due to the presence of a polymorphic 13 amino acid tandem repeat sequence which results in a longer GPIbα molecule (one tandem repeat in the most well-characterized GenBank:J02940.1 human GPIbα sequence (Lopez et al. 1987), See table I, and three repeats in the NCBI NP000164.5 human GPIbα reference sequence) . Interestingly, it appears that both reference sequences may not reflect the most frequent number of polymorphic repeat sequences found in humans, which appears to be two tandem repeats in this region. Therefore, in most humans, the length and rod-like structure of the macroglycopeptide region extends the globular ligand binding domain approximately 440 angstroms above the lipid bilayer (Lopez, Ludwig, and McCarthy 1992, Fox, Aggerbeck, and Berndt 1988). The cytoplasmic domain of GPIbα is 96 residues long and phosphorylated on Ser^{587}, Ser^{590} and Ser^{609} (Mangin et al. 2004, Bodnar et al. 1999).

The β-chain of the GPIb-IX-V complex, GPIbβ, is approximately 24 kDa (Berndt et al. 1985). The mature polypeptide is 181 amino acids long and contains a single 25 amino acid transmembrane domain (Trp^{123}-Leu^{147}) (Lopez et al. 1988). As mentioned above, in contrast to some of the other subunits of GPIb-IX-V, GPIbβ contains only a single extracellular LRR,

and the amino acid sequences flanking this region are conserved with the LRR flanking sequences found in GPIbα. GPIbβ is disulfide bonded to GPIbα, to form the heterodimer known as GPIb, via an extracellular membrane proximal Cys ^{122} on GPIbβ (McEwan et al. 2011, Luo et al. 2007). Four intramolecular disulfide bonds are also found in the extracellular domain of GPIbβ; two disulfide bonds are found on each side of the LRR. GPIbβ also contains an N-linked glycosylation site at Asn^{41} (McEwan et al. 2011). The cytoplasmic region of GPIbβ is 34 amino acids in length; and intracellular palmitoylation and phosphorylation occurs on Cys^{148} and Ser^{166}, respectively (Wardell et al. 1989, Fox, Reynolds, and Johnson 1987, Muszbek and Laposata 1989).

GPIbα Protein Sequence (GenBank:J02940.1)	
Region or Modification	Amino Acid Location/Numbering
Extracellular	17-485
Cytoplasmic	515-610
Transmembrane	486-514
Sulfated Tyrosines	276,278,279
N-linked Glycosylation	21, 159
O-linked Glycosylation	292-428
Disulfides	4-17,209-248,211-264, 484,485
Polymorphic Repeat Sequence	415-428

TABLE I: GPIbα protein organization, domain localization, and modifications. Numbering according to the mature form of full-length human GPIbα. Amino acid residues with posttranslational modifications are also indicated.

GPIX is approximately 22 kDa and, like GPIbβ, contains a single LRR and conserved LRR flanking sequences (Berndt et al. 1985, Hickey, Williams, and Roth 1989). The mature GPIX polypeptide contains 160 amino acids, which includes a 20 amino acid transmembrane domain (Val^{135}-Ala^{154}) and a short 6 amino acid cytoplasmic domain. The extracellular intramolecular disulfide bond arrangement for GPIX is unclear, but may involve Cys^{73}-Cys^{97} (Kunishima et al. 1999, Noda et al. 1996). GPIX may be myristoylated or palmitoylated on the transmembrane cysteine (Schick and Walker 1996, Muszbek and Laposata 1989).

GPV is an approximately 83 kDa polypeptide composed of 544 amino acids, a 24 amino acid transmembrane domain (505-528), and a 16 amino acid cytoplasmic domain (Lanza et al. 1993, Hickey et al. 1993). As mentioned above, GPV contains 15 tandem LRR (I^{39}-E^{399}) and these are flanked by conserved sequences found in all other subunits of the GPIb-IX complex. GPV also contains eight N-linked glycosylation sites. Interestingly, GPV is cleaved by thrombin between Arg^{460} and Gly^{461} to release an approximately 69 kDa extracellular fragment (Berndt and Phillips 1981, Mosher et al. 1979, Phillips and Agin 1977b), however, the role of GPV in thrombin-induced platelet activation is unclear. GPIbα is the only subunit of the GPIb complex known to contain binding sites for ligands, such as von Willebrand factor (VWF), Factor XI, leukocyte Mac-I, and thrombin (Clemetson 2007).

1.2.1.2 Regulation of the VWF-binding function of GPIb-IX

The binding of VWF to platelet GPIb-IX is regulated in two ways: conformation-dependent changes of the ligand itself and changes in receptor affinity. Of the two, the regulation of VWF is better understood. Under physiological or pathological conditions, the conformation of VWF is modified by its immobilization onto surfaces or when bound to

subendothelial collagen, and by its exposure to shear stress in flowing blood. Shear-dependent conformational changes cause VWF to unfold exposing receptor-binding domains that allow it to bind platelets. The VWF-GPIb-IX-V interaction involves the A1 domain of VWF, the concave face of GPIbα, and β-switch region of GPIbα, which undergoes conformational changes under flow conditions that are stabilized by binding VWF (Huizinga et al. 2002, Dumas et al. 2004, Lou and Zhu 2008, Yago et al. 2008). In vitro, the addition of the non-physiological modulators, ristocetin (an antibiotic, no longer used clinically because it causes thrombocytopenia and platelet agglutination) or botrocetin (a snake venom protein), are used to induce conformational changes in soluble VWF that enable it to bind to GPIb-IX, which induces agglutination (cross-linking of GPIb-IX receptor complexes, and thus adjacent platelets, via intervening receptor-bound VWF molecules) and aggregation. GPIb-IX is also sensitive to increases in shear force and will undergo conformational changes that enhances its binding affinity for VWF (López and Dong 1997, Dumas et al. 2004, Ruggeri and Mendolicchio 2007). In addition, intracellular signaling regulates the affinity of GPIb-IX for VWF. Treatment of platelets with cAMP-elevating agents such as PGE_1 induces PKA-dependent phosphorylation of GPIbβ Ser ^{166}. This phosphorylation is believed to inhibit the binding of VWF to GPIb-IX (Du 2007).

PKA-mediated phosphorylation of GPIbβ on Ser ^{166} regulates the binding of a signaling and adaptor protein 14-3-3ζ (Feng et al. 2000, Fox, Reynolds, and Johnson 1987, Wardell et al. 1989, Calverley, Kavanagh, and Roth 1998), which in turn regulates GPIb-IX binding to VWF (Bodnar et al. 2002). 14-3-3 proteins are a ubiquitously expressed family of proteins involved in many cellular functions such as signal transduction, protein trafficking, cell cycle regulation, and apoptosis (Morrison 2009). 14-3-3 proteins are expressed as homodimers or

heterodimers that function by binding to and regulating serine- or threonine-phosphorylated proteins. There are six isoforms of 14-3-3 expressed in platelets, each of which were demonstrated to bind to GPIb-IX (Mangin et al. 2009). It has previously shown that 14-3-3ζ binding to the α-chain of GPIb promotes GPIb-signaling (Gu et al. 1999). A site in the cytoplasmic domain of GPIbα, Ser^{605}-Leu^{610}, was previously found to be critical for 14-3-3 binding (Du, Fox, and Pei 1996), and moreover, 14-3-3ζ binding to this region has been shown to require a constitutive phosphorylation site on Ser^{609} (Bodnar et al. 1999). The binding of 14-3-3 to other regions on GPIbα upstream of this site has also been reported (Mangin et al. 2004, Yuan et al. 2009). Using a cell-permeable peptide inhibitor that mimics the C-terminal GPIbα-14-3-3-binding sequence it was shown that 14-3-3-binding to GPIb was critical for the ability of platelets to bind VWF and for VWF-dependent platelet activation in human platelets. Based on these observations, a ‘toggle switch’ model was put forward that suggests that 14-3-3 binds to both GPIbα and GPIbβ under resting conditions when GPIbβ Ser^{166} is phosphorylated (i.e., when PKA/cAMP activity is increased) placing GPIb-IX in a low affinity state for VWF (Du 2007). Upon platelet activation, GPIbβ is then dephosphorylated causing the 14-3-3 dimer to bind only to two sites on GPIbα (or to one site on GPIbα and another site on a different protein). Recently, it has been shown that GPIbα forms disulfide bonds with two GPIbβ molecules (Luo et al. 2007), however, how this fits in with the model of a single 14-3-3 dimer constitutively bound to GPIbα and GPIbβ in resting platelets requires further investigation.

1.2.1.3 VWF/GPIb-IX-mediated platelet activation

The platelet glycoprotein GPIb-IX-V complex, a member of the leucine-rich repeat family of proteins, is the receptor for the adhesive ligand von willebrand factor (VWF). Under high shear rate blood flow conditions present in arteries and arterioles, the initial adhesion of platelets to immobilized VWF, exposed on the surface of damaged endothelium or within the subendothelial matrix, is dependent on the platelet GPIb-IX-V receptor complex (López and Dong 1997, Du 2007, Li et al. 2010). The major ligand binding subunit of the GPIb-IX-V receptor complex, GPIbα, contains binding sites for the A1 domain of VWF (Vicente, Houghten, and Ruggeri 1990, Cruz et al. 2000, Matsushita and Sadler 1995, Huizinga et al. 2002, Marchese et al. 1995). VWF and GPIbα respond to increasing shear force by undergoing conformational changes that increase their binding affinity for one another (Schneider et al. 2007, Miura et al. 2000, Lou and Zhu 2008); these shear-induced increases in receptor/ligand affinity enable the formation of long-lived, but reversible, GPIbα/VWF ‘catch bonds’ or ‘flex bonds’ that promote transient initial platelet adhesion (Kim et al. 2010, Yago et al. 2008, Huizinga et al. 2002, Lou and Zhu 2008). Interestingly, using single molecule measurements of the pulling force of the VWF A1 domain on immobilized recombinant full-length GPIb-IX complex, a region within the extracellular juxtamembrane stalk of GPIbα, called the mechanosensitive domain (MSD), was shown to unfold and extend upon VWF A1-dependent pulling (Zhang et al. 2015). The MSD (Ala^{417}-Phe^{483}) appears to be responsible for enabling unfolding and extension of GPIbα, because mutations within the MSD abolished the ability of the GPIb-IX complex to undergo force-induced unfolding and extension. However, whether and how this region regulates VWF/GPIb-IX-dependent platelet function is unclear. In addition to promoting initial adhesion, GPIbα binding to immobilized VWF elicits a cascade of

intracellular signaling events that promote platelet activation, granule secretion and activation of another major platelet surface receptor, integrin αIIbβ3, which leads to stable platelet adhesion and platelet aggregation (Du 2007, Li et al. 2010, Ruggeri and Mendolicchio 2007) (Figure 1). Transduction of GPIb-IX-V signaling is facilitated by the association of the cytoplasmic domain of GPIbα with several signaling molecules, such as Src family kinases (SFKs), phosphoinositide 3-kinase (PI3K), adapter molecule 14-3-3ζ, and actin cytoskeleton-associated protein filamin (Wu et al. 2003, Andrews and Fox 1991, 1992, Okita et al. 1985, Mangin et al. 2004, Munday, Berndt, and Mitchell 2000). It has been shown that SFKs and PI3K are critical mediators of VWF/GPIb-IX signaling leading to calcium mobilization, granule secretion and thromboxane A_2 (TXA_2) production that induces integrin activation and subsequent platelet aggregation (Liu et al. 2005, Yap et al. 2002, Kasirer-Friede et al. 2004, Liu et al. 2006). The filamin/GPIbα interaction has been shown to play a role in regulating platelet adhesion and maintaining platelet membrane integrity under high shear conditions (Cranmer et al. 2011).14-3-3ζ/GPIb interaction has been shown to promote VWF/GPIb-dependent signaling leading to integrin activation, and to regulate the VWF binding function of GPIbα (Du, Fox, and Pei 1996, Gu et al. 1999, Dai et al. 2005, Yuan et al. 2009).

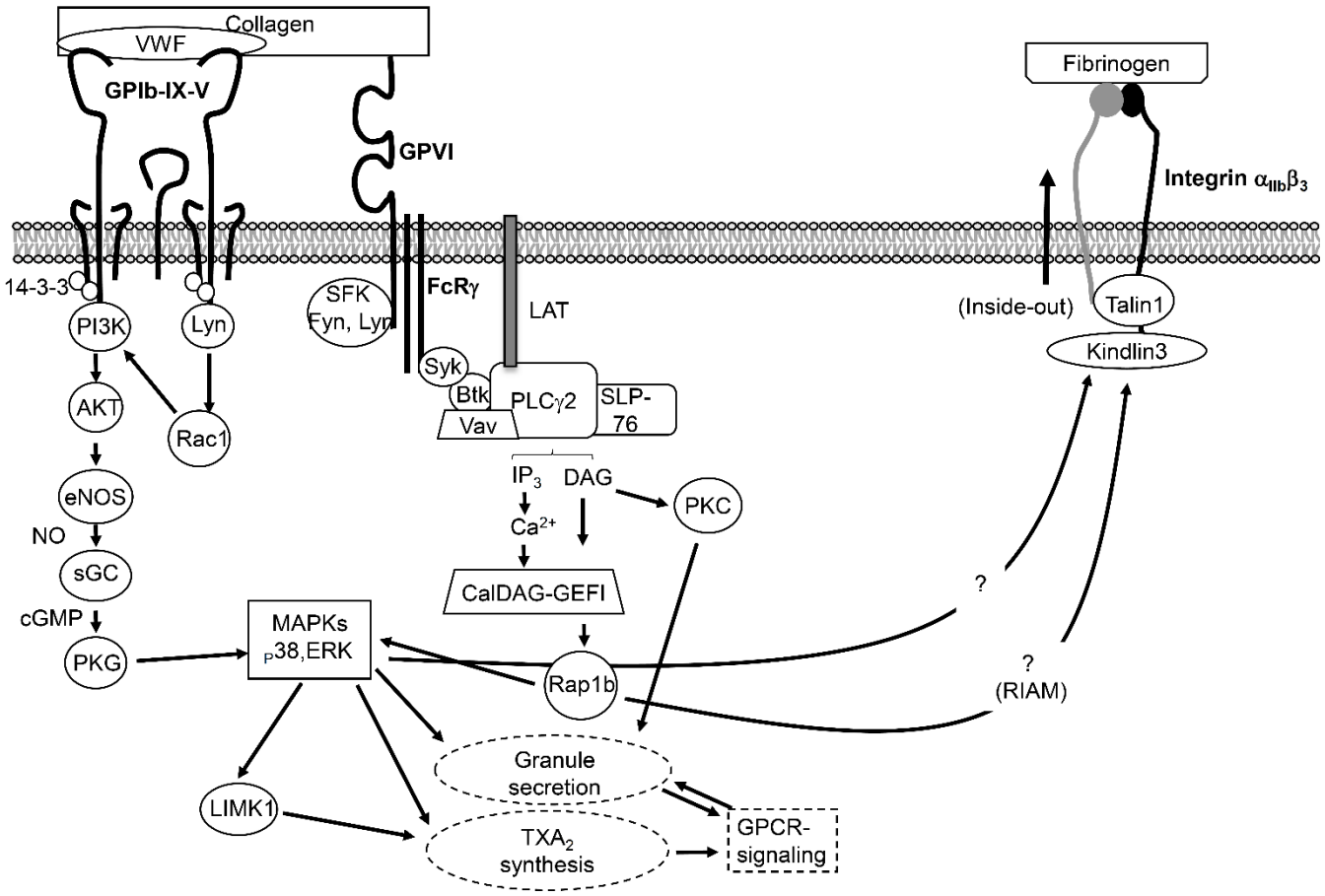


Figure 1. Platelet adhesion receptor signaling pathways. A schematic of the major platelet adhesion receptors that shows critical molecules involved in signal transduction leading to platelet activation and secondary signaling events, such as thromboxane A2 (TXA2) synthesis and granule secretion, which are important for amplification of the initial stimulus.

In the past decade, the downstream VWF/GPIb-IX-induced signaling events have been further dissected primarily by studies using knockout mice and isolated human and mouse platelets. The SFKs, particularly Lyn, are the most upstream signaling molecules activated upon VWF binding to GPIbα (Liu et al. 2005, Yin, Liu, et al. 2008, Delaney et al. 2012). The SFK Lyn is required for VWF/GPIb-IX-induced activation of the PI3K/AKT pathway leading to integrin activation and subsequent integrin-dependent stable platelet adhesion and platelet aggregation (Yin, Liu, et al. 2008). The Rho family small GTPase, Rac1, functions downstream of Lyn and is a critical link between VWF/GPIb-IX-induced Lyn activation and the PI3K/AKT pathway (Delaney et al. 2012). The VWF/GPIb-IX Lyn/PI3K/AKT pathway has also been shown to induce cyclic guanosine monophosphate (cGMP) production (Yin, Liu, et al. 2008). cGMP production is regulated by the nitric oxide activated soluble guanylyl cyclase (sGC), and the NO/sGC/cGMP pathway stimulates platelet activation as demonstrated by the defects in in vitro platelet function, as well as the defective hemostasis and thrombosis observed in vivo in the platelet-specific sGC knockout mice (Zhang et al. 2011). cGMP activates the cGMP-dependent protein kinase (PKG), which has also been shown to be important for stimulating platelet activation and promoting hemostasis (Li et al.); and furthermore, PKG was found to stimulate activation of mitogen-activated protein kinases (MAPKs), p38 and extracellular signal-regulated kinases 1 and 2 (ERK1/2). p38 mediates VWF-induced ERK activation which leads to thromboxane production and integrin activation (Li, Zhang, et al. 2006, Li, Xi, and Du 2001, Garcia et al. 2005) (Figure 1). Downstream of MAPKs, the serine/threonine kinase, LIM kinase-1 (LIMK1), promotes VWF-induced platelet activation by promoting thromboxane production and phosphorylation of the rate-limiting enzyme in thromboxane production,

cytosolic phospholipase A2 ($cPLA_2$) (Estevez et al. 2013). Interestingly, platelets from mice null for LIMK1 display enhanced responses to agonists that act independent of GPIb-IX-V. In vivo studies in mice have supported the physiological importance of VWF/GPIb-dependent signaling for promoting arterial thrombosis using the ferric chloride ($FeCl_3$) carotid artery injury model (Liu et al. 2008). In light of the recent identification of a mechanosensitive domain in GPIbα, it would be of interest to the field to determine whether this domain plays a role in GPIb-IX signaling or perhaps some alternative function. Future research in this area may aid in identification of novel regulators important for transmission of shear-dependent platelet activation signals or in development of a diagnostic tool for directly detecting GPIb-IX receptor activation.

1.2.2 Integrin α2β1 and Glycoprotein VI

The primary platelet collagen receptors, integrin α2β1 and glycoprotein VI, play important roles in collagen-induced platelet activation leading to stable platelet adhesion and aggregate formation; these essential platelet functions are dependent on glycoprotein VI (GPVI)-mediated signaling and integrin $\alpha_2\beta_1$-dependent stable adhesion (Sarratt et al. 2005) (Figure 1). GPVI, a member of the immunoglobulin superfamily of proteins, is the major receptor for platelet activation signaling in response to collagen (Kato et al. 2003, Moroi et al. 1989, Kehrel et al. 1998). On the platelet surface GPVI is in a noncovalent complex with the Fc receptor γ-chain (FcR γ-chain) (Gibbins et al. 1997, Tsuji et al. 1997, Ezumi et al. 1998). The Ig-like domains on the extracellular domain of GPVI interact with collagen (Horii, Kahn, and Herr 2006, Smethurst et al. 2004), but the GPVI-associated dimeric FcR γ-chain is necessary for converting collagen binding to GPVI into intracellular signaling events via its cytoplasmic

immunoreceptor tyrosine-based activation motif (ITAM) (Berlanga et al. 2002, Nieswandt et al. 2000, Poole et al. 1997, Wu et al. 2001), which contains two conserved YxxL motifs separated by 7 amino acids (in mouse and human FcRγ-chain) (Watson, Herbert, and Pollitt 2010).

1.2.2.1 Collagen/ GPVI-mediated platelet activation signaling

Upon collagen-induced cross-linking of GPVI receptors, the FcR γ-chain ITAM is tyrosine phosphorylated by GPVI-associated SFKs, Lyn and Fyn, that bind the proline-rich domain of GPVI (Ezumi et al. 1998, Schmaier et al. 2009) and play a critical role in promoting GPVI signaling, however, Lyn- and Fyn-independent GPVI-mediated platelet activation has also been reported (Quek et al. 2000). SFK activation is regulated by a membrane protein tyrosine phosphatase, CD148, which relieves the inhibitory phosphorylation on SFKs and thereby promotes platelet activation (Senis et al. 2009). SFK-mediated FcRγ-chain phosphorylation enables binding and activation of the non-receptor tyrosine kinase Syk (Gibbins et al. 1996, Ezumi et al. 1998, Quek et al. 2000), whose ITAM-binding N-terminal SH2 domain appears to be required for transduction of GPVI signaling (Hughes et al. 2015). Activated Syk initiates a cascade of events involving adapter molecules, Src homology (SH2 domain-containing leukocyte phosphoprotein of 76kDa (SLP-76) (Gross et al. 1999, Clements et al.), linker for activated T-cells (LAT)(Pasquet et al. 1999), Grb2 (Dütting et al. 2014), Gads (Hughes et al. 2008), Tec family kinases, Btk, Tec (Atkinson, Ellmeier, and Watson 2003, Quek, Bolen, and Watson), and lipid kinase phosphatidylinositol 3-kinase (PI3K) (Bobe et al. 2001, Canobbio et al. 2009, Watanabe et al. 2003), which leads to translocation to the plasma membrane, phosphorylation and activation of phospholipase C-γ2 (PLCγ2) (Poole et al. 1997, Bobe et al. 2001, Suzuki-Inoue et al. 2003, Wang et al. 2000, Watson et al. 2005, Watson,

Herbert, and Pollitt 2010) (Figure 1). PLCγ2 hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP_2) into 1,2-diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3). DAG and IP_3 activate protein kinase C and release calcium into the cytosol from intracellular stores, respectively, promoting thromboxane production and granule secretion, which leads to integrin activation (Lecut et al. 2004, Watson et al. 2005, Watson, Herbert, and Pollitt 2010). Interestingly, mouse platelets null for an intracellular GPIbα-binding protein, Filamin A, showed defective Syk and PLCγ2 phosphorylation, as well as diminished platelet activation responses to GPVI-specific agonists, CRP and convulxin; this early block in GPVI signaling appears to be mediated by direct association with Syk through FlnA Ig repeat 5 (Falet et al. 2010). Integrin $\alpha_2\beta_1$ activates many of the same signaling intermediates as the GPVI/ITAM pathway (Inoue et al. 2003, Petzold et al. 2013). Collectively, these studies show that the major platelet collagen receptors, GPVI and integrin $\alpha_2\beta_1$, both promote platelet adhesion and activation in response to collagen (Kuijpers et al. 2003, Auger et al. 2005, Lecut et al. 2004, Sarratt et al. 2005). Although, there are conflicting reports regarding whether these platelet collagen receptors are required for hemostasis in mice (Grüner et al. 2004, Kato et al. 2003, Nieswandt et al. 2001, Petzold et al. 2013, Sarratt et al. 2005), investigations using in vivo thrombosis models in mice suggest that the GPVI/ITAM pathway plays a role in promoting both $FeCl_3$-induced arterial thrombosis and collagen/epinephrine-induced pulmonary thromboembolism (Nieswandt et al. 2001, He et al. 2003, Massberg et al. 2003, Dubois et al. 2006).

In addition to transduction of collagen-induced signaling, the FcRγ/ITAM pathway plays a role in amplification of VWF/GPIb-dependent platelet activation. The platelet GPIb-IX-V receptor complex has been shown to associate with the FcRγ-chain (Falati, Edmead, and Poole 1999, Wu et al. 2001), and also reportedly associates with another ITAM-containing

molecule the low affinity receptor for immunoglobulin, FcγRIIA (Sullam et al. 1998). VWF binding to GPIbα stimulates the activation of the ITAM signaling pathway (Wu et al. 2001, Liu et al. 2006, Liu et al. 2005), and FcRγ null mouse platelets have been shown to display defective VWF/GPIb-dependent platelet activation signaling (Wu et al. 2001). Deletion of the FcRγ-chain or LAT in mouse platelets resulted in reduced VWF/botrocetin-induced platelet aggregation and aggregation-dependent signaling, however, early VWF/GPIb-dependent signaling was not affected (Liu et al. 2006, Liu et al. 2005). Similarly, other reports have shown that early VWF/GPIb-dependent intracellular calcium mobilization, shape change, and TXA_2 production leading to integrin αIIbβ3 activation does not require the ITAM pathway (Kasirer-Friede et al. 2004, Mangin et al. 2003, Yuan et al. 1999, Canobbio et al. 2001) (Figure 1).

1.2.3 Integrin αIIbβ3

Integrins are a large family of transmembrane adhesion receptors that are important for a wide range of processes in other cells including cell migration, cell-cell and cell-matrix contact during development, cancer metastases, and cell proliferation (Hynes 2002, Desgrosellier and Cheresh 2010). In platelets, integrins mediate platelet-platelet and platelet-matrix interactions, which are required for stable platelet adhesion and platelet aggregation. Integrins are a heterodimeric receptor complex that is composed of single-pass transmembrane α and β-subunits. At 80,000 copies per platelet (Wagner et al. 1996), integrin αIIbβ3 is the most abundant transmembrane protein on the platelet surface. The major ligands for αIIbβ3 are fibrinogen and VWF. αIIbβ3 engages fibrinogen via a HHLGGAKQAGDV sequence in the C-terminus of the fibrinogen γ-chain and RGD sequences in the α-chain (Estevez, Shen, and Du 2015, Shen, Delaney, and Du 2012), and also binds to VWF via the RGD sequence. The physiological importance of platelet integrin αIIbβ3 is demonstrated by the excessive bleeding observed in Glanzmann thrombasthenia patients, which is a rare autosomal recessive bleeding disorder caused by functional abnormalities in αIIbβ3 or deficiencies of its expression. Glanzmann thrombasthenia (GT) patients do not display low platelet counts or altered platelet morphology. Some patients present with gastrointestinal bleeding but this appears to be infrequent. In general, these patients' platelets do not aggregate in response to physiological agonists, and in addition some patients' platelets also display defective clot retraction (Nurden 2006). The variance in disease presentation is sometimes due to variant types of αIIbβ3 mutations that produce different αIIbβ3 molecules and current sequencing methodologies and computational approaches appear to have

increased the number of avenues for predicting and then dissecting the impact of known patient mutations on αIIbβ3 function (Buitrago et al. 2015). Mice null for β3 show many of the characteristics (e.g., prolonged bleeding and platelet aggregation defects) associated with human GT and have been a useful model for understanding the role of $\alpha_{IIb}\beta_3$ in platelet function (Hodivala-Dilke et al. 1999). Importantly, $\alpha_{IIb}\beta_3$ is essential for promoting thrombosis. Thus, several αIIbβ3 antagonists have been developed some of which are used clinically to prevent thrombosis, but due to risk of hemorrhage and thrombocytopenia they are primarily used acutely during percutaneous coronary interventions (Estevez, Shen, and Du 2015, Cox, Brennan, and Moran 2010).

Integrin αIIb and β3 subunits are single pass transmembrane proteins with long extracellular domains and short cytoplasmic tails. The N-terminal region formed between the α and β-subunits is known as the headpiece of αIIbβ3 and it contains the binding site for ligands. Below the head region are two separate legs that contain upper and lower regions. Thus, from N- to C-terminus αIIb contains: seven β-propeller repeats, a thigh domain, and two calf domains. The β3 subunit contains: a β-I-domain (β interactive domain), two hybrid domains, a plexin semaphorin integrin domain, and four integrin epidermal growth factor-like domains. Platelet integrin αIIbβ3 is thought to exist in three states: a resting state found on quiescent circulating platelets, in which αIIbβ3 maintains a bent conformation with low-affinity for ligands; an intermediate state that has extended its extracellular domain but still has a closed ligand binding domain; and an extended activated state that occurs upon agonist induced platelet stimulation, wherein the β3 hybrid domain in the thigh is thought to swing away from the α-subunit altering the headpiece into a configuration that has high-affinity for ligands (Figure 2).

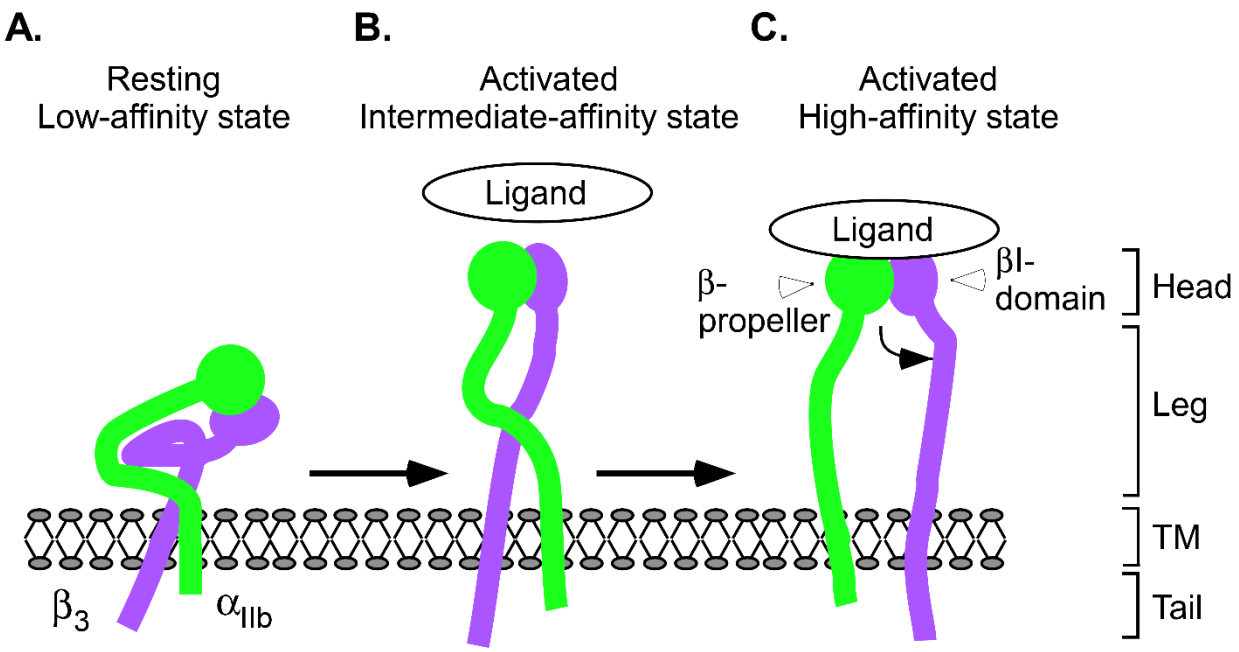


Figure 2. Conformational states during integrin αIIbβ3 activation. Schematic emphasizing large-scale movements of the extracellular, transmembrane and cytoplasmic regions that occur during integrin activation. The N-terminal region formed between the α and β-subunits is known as the headpiece of αIIbβ3 and on top it contains the binding site for ligands. Below the head region each subunit constitutes two separate legs that are further subdivided into upper (thigh) and lower (calf) regions. In the extracellular region going N- to C-terminus αIIb contains: seven β-propeller repeat domains, a thigh domain, and two calf domains. In contrast, the β3 subunit contains: a βI-domain (interactive domain), two hybrid domains, a plexin semaphorin integrin domain, and four integrin epidermal growth factor-like domains (See these reviews for domain structure details (Cox, Brennan, and Moran 2010, Bledzka, Smyth, and Plow 2013)). Platelet integrin αIIbβ3 is thought to exist in three states: **A,** a resting state found on quiescent circulating platelets, in which αIIbβ3 maintains a bent conformation with low-affinity for ligands; **B,** an intermediate state that has extended its extracellular domain but still has a closed ligand binding domain; and **C,** an extended activated state that occurs upon ligand binding, wherein the β3 hybrid domain in the thigh is thought to swing away from the α-subunit altering the headpiece into a high-affinity configuration. Although not depicted here (see figure 3), talin and kindlin bind the β3 cytoplasmic tail in the first step to initiate these conformational changes. TM denotes transmembrane domain.

1.2.3.1 Inside-out signaling

The affinity of integrin αIIbβ3 for its ligands is regulated by agonist elicited intracellular signaling events. Although many platelet agonists engage distinct surface receptors and utilize different receptor-proximal signaling molecules, all agonist pathways induce intracellular signaling events that result in separation of the cytoplasmic and transmembrane regions of the αIIbβ3 complex, which transforms αIIbβ3 into a high-affinity activated state (Figure 3). Agonist-induced intracellular signaling events that transform resting αIIbβ3 integrins into an activated high-affinity state are collectively known as inside-out signaling. A key final step of inside-out signaling downstream of all agonist pathways is the binding of cytosolic adapter protein talin to the β3 cytoplasmic tail. Talin binds to a region on the β3 cytoplasmic tail containing an NPXY motif, which is thought to facilitate a second interaction with a membrane proximal binding site on β3, and both are critical for integrin activation (Wegener et al. 2007). These interactions disrupt inner and outer membrane clasps held between α- and β- chain cytoplasmic and transmembrane regions triggering αIIbβ3 activation. Consistent with the important role for talin in regulating integrin activation in reconstituted cell models, structural studies, and in vitro biochemical studies, deletion of talin1 in mice has been shown to impair agonist-induced α2β1 and αIIbβ3 integrin activation (Petrich et al. 2007, Nieswandt et al. 2007). Talin deletion also causes severe defects in platelet aggregation in response to various agonists (Nieswandt et al. 2007). Similarly, platelets from mice expressing mutant β3 (L746A) with selective disruption of talin binding to β3 show defective fibrinogen binding and platelet aggregation upon agonist stimulation. However, a notable difference between different studies of talin1 null and talin binding defective β3 L746A platelets was the demonstration of a role for talin in platelet

spreading on fibrinogen, wherein talin1 null platelets showed severe defects in thrombin-induced spreading (Nieswandt et al. 2007), and mutant β3 L746A-expressing platelets displayed only a minor reduction in response to certain agonists (Petrich et al. 2007). Defective agonist-induced platelet spreading phenotypes were also recently reported for platelets expressing distinct talin mutations that selectively disrupt binding to the β3 NPXY motif (W359A) or membrane proximal region (L325R) (Stefanini et al. 2014). Interestingly, although the W359A talin mutant mice did show the expected impairment of agonist-induced integrin activation, these mice displayed less profound bleeding phenotypes than talin null or L325R talin mutant mice, which exhibit spontaneous gastrointestinal bleeding of different severities. In addition, integrin-talin-cytoskeleton linkage also appears to be important for clot retraction (Haling et al. 2011). Future, studies may elucidate the mechanisms by which talin plays roles in platelet spreading and clot retraction. In addition to talin, kindlins are known to be important regulators of integrin function. Kindlins bind to β3 cytoplasmic tails at a membrane distal NXXY motif distinct from the talin binding site. Agonist-induced integrin activation is defective in kindlin-3 null platelets, and platelet aggregation response to various platelet agonists is also severely inhibited. Kindlin-3 null platelets display defective thrombin-induced spreading on fibrinogen which cannot be rescued by pretreatment with manganese (Moser et al. 2008). Essentially, kindlin-3 null mice phenocopy the platelet defects reported in talin1 null mice. Although, both talin and kindlin play critical roles in integrin activation, in the absence of kindlin or talin, the presence of the other allows for some residual integrin activation in response to high doses of agonist, but this low level of αIIbβ3 activation appears insufficient to support stable platelet aggregation (Klapproth et al. 2015). Importantly, in cell culture model systems only when co-expressed with talin head domain do kindlins enhance αIIbβ3 activation. Kindlins

have been shown to play a role in promoting integrin binding to multivalent ligands, which also appears to be dependent on synergizing with talin-induced integrin activation. However, kindlins appear to be dispensable for integrin binding to monovalent ligands and do not synergize with talin to promote binding to such ligands. Accordingly, in kindlin-3 null platelets agonist-induced αIIbβ3 binding to multivalent ligand fibrinogen was diminished, whereas binding to a monovalent ligand (fibronectin tenth type III repeat) was unaffected. By contrast, talin plays an important role in regulating $\alpha_{IIb}\beta_3$ binding to both types of ligand. Emerging evidence suggests kindlins regulate binding of multivalent ligands to αIIbβ3 by promoting clustering of activated integrins (Ye et al. 2013, Morse, Brahme, and Calderwood 2014, Ye, Snider, and Ginsberg 2014). Upstream of talin-dependent αIIbβ3 activation, it had been previously suggested that a Rap1 effector, Rap1 GTP-interacting adaptor molecule (RIAM), played an important role, however, a recent study casts doubt on the importance of RIAM in mouse platelets. In contrast to data primarily obtained in cellular model systems (Watanabe et al. 2008, Han et al. 2006, Lee et al. 2009), RIAM null platelets showed no defect in integrin activation nor in any other parameters of integrin-dependent platelet function (e.g., cell spreading, clot retraction) (Stritt et al. 2015). In light of these recent findings, and previous studies showing partial inhibition of integrin activation in CalDAG-GEF1 or Rap1b null platelets, future studies should identify alternative Rap1 effectors in platelets and investigate Rap1b-independent αIIbβ3 activation.

1.2.3.2 Outside-in signaling

Ligation of activated integrin $\alpha_{IIb}\beta_3$ by extracellular adhesive proteins, such as fibrinogen, induces conformational changes within αIIbβ3 and receptor clustering, which triggers formation and activation of intracellular signaling networks at the β3 cytoplasmic tail. Collectively this process, known as outside-in signaling, enables stable adhesion, platelet spreading, granule secretion, and clot retraction to occur, and it is critical for promoting platelet aggregation and thrombosis. The earliest known outside-in signaling event induced by fibrinogen binding to αIIbβ3 is the binding of Gα13 to a conserved EXE motif on the β3 cytoplasmic tail (Figure 3). Although this EXE motif overlaps with a membrane proximal talin binding site it is not required for talin binding, and Gα13 and talin appear to bind in opposing waves to the β3 cytoplasmic tail to control the direction of integrin signaling. Gα13 binding to β3 is required c-Src activation and src-dependent outside-in signaling, which involves transient src-dependent inhibition of RhoA that is important for platelet spreading. Consistent with this notion, knockdown of platelet Gα13 abolished integrin-induced c-Src activation, reversed RhoA inhibition, and thereby inhibited platelet spreading on fibrinogen. In addition, clot retraction is enhanced by depletion of platelet Gα13, which further lends support to a role for Gα13 in mediating integrin-dependent inhibition of RhoA during outside-in signaling. Thus, when $\alpha_{IIb}\beta_3$ is ligated Gα13 binds to β3 to regulate platelet spreading and clot retraction. As previously mentioned, a key early outside-in signaling mediator is c-Src, particularly β3-bound c-Src (Arias-Salgado et al. 2003, Ablooglu et al. 2009). Activated c-Src mediates phosphorylation of two NXXY motifs on the β3 tail which are important for regulating the outcome of integrin outside-in signaling. Specifically, phosphorylation at Tyr747 and Tyr 759 inhibits talin binding

and prevents calpain cleavage, respectively, which are essential for regulating the switch between platelet spreading and retraction. β3 tail phosphorylation may also promote docking sites for other proteins, including adaptor protein SHC and myosin, the latter of which is important in clot retraction. In addition, SFKs promote activation of PI3K and ITAM pathways, which appear to serve as amplification mechanisms for promoting spreading, granule secretion, and thrombus formation (O'Brien et al. 2012, Zhi et al. 2013). In agreement with an important role for tyrosine phosphorylation of the β3 tail in regulating outside-in signaling, mice expressing β3 with both tyrosines (Tyr747 and Tyr 759) mutated to alanine exhibit defective platelet aggregation and clot retraction, but they do not display impaired integrin activation (Law et al. 1999). Interestingly, knock-in mice with a deletion in the C-terminal β3 RGT sequence important for c-Src binding to β3 display defective platelet spreading, but surprisingly they display normal clot retraction, suggesting c-Src binding to β3 is dispensable for some outside-in signaling pathways. Moreover, it is unclear why the β3 RGT deletion causes a minor reduction in inside-out signaling (Ablooglu et al. 2009). In addition to SFKs, other tyrosine kinases have been reported to play a role in outside-in signaling, including FAK, ILK and Syk. Syk is activated by SFKs (Obergfell et al. 2002), and may also be activated by interaction with the β3 tail (Woodside et al. 2001). Syk has also been shown to be important for spreading on fibrinogen (Abtahian et al. 2006, Obergfell et al. 2002). In human platelets, $\alpha_{IIb}\beta_3$ outside-in signaling appears to utilize the ITAM-containing FcγRIIa receptor to activate the Syk/PLCγ2 pathway, which promotes platelet spreading, secretion, and thrombosis (Boylan et al. 2008, Zhi et al. 2013). Recently, vacuolar protein sorting-associated protein 33B (VPS33B), previously shown to be important in megakaryocyte and platelet α-granule biogenesis (Lo et al. 2005), was found to bind to the β3 tail. Mouse platelets null for VPS33B were generated and

were found to display defective outside-in signaling and endocytosis of fibrinogen. Interestingly, VPS33B appears to promote outside-in signaling upstream of RhoA and Rac1-dependent pathways (Xiang et al. 2015).

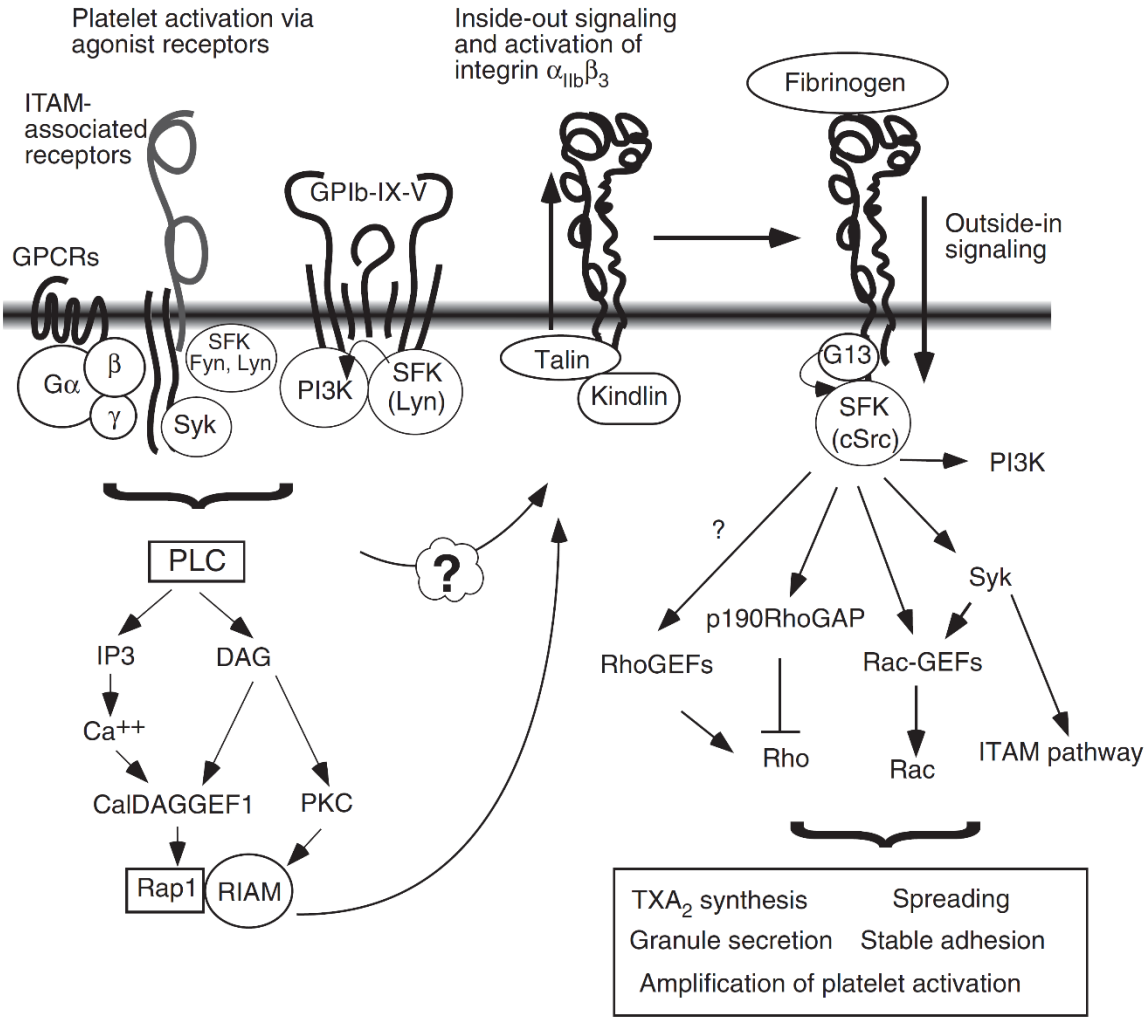


Figure 3. Inside-out and outside-in signaling of the platelet integrin αIIbβ3. Agonist receptors (e.g., GPCRs) elicit intracellular signaling events which converge on the cytoplasmic tail of the β3 subunit via the binding of talin and kindlin, which in turn activates integrin αIIIbb3 enabling it to bind its ligands. Ligand binding to αIIbβ3 induces conformational changes within αIIbβ3 that it then transforms into intracellular signals via its association with intracellular signaling molecules (e.g., Gα13 and SFKs), and this process is critical for various platelet responses, including spreading, stable adhesion, and granule secretion.

1.3 Platelet G protein-coupled receptors

Circulating platelets are kept in a resting state by endothelium-derived soluble molecules such as prostacyclin (PGI_2). However, upon blood vessel injury and subsequent platelet activation platelets rapidly produce several soluble agonists which greatly amplify the growth of the thrombus and recruit circulating platelets. Activated platelets release several soluble agonists such as adenosine diphosphate (ADP), thromboxane A_2 (TXA_2), and serotonin. Damaged endothelium and activated platelets also promote formation of the potent platelet agonist thrombin, which is the effector protease of the coagulation system (Coughlin 2005). In addition, platelets also produce chemokines (e.g. RANTES and platelet factor 4, PF4), and bioactive lipids (e.g. sphingosine 1-phosphate, S1P and lysophosphatidic acid, LPA) which play roles in atherosclerosis and inflammation (Ramji and Davies 2015, Huo et al. 2003, Koenen and Weber 2010, Morrell et al. 2014, Schober and Siess 2012, Urtz et al. 2015, Maceyka et al. 2012). Each of these soluble agonists regulates platelet reactivity by activating members of a family of seven-transmembrane domain containing receptors known as G-protein-coupled receptors (GPCRs) (Offermanns 2006) . Platelets express several GPCRs and upon activation by soluble agonists they elicit intracellular signaling which is mediated by heterotrimeric guanine nucleotide-binding proteins (G proteins) (Offermanns 2006) (Figure 4).

Heterotrimeric G proteins consist of an α-subunit in complex with the tightly associated β- and γ-subunits. The heterotrimeric α, β, and γ complex exists in two states: an inactive state where the α-subunit is bound to guanosine diphosphate (GDP) and complexed with $\beta\gamma$-subunits; and an active state where the $G\alpha$-subunit is bound to guanosine triphosphate (GTP) and dissociated from $\beta\gamma$-subunits. Agonist bound GPCRs undergo conformational changes that

catalyze the exchange of GDP for guanosine triphosphate (GTP) on the Gα-subunit (Venkatakrishnan et al. 2013, Kobilka and Deupi). Consequently, the GTP-bound Gα-subunit also undergoes conformational changes within the switch region causing it to dissociate from the $\beta\gamma$-subunits. All Gα-subunits contain a conserved GTPase domain which is involved in binding $\beta\gamma$-subunits, GPCRs, and effector proteins (Oldham and Hamm 2008). Upon activation Gα-GTP- and $\beta\gamma$-subunits transduce GPCR signaling by binding to downstream effector proteins such as phospholipase Cβ and PI3 kinase. Gα-GTP signal propagation is then terminated by GTP hydrolysis, which is mediated by the relatively slow intrinsic GTPase activity of Gα-subunits or by the binding of GTPase-activating proteins (GAP). Based on primary sequence homology of the Gα-subunit, heterotrimeric G proteins are grouped into four classes: G$\alpha_{i/o}$, Gα_s, G$\alpha_{q/11}$ and G$\alpha_{12/13}$ (Oldham and Hamm 2008). Each class of G protein binds to a distinct effector protein, except for Gα_i and Gα_s that both bind to adenylyl cyclase. Platelets express $G_{q/11}$, $G_{i/z}$, $G_{12/13}$ and G_s α-subunits which couple to various platelet GPCRs (Offermanns 2006) (Figure 4). Physiological platelet inhibitors, such as prostaglandin and adenosine, bind the prostacyclin receptor (PGI_2) (Murata et al. 1997) and adenosine receptor ($A2_a$) (Ledent et al. 1997), respectively, both of which couple to Gα_s. Gα_s stimulates adenylyl cyclase to raise intracellular cyclic AMP which inhibits platelet function (Offermanns 2006). Gαi also binds adenylyl cyclase, but inhibits its activity, and is activated by the platelet agonist ADP which binds to the Gα_i-coupled $P2Y_{12}$. ADP also binds $P2Y_1$ which is coupled to Gα_q (Jantzen et al. , Léon et al. 1999, Jin and Kunapuli 1998). The potent platelet agonist thrombin stimulates platelets via cleavage and activation of protease activated receptors (PAR), and at low doses of thrombin platelet response requires a cooperative signaling mechanism mediated

by PAR and GPIb-IX-dependent signaling to promote platelet activation (Estevez et al. 2016). PAR1 and PAR4 are expressed in human platelets, and PAR3 and PAR4 are expressed in mouse platelets (Kahn et al. 1998, Sambrano et al. 2001, Kahn et al. 1999). PAR3 is believed to function as a cofactor for PAR4 activation and not to induce platelet activation signaling itself (Nakanishi-Matsui et al. 2000). PAR1 and PAR4 are coupled to both $G\alpha_q$ and $G\alpha_{13}$ (Offermanns et al. 1997, Moers et al. 2003, Moers et al. 2004). TXA_2 activates platelets by binding to the thromboxane-prostanoid (TP) receptor (Thomas et al. 1998), which couples to both $G\alpha_q$ and $G\alpha_{13}$ (Offermanns et al. 1997, Knezevic, Borg, and Le Breton 1993, Moers et al. 2003, Offermanns et al. 1994, Moers et al. 2004). The weak platelet agonist epinephrine binds to the α_{2A} adrenergic receptor (α_{2A}) that is believed to couple to $G\alpha_z$, which is a member of the $G\alpha_i$ family (Yang et al. 2000, Offermanns 2006, Požgajová et al. 2006) (Figure 4).

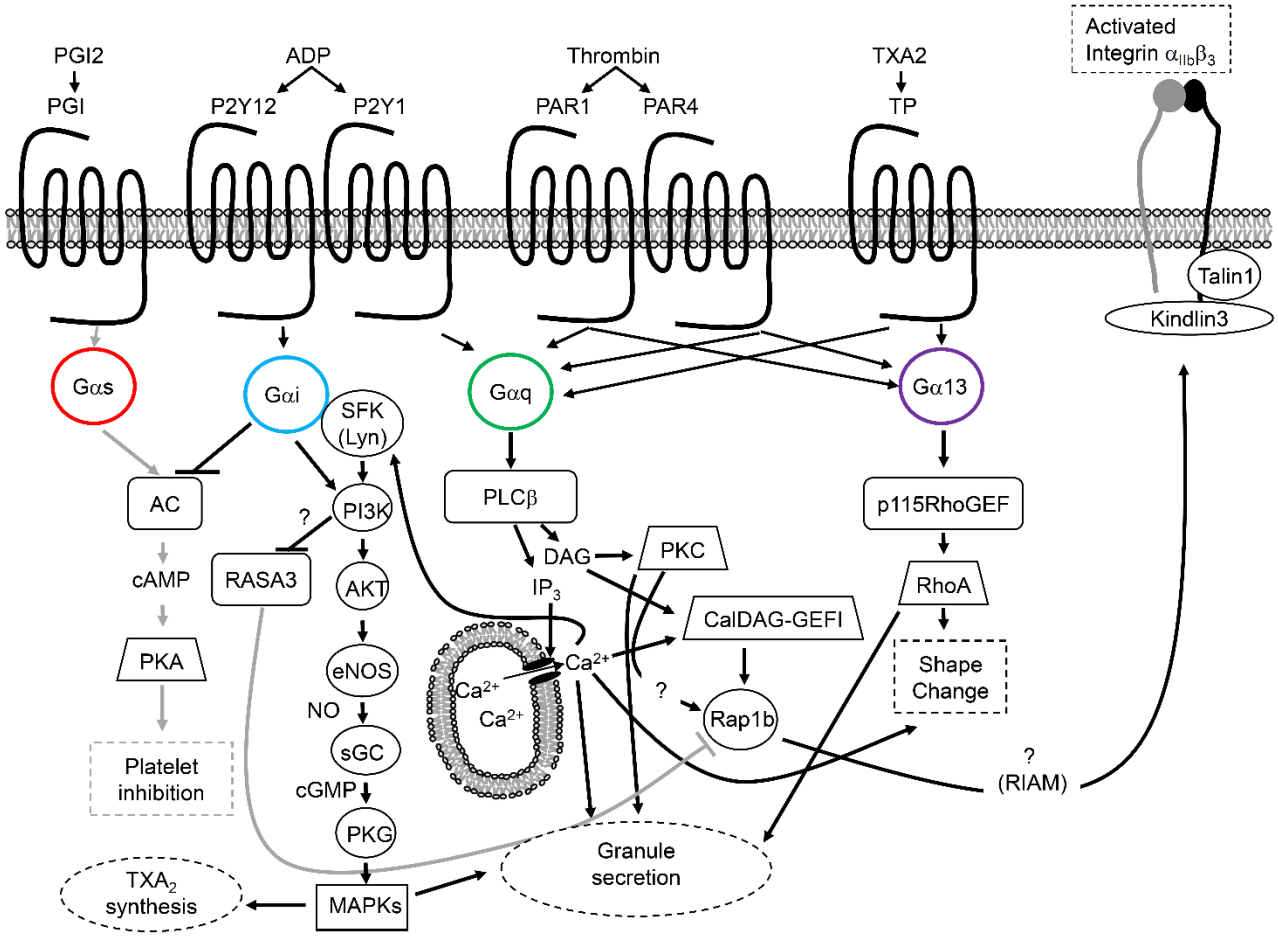


Figure 4. Platelet G protein-coupled receptor signaling in platelets. The major GPCRs expressed in platelets which couple to different G proteins that elicit signal transduction leading to either platelet activation (Gαi, Gαq , Gα13) or inhibition (Gαs), depicted with blue, green, purple or red outline, respectively. Grey colored lines/arrows are used to show inhibitory signaling pathways, whereas black lines/arrows show stimulatory pathways.

1.3.1 Gq-mediated signaling

Upon activation of Gαq-coupled GPCRs, Gαq binds and activates PLCβ isozymes that catalyze hydrolysis of PIP_2 to form second messengers IP_3 and DAG. Essential interfaces for Gαq binding and activation of PLCβ are thought to involve the proximal and distal C-terminal domains on PLCβ and the switch and N-terminal regions of Gαq (Lyon and Tesmer 2013, Nance et al. 2013, Waldo et al. 2010, Baltoumas, Theodoropoulou, and Hamodrakas 2013). Although in other cells PLCβ can be activated by Gβγ or Rac1, in platelets PLCβ activity appears to be primarily dependent on Gαq, because Gαq null platelets do not release IP_3 or mobilize calcium in response to GPCR agonist stimulation (Offermanns et al. 1997). Gαq has also been shown to be critical for platelet aggregation in response to a variety of platelet agonists. Mice null for Gαq display severe aggregation defects in response to stimulation by GPCR agonists (thrombin, ADP and TXA2) or collagen (Offermanns et al. 1997). The latter defect is due the importance of TXA_2 production in promoting collagen-induced platelet activation. Although Gαq null platelets undergo normal shape change upon thrombin, collagen or U46619 stimulation, they do display defective ADP-induced platelet shape change (Offermanns et al. 1997), which may involve a Ca^{2+}/calmodulin-dependent myosin light chain kinase pathway (Paul, Daniel, and Kunapuli 1999, Bauer et al. 1999). In addition, $G\alpha_q$ has also been reported to regulate ADP-induced RhoA activation (Moers et al. 2004); and this effect may involve other $G\alpha_q$ effectors including guanine exchange factors (GEF), such as p63RhoGEF or LARG (Rojas et al. 2007, Vogt et al. 2003).

1.3.2 G13-mediated signaling

Platelets express both $G\alpha_{12}$ and $G\alpha_{13}$, however, $G\alpha_{12}$ appears to be dispensable for GPCR-induced platelet shape change and platelet activation. By contrast, $G\alpha_{13}$ is critical for thrombin- or U46619 (a TXA_2 analog)-induced platelet aggregation and secretion, particularly at low doses of agonist. $G\alpha_{13}$ null platelets show defects in thrombin and U46619-induced shape change, and this is consistent with the finding that low dose RhoA activation and subsequent MLC phosphorylation was abolished by deletion of $G\alpha_{13}$ (Moers et al. 2003, Offermanns et al. 1994, Moers et al. 2004). Activation of $G\alpha_{13}$-coupled GPCRs elicits signaling through the small monomeric GTP-binding protein Rho. The link from GPCR-activation to Rho signaling is mediated by $G\alpha_{13}$-binding to p115RhoGEF. The $G\alpha_{13}$-p115RhoGEF binding interface involves residues in the switch region and α3 helix of $G\alpha_{13}$ that interact with RGS homology (RH) and DH domains of p115RhoGEF (Kozasa et al. 2011, Hajicek et al. 2011); and this interaction stimulates GDP to GTP exchange on Rho via the DH domain of p115RhoGEF (Kozasa et al. 2011, Baltoumas, Theodoropoulou, and Hamodrakas 2013). GTP-bound RhoA activates its effectors such as Rho-associated protein kinases (ROCK). ROCK phosphorylates myosin light chain phosphatase, and thereby inhibits its activity toward myosin light chain (MLC), which is the regulatory subunit of myosin IIA (Suzuki et al. 1999, Klages et al. 1999). MLC phosphorylation is critical for platelet shape change and clot retraction, and promotes secretion and aggregation (Léon et al. 2007, Bauer et al. 1999, Paul, Daniel, and Kunapuli 1999, Getz et al. 2010, Johnson et al. 2007). In agreement with an important role for the RhoA/ROCK/MLC pathway in platelet activation, studies have shown that nonmuscle myosin heavy chain IIA (NMHC-IIA) or RhoA deficient platelets display defective shape change and partial reductions in aggregation and secretion in response to low dose

stimulation of Gα13-coupled receptors. In addition, although RhoA and NMHC-IIA appear dispensable for spreading on fibrinogen, platelets deficient for either of these proteins displayed defective clot retraction. Another interesting finding from these studies was that RhoA null platelets displayed enhanced aggregation responses to collagen and CRP. By contrast, NMHC-IIA-deficient platelets were suggested to display comparable collagen response to wildtype controls, but in fact, the data do seem to indicate partial enhancement of aggregation at low doses of collagen (Pleines et al. 2012, Léon et al. 2007). Remarkably, in platelets null for RhoA effector, ROCK1, no defects in collagen-induced shape change, platelet aggregation, or secretion were reported (Dasgupta et al. 2013). Pharmacological evidence suggests that ROCK promotes thrombin-induced shape change, aggregation, secretion, and LIMK1 activation (Pandey et al. 2006). In addition, LIMK1 null platelets also do not phenocopy RhoA null platelets (Estevez et al. 2013). Thus, future studies are necessary to elucidate the mechanisms responsible for differential roles of RhoA and its effectors in different signaling pathways.

1.3.3 Gi-mediated signaling

Despite the lack of aggregation response to GPCR agonists in platelets lacking both Gαq and Gα13, Gαi family members also play important roles in promoting platelet activation. Gαi binds adenylyl cyclase, via an interface that is suggested to involve the α4-β6 loop on Gαi and C1 domain on adenylyl cyclase (AC), and this interaction inhibits synthesis of cyclic adenosine monophosphate (cAMP) (Dessauer et al. 1998, Baltoumas, Theodoropoulou, and Hamodrakas 2013, Taussig, Iniguez-Lluhi, and Gilman 1993). Elevated cAMP activates cAMP-

dependent protein kinase (PKA) signaling which inhibits platelet activation. Thus, by inhibiting AC Gαi relieves the inhibitory effects of the cAMP/PKA pathway on platelet function (Raslan and Naseem 2014, Siess and Lapetina 1990, Beck et al. 2014, Aburima et al. 2013, Best et al. 1977). Gαi signaling is particularly important for promoting platelet responses to threshold doses of GPCR agonists or collagen. This is largely due to positive feedback from agonist-induced secretion of ADP which then activates Gαi-coupled P2Y12 receptors. In fact, P2Y12 null platelets display significantly diminished aggregation response at low doses of GPCR agonists or collagen (Foster et al. 2001, Jin and Kunapuli 1998, Nurden et al. 1995). Interestingly, Gαi2 null mice display only a partial defect in agonist-induced inhibition of cAMP synthesis (Devanathan et al. 2015, Jantzen et al. , Yang et al. 2002), which is abolished in ADP-stimulated P2Y12 null platelets (Nurden et al. 1995, Hollopeter et al. 2001, Foster et al.). A recent study suggests that in the absence of Gαi2, Gαi3 may partially compensate in promoting ADP-induced platelet activation, because when Gαi2 null platelets were pretreated with P2Y12 antagonist, ARC69931, the residual ADP-induced aggregation was abolished; and furthermore, each Gαi protein was shown to be upregulated when the other was absent (Devanathan et al. 2015). In agreement with this notion, pertussis toxin-treatment of wildtype (WT) platelets resulted in greater inhibition of platelet function than that observed in Gαi2 null platelets (Devanathan et al. 2015). In addition, integrin activation is also diminished in Gαi2 null platelets, suggesting that Gαi signaling is important for integrin activation (Devanathan et al. 2015, Jantzen et al.). To resolve issues due to compensation future studies should investigate double $G\alpha i_{2/3}$ knockout platelets. Gαi signaling not only reduces cAMP levels but also promotes platelet activation via cAMP-independent mechanisms. Importantly, direct inhibition of the major Gαi family effector, adenylyl cyclase, appears to be insufficient to rescue

the defective aggregation in Gαi2 or Gαz null platelets (Yang et al. 2002, Lova et al. 2003). The Gαi signaling pathway has been shown to involve PI3K (Hirsch et al. 2001, Lian et al. 2005, Kauffenstein et al. 2001, Cosemans et al. 2006, Schoenwaelder et al. 2007, Canobbio et al. 2009), particularly Gβγ-regulated PI3Kβ/γ family members (Kurosu et al. 1997, Thomason et al. 1994, Vanhaesebroeck and Waterfield 1999, Maier, Babich, and Nürnberg 1999), and the small GTP-binding RAS-related protein 1b (Rap1b) (Lova et al. 2003, Lova et al. 2002, Chrzanowska-Wodnicka et al. , Woulfe et al. 2002), which promote integrin activation and platelet aggregation. Recently, a GTPase-activating protein (GAP) for Rap1b, RASA3, was shown to be regulated downstream of PI3K/Gαi-signaling, and inhibition of RASA3 by the Gαi-coupled P2Y12 pathway appears to be important for promoting sustained Rap1b signaling leading to integrin activation and thrombus formation (Stefanini et al. 2015, Stefanini and Bergmeier 2016).

1.4 Common platelet activation pathways

Downstream of various platelet surface receptors, PLC isozymes serve as hubs upon which numerous platelet activation signaling pathways converge. Platelets express at least three distinct families of PLC: PLCβ, PLCγ and PLCδ. In human platelets PLCγ2, PLCβ2, and PLCβ3 appear to be the predominantly expressed PLC family members (Lee et al. 1996, Lian et al. 2005). PLCβ and PLCγ are activated via different mechanisms (Han et al. 2011, Lyon and Tesmer 2013, Rhee 2001). The former is activated by binding Gαq proteins and the latter by ITAM-dependent protein tyrosine phosphorylation (Lyon and Tesmer 2013, Mangin et al. 2003, Gross et al. 1999, Pasquet et al. 1999, Suzuki-Inoue et al. 2003). This difference in

regulation is imparted by a C-terminal coil-coil domain found in PLCβ that is not present in PLCγ. By contrast, PLCγ contains SH2, SH3 and split PH domains that are not found in PLCβ (Rhee 2001). Upon activation, PLC catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) into diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3). IP_3 binds the IP_3 receptor resulting in Ca^{2+} release from the dense tubular system into the cytosol. In addition, DAG activates protein kinase C. Elevations in intracellular Ca^{2+} concentrations and activated protein kinase C (PKC) lead to integrin activation, granule secretion, and platelet aggregation via the Ca^{2+}- and - DAG-regulated guanine nucleotide exchange factor I (CalDAG-GEFI), a GEF for Rap1b.

1.4.1 Calcium signaling

Regardless of the agonist receptor engaged, nearly all platelet agonists elicit elevations in intracellular calcium. Elevated calcium levels regulates the activity of several signaling molecules including PKC, calmodulin, CalDAG-GEFI, and proteases (e.g. calpain, gelsolin); and these molecules play roles in several processes, such as granule secretion, shape change, and integrin activation(Li et al. 2010). There are two components that give rise to increases in intracellular calcium: (1) release of calcium from intracellular stores in the dense tubular system (DTS) and (2) influx of extracellular calcium through plasma membrane (Varga-Szabo, Braun, and Nieswandt 2009). IP_3 binds and opens the intracellular IP_3 receptor (IP_3R), a calcium channel, thus triggering intracellular calcium release. Upon depletion of intracellular Ca^{2+} stores, plasma membrane Ca^{2+} channels open and extracellular Ca^{2+} enters the cytosol, and also refills the DTS stores. The two major proteins that are critical in this process, known

as store-operated calcium entry (SOCE), are stromal interaction molecule 1 (STIM1) and Orai1(Varga-Szabo, Braun, and Nieswandt 2009). STIM1 functions as a Ca^{2+} sensor in the DTS and Orai1 is the SOC channel in the plasma membrane. SOC-induced calcium influx is nearly abolished in STIM1 null platelets (Varga-Szabo et al. 2008, Grosse et al. 2007). Interestingly, despite a general defect in calcium elevation induced by distinct agonists (GPCR and GPVI agonists), STIM1 null platelets displayed a selective platelet activation defect only in the GPVI/ITAM pathway (Varga-Szabo et al. 2008, Grosse et al. 2007). Although one report suggested that Orai1 null platelets mimic the defective SOC-induced and agonist-induced calcium elevation responses reported for STIM1 null platelets, in particular the selective defect in GPVI/ITAM-induced platelet activation (Braun et al. 2009), another report using R93W ORAI1 mutant mice (a loss of function mutation i.e., does not block expression only function) suggests a minor role for ORAI1 also in PAR-induced platelet activation. Nonetheless, how STIM1 and Orai1 play a role in the GPVI/ITAM pathway is unclear (Braun et al. 2009, Varga-Szabo et al. 2008), and how STIM1, but not ORA1 affects intracellular calcium elevation is unclear (Bergmeier et al. 2009, Braun et al. 2009, Varga-Szabo et al. 2008). There may also be other SOC or receptor-operated channels whose role in platelet activation are yet to be elucidated (Gilio, van Kruchten, et al. 2010, Ramanathan et al. 2012).

1.4.2 <u>Protein kinase C</u>

Platelets express at least seven isozymes of the serine/threonine PKC family, which are grouped into conventional (α,β), novel (δ,θ,η,ε), and atypical(ζ) subfamilies. The groupings are based on the presence or absence of DAG- and Ca^{2+}-binding domains: the conventional PKCs

contain both DAG and Ca^{2+}-binding domains, the novel PKCs contain only DAG-binding, and the atypical PKCs are lacking both of these domains (Heemskerk et al. 2011, Murugappan et al. 2004). Deciphering the roles of individual PKC isoforms in platelet function is complex: a single PKC isozyme can have opposite roles in platelet activation in response to different agonists, and multiple PKC isozymes can support or antagonize each other in the same pathway. PKC α/β have been shown to play an important role in promoting granule secretion, integrin activation, and platelet aggregation induced by GPVI and PAR stimulation (Konopatskaya et al. 2009). On the other hand, in the ADP/P2Y1 pathway, PKCα/β isozymes appear to negatively regulate TXA_2 production, and phosphorylation of Lyn and PKCδ (Bhavanasi et al. 2015). By contrast, PKCδ appears to promote ADP-induced granule secretion and TXA_2 production downstream of Lyn; and furthermore, Lyn regulates PKCδ Tyr^{311} phosphorylation, and PKCδ may promote this ADP pathway independent of its catalytic activity (Bhavanasi et al. 2015). It is unclear how Lyn is inhibited by PKCα/β, and how PKCδ can signal independent from its kinase activity. In the GPVI pathway, PKCδ negatively regulates platelet aggregation, and possibly also dense granule secretion, but in the PAR pathway it promotes aggregation and secretion (Pula et al. 2006, Bhavanasi et al. 2015, Murugappan et al. 2004). Interestingly, PKCδ is suggested to promote PAR- and GPVI-induced phosphorylation of protein kinase D2 (PKD2) (Bhavanasi et al. 2011), a PKC substrate. In contrast, a study using PKD2 knock-in mouse platelets, expressing catalytically inactive PKD2, showed defective responses to both CRP and thrombin stimulation (Konopatskaya et al. 2011); and in addition, PKD2 can still be phosphorylated in GPVI or PAR-stimulated PKCδ null platelets (Bhavanasi et al. 2011), suggesting PKCδ-independent functions of PKD2. PKCθ appears to play a stimulatory role in PAR-induced platelet

aggregation, secretion, and integrin activation (Cohen et al. 2009, Nagy et al. 2009). However, there is controversy over the role of PKCθ in GPVI-dependent platelet activation with two groups reporting opposite results (Hall et al. 2008, Gilio, Harper, et al. 2010, Nagy et al. 2009). PKCε is only expressed in mouse platelets where it appears to play an important role in promoting GPVI-induced platelet activation (Pears et al. 2008). PKCs are known to directly phosphorylate several substrates, such as pleckstrin, myristoylated alanine-rich C kinase substrate (MARCKS), and SNAP-23; however, it is mechanistically unclear whether and how individual PKC isozymes display selectivity for these substrates in intact platelets (Hartwig et al. 1992, Polgár et al. 2003, Tyers et al. 1988, Heemskerk et al. 2011, Lian et al. 2009, Pula et al. 2006). Nonetheless, PKC-dependent phosphorylation of these substrates regulates granule secretion, actin cytoskeletal dynamics, and integrin activation. Future studies may clarify the differential roles of various PKCs, and also reveal how individual PKC isozymes, perhaps spatially constrained to act on specific substrates, can give rise to their distinct roles in platelet activation.

1.5 GPIb-IX-V and PARs in thrombin signaling

Since the mid1970s there have been numerous reports suggesting that the platelet GPIb-IX receptor complex functions as a thrombin receptor. Early evidence for the functional role of GPIb-IX in platelet thrombin response was the demonstration that glycocalicin could inhibit the binding of thrombin to platelets. The level of thrombin binding to platelets was later found to be proportional to the amount of intact GPIb on the platelet surface, and the thrombin binding site resided within an extracellular 45 kDa trypsin-sensitive fragment of GPIbα

(Okumura and Jamieson 1976a, Okumura, Hasitz, and Jamieson 1978). The clinical significance of thrombin-GPIb interaction was first observed in patients with Bernard-Soulier syndrome (BSS), a disease resulting in defective hemostasis. BSS platelets display diminished thrombin binding and thrombin-induced platelet aggregation, but only at low doses of thrombin (Jamieson and Okumura 1978), and this phenotype is not due to defective expression of the "moderate affinity receptors" (prior to cloning of the platelet protease-activated receptors they were referred to as moderate and low-affinity receptors) (McNicol et al. 1996). It was later determined that patients with BSS have absent or abnormal expression of GPIbα, GPIbβ, or GPIX (Kenny et al. 1999, Moran et al. 2000, Ware et al. 1993). Studies of the affinity of thrombin for GPIb using radio-labeled thrombin, anti-GPIbα monoclonal antibodies, and GPIb peptide mimetics demonstrated that platelets express different sub-populations of thrombin receptors with varying affinities for thrombin (Greco et al. 1996, Gralnick et al. 1994). GPIb-IX was classified as a high affinity thrombin receptor and moderate and low affinity thrombin receptors were also present contributing only at high concentrations of thrombin. Interestingly, the number of GPIb-IX receptors expressed on platelets is far greater than the number of high affinity thrombin-binding sites suggesting that the high affinity thrombin-binding site of GPIb is regulated. The moderate affinity receptors are now understood to be the protease activated receptors (PARs), a family of G-protein coupled receptors directly activated by thrombin via a tethered ligand mechanism (Vu et al. 1991). In platelets, the PARs have been shown to be critical for thrombin signaling (Sambrano et al. 2001, Kahn et al. 1999). Interestingly, PAR-3 appears not to signal itself, but serves as a cofactor for PAR-4 activation (Nakanishi-Matsui et al. 2000). In a functionally analogous manner to PAR3, GPIb-IX has been reported to function as a cofactor promoting thrombin-mediated PAR-1 cleavage (De Candia et al. 2001). However,

there is evidence suggesting GPIb-IX may itself signal when thrombin is immobilized (Adam, Guillin, and Jandrot-Perrus 2003), or in combination with PARs (Lova et al.) or GPV, a thrombin substrate and subunit of the GPIb complex, may unmask a thrombin receptor function of GPIb (Bienz, Schnippering, and Clemetson 1986).

1.6 LIM kinases

In search of novel protein kinases a genomic fragment of chick c-sea (cellular sarcoma, erythroblastosis, and anemia), a cellular homologue of the transforming oncogene v-sea (encoded by the avian S13 retrovirus) receptor tyrosine kinase (Huff et al. 1993), was used to screen a human liver cancer cell line cDNA library. From this search human LIMK1 (Mizuno et al. 1994), and subsequently human LIMK2 (Nunoue et al. 1995), were discovered and characterized as LIM domain-containing protein kinases. Lim kinase 1 (LIMK1) and Lim kinase 2 (LIMK2), form the Lim kinase family (LIMK) of serine/threonine kinases. In humans, LIMK1 is found on chromosome 7 and spans 16 exons; whereas LIMK2 is found on chromosome 22 and spans 18 exons. LIMK1 and LIMK2 have 50% overall amino acid sequence homology with greater homology within the kinase domains (Nunoue et al. 1995, Okano et al. 1995). In mice, LIMK1 was also identified using mRNA from an epithelial cell line, which the authors called protein kinase and zinc finger domains, Kiz-1. Later, LIMK2 was also identified in mice (Bernard et al. 1994, Ikebe et al. 1997). It is now known that the LIMK family, in particular LIMK1, is evolutionarily conserved and found in several fish, amphibian, primate, marsupial, reptile, bird, and insect species (Scott and Olson 2007).

1.6.1 LIM kinase structure and function

The number of amino acids in LIMK1 is 647 and in LIMK2 638, and both are approximately 72 kDa. The name 'LIM' is derived from the homeodomain containing transcription factors, Lin-11, Isl-1, and Mec-3, where the double zinc finger LIM motif was first characterized (Kadrmas and Beckerle 2004, Bach 2000). Individual LIM domains contain eight conserved zinc-binding residues: two independent zinc-binding modules with a two amino acid spacer in between, and each is composed of three cysteines and a histidine (or fours cysteines) which together coordinate two zinc atoms, and because they occur in tandem they form a two-finger-like topology (i.e. two zinc fingers) (Kadrmas and Beckerle 2004, Dawid, Breen, and Toyama 1998). In LIMK there are two of these LIM domains at the N-terminus. The full-length LIMK protein organization consists of, two N-terminal LIM domains, a PDZ domain, a serine/proline rich region and a C-terminal kinase domain (Figure 5). The LIM domains are important for intermolecular protein-protein interactions which regulate LIMK catalytic activity (Edwards and Gill 1999, Nagata, Ohashi, and Mizuno 1999).

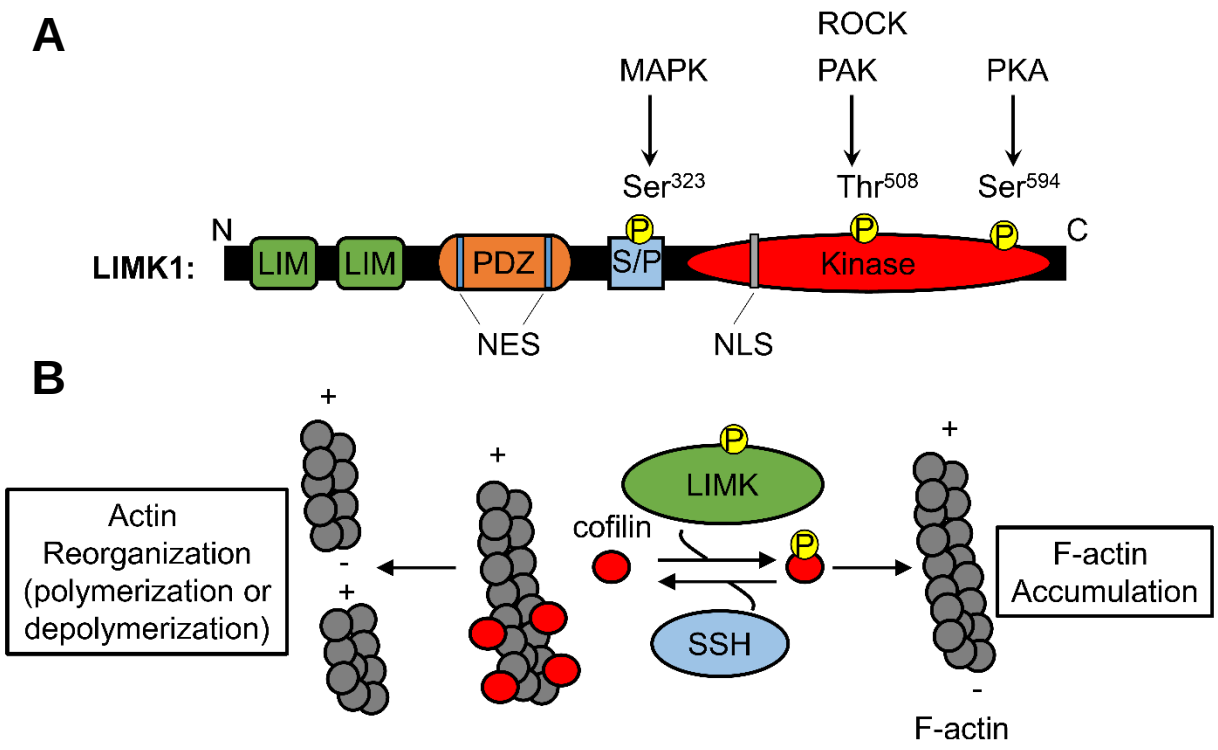


Figure 5. LIMK1 structure and function. A, Protein domain structure of LIMK1, and phosphorylation sites for kinases that have been shown to activate the catalytic activity of LIMK1 toward its major substrate cofilin. NES, Nuclear export signal; NLS, Nuclear localization signal; S/P, serine and proline-rich region. **B,** A schematic showing the most well-established function of LIMK1 in regulating actin reorganization. LIMK1 phosphorylates and inactivates cofilin inhibiting its binding to F-actin, and thereby enabling F-actin to accumulate. However, cofilin is dephosphorylated by phosphatases(e.g, slingshot, SSH) enabling it to bind and sever F-actin filaments exposing new barbed ends. +, growing ends or barbed ends; -, pointed ends.

In cells, LIMK overexpression promotes actin filament (F-actin) accumulation. Conservative mutations in the second LIM domain (LIM2) or PDZ domain appear to enhance LIMK-dependent actin filament accumulation activity, as per formation of punctate fluorescent antibody conjugated phalloidin staining, suggesting that these domains negatively regulate LIMK-dependent F-actin accumulation. However, single amino acid and/or conservative LIM2 or PDZ domain mutations do not affect in vitro activity of LIMK, in terms of its ability to phosphorylate its substrate cofilin. By contrast, deletion or mutation of both N-terminal LIM domains increases the catalytic activity of LIMK in vitro. Thus, the LIM domains negatively regulate the kinase domain of LIMK, whereas the negative regulatory role of the PDZ domain on LIMK-dependent F-actin accumulation is less clear but may be due to indirect effects, such as alterations in LIMK dimerization or modulating LIMK binding to other proteins. In agreement with the former, LIMK have been shown to form homo- and heterodimers (e.g., LIMK1-LIMK1 or LIMK1-LIMK2), and these appear to form in an anti-parallel fashion. In fact, GST-tagged N-terminal LIMK1 LIM domains (amino acids 22-140) were used to precipitate the C-terminal LIMK protein kinase domain lacking the LIM domains. In contrast, GST-PDZ (amino acids 144-296) could not precipitate the C-terminal kinase domain (Hiraoka et al. 1996, Nagata, Ohashi, and Mizuno 1999). Accordingly, it is now believed that LIMK monomers form intramolecular interactions and also dimerize via N- and C-terminal domain interactions: N-terminal LIM domains of one LIMK monomer interact with the C-terminal domain of another LIMK molecule (or with its own C-terminal domain), and the C-terminal kinase domain of a LIMK monomer interacts with the N-terminal LIM domains of another LIMK molecule (or with its own N-terminal domain) (Hiraoka et al. 1996). The following findings are consistent with the notion of LIMK

self-association and homo- and heterodimer formation: transfection of LIM domains (or full-length kinase dead LIMK1) into cells inhibits the actin filament accumulation activity of both full-length LIMK1, as well as that induced by the kinase domain alone (Edwards and Gill 1999); GST-tagged N-terminal (amino acids 4-215) or LIM domains (amino acids 1-144) can bind to LIMK fragments in vitro as long as they maintain an intact kinase domain (amino acids 297-610), and adding exogenous GST-LIM domain inhibits in vitro LIMK1 mediated phosphorylation of cofilin (Nagata, Ohashi, and Mizuno 1999); and LIMK2-specific monoclonal antibodies can Co-IP LIMK1 (Acevedo et al. 2006), which is similar to the finding that immunoprecipitation of untagged LIMK1 can be used to detect LIMK1-LIMK2 dimers in cells expressing tagged LIMK2 (Hiraoka et al. 1996). Interestingly, no consensus motif for binding to the LIM domain has yet emerged, although there are several proteins known to bind to LIM domains (Dawid, Breen, and Toyama 1998, Bach 2000, Kadrmas and Beckerle 2004).

In addition to modulating LIMK-dependent actin filament accumulation in cells (Edwards and Gill 1999), the LIMK PDZ domain has been shown to regulate LIMK localization. The PDZ domain contains two leucine-rich nuclear export signal (NES) sequences (amino acids 231-242 and 243-255). Normally, LIMK1 is located in the cytoplasm, however, upon mutation of both its PDZ NES motifs LIMK1 appears to be primarily retained in the nucleus. Nuclear localization of LIMK1 may be regulated by a highly basic motif in the kinase domain(amino acids 499-503, KKPDRKKR), because deletion of this putative nuclear localization signal (NLS) appears to reduce nuclear accumulation of LIMK1 (Yang and Mizuno 1999). LIMK family members have some other notable features within the kinase domain: an Asp-Leu-Asn-Ser-His-Asn (DLNSHN) sequence in subdomain VIB, which is not characteristic of serine/threonine (DLKXXN) or tyrosine kinases (DLAARN or DLRAAN); and a highly basic RSLKKPDRKKR

sequence in the activation loop between subdomains VII and VIII. As discussed above, the basic insert sequence may be important in LIMK1 localization. However, another group did not report a role for this basic sequence in LIMK localization. Instead, the basic insert sequence was shown to promote LIMK-dependent F-actin accumulation, which the authors suggested could be due to its importance in recruiting LIMK activating kinases (Edwards and Gill 1999). The Asp^{460} (in an IHRDL motif) is the catalytic active site of LIMK1 (or in LIMK2 Asp^{451}) and is required for phosphorylation of substrates. LIMK kinase domain catalytic activity is required for promoting actin filament accumulation (Arber et al. 1998, Edwards and Gill 1999). Expression of only the kinase domain of LIMK is sufficient to exert actin filament accumulation activity (Edwards and Gill 1999, Nagata, Ohashi, and Mizuno 1999). One important caveat of these studies would be that there is marked contrast between the cellular actin accumulation morphology induced by overexpression of the kinase domain alone to that induced by full-length LIMK (Arber et al. 1998, Nagata, Ohashi, and Mizuno 1999). High, and perhaps non-physiological, levels of LIMK activity are likely balanced by cellular regulatory mechanisms (e.g., increased phosphatase activity) that may prevent continuous stabilization of actin filaments, which would be disruptive for normal cellular processes such as cell division. Moreover, full-length LIMK contains regulatory N-terminal LIM domains that repress kinase activity. Therefore, punctate actin filament accumulation occurring simultaneously throughout the cytoplasm, particularly that shown in studies using kinase domain alone, may not represent physiological actin cytoskeletal reorganization.

Based on amino acid sequence differences between LIMK PDZ domains monoclonal antibodies were generated that specifically recognize the PDZ domain of mouse LIMK1 or LIMK2. In mice, both LIMK1 and LIMK2 appear to be ubiquitously expressed, however, their

expression levels may be distinct in different tissues (Foletta et al. 2004, Acevedo et al. 2006). In humans, a similar LIMK expression pattern may also be true (Okano et al. 1995, Foletta et al. 2004, Acevedo et al. 2006). There is also evidence, primarily at the mRNA level, for splice variants of LIMK1 and LIMK2 (Okano et al. 1995, Ikebe et al. 1997, Ikebe, Ohashi, and Mizuno 1998, Edwards and Gill 1999). Thus, besides full-length LIMK1 and LIMK2 (LIMK2a) there appears to be several other LIMK splice variants: dLIMK1, LIMK1-s, LIMK2b, tLIMK2, and LIMK2c. dLIMK1 contains a 12 amino acid insertion at Phe^{294}(FARTWVALSPSA) and is missing the entire kinase domain (Edwards and Gill 1999); whereas LIMK1-s (LIMK1-short) lacks 20 amino acids in the kinase domain(Bernard et al. 1994). LIMK2b is only missing part of the first LIM domain. tLIMK2 is missing both LIM domains and part of the PDZ domain. LIMK2c contains a 6 amino acid insertion in the kinase domain (Ikebe et al. 1997, Ikebe, Ohashi, and Mizuno 1998). Interestingly, the mRNA expression pattern of tLIMK2 and LIMK2c appears to be tissue specific (Ikebe, Ohashi, and Mizuno 1998, Ikebe et al. 1997). The role of these LIMK isoforms is unclear. In mouse testes LIMK2 appears to be exclusively expressed in elongated spermatids, whereas LIMK1 is expressed in spermatocytes, sertoli cells, and early spermatids. Thus, in mice there may be distinct developmental roles for LIMK1 and LIMK2 in the testes. In mouse fibroblasts, LIMK1 is localized to the nuclear and cytoplasmic compartments and enriched in focal adhesions. By contrast, LIMK2 appears localized in the nucleus and throughout the cytoplasm, but does not appear to be enriched in focal adhesions. Interestingly, LIMK1, but not LIMK2, was shown to associate with paxillin by co-immunoprecipitation in NIH3T3 cells, suggesting functional differences in these cells.

The most well-established function of LIMK is in the regulation of F-actin accumulation (Bernard 2007, Scott and Olson 2007). Due to prior knowledge of the importance of the small

Rho GTPases, Rac, Rho, and Cdc42, in regulating actin cytoskeleton reorganization (Hall 1998), in the late 1990s several groups reported that Rho GTPases function as key upstream activators of LIMK (Arber et al. 1998, Yang et al. 1998, Edwards et al. 1999, Maekawa et al. 1999, Sumi et al. 1999). Lamellipodia formation induced by overexpression of constitutively active Rac (RacV12) in cells was found to be inhibited by cotransfection with kinase inactive LIMK1 (Arber et al. 1998, Yang et al. 1998). Interestingly, in fibroblasts stimulated with phorbol 12-myristoyl-13-acetate (PMA), a PKC activator, LIMK1 phosphorylation was inhibited, suggesting PKC signaling may negatively regulate LIMK1 (Arber et al. 1998). Constitutively active Cdc42 (Cdc42V12) was also found to stimulate LIMK1 activation. Although Cdc42-induced filopodia formation was not inhibited by cotransfection with kinase inactive LIMK1, actin reorganization and accumulation appears altered compared to cells cotransfected with wildtype LIMK1, suggesting at least a partial role for LIMK1 in Cdc42-dependent actin reorganization (Arber et al. 1998, Edwards et al. 1999). By contrast, LIMK2 was shown to mediate Cdc42-induced filopodia and Rho-induced stress fiber formation, but was not required for lamellipodia formation induced by Rac (Maekawa et al. 1999, Sumi et al. 1999). However, LIMK1 was also shown to play a role downstream of Rho signaling leading to stress fiber formation (Maekawa et al. 1999). Taken together, LIMK2 may have a specific role in mediating Cdc42-dependent actin reorganization, whereas LIMK1 may play a specific role in mediating Rac-dependent actin reorganization, and Rho signaling may activate both LIMKs. How small Rho GTPases accomplish specific activation of LIMK1 or LIMK2 is still unclear (Figure 6).

LIMK1 null mice display altered hippocampal neuron function, abnormal neuronal actin morphology and impaired learning behaviors (Meng et al. 2002). Similarly, LIMK1/2 double knockout mice also display abnormal actin accumulation and hippocampal neuron function

(Meng et al. 2004). Surprisingly, LIMK1/2 double knockout mice (LIMK 1/2 KO) are fertile; especially, in light of a report of defective spermatogenesis in LIMK2 KO mice (Takahashi et al. 2002). Additionally, LIMK1 KO mice display reductions in bone density; the number of osteoblast colony forming units in their bone marrow appears to be reduced, and osteoclasts show enhanced spreading in response to stimulation (Kawano et al. 2013). With the exception of the above mentioned studies, LIMK null mice have not been reported to exhibit any other developmental defects or abnormalities. In humans, hemizygosity of LIMK1 is implicated in a rare cognitive disorder known as Williams syndrome (WS) or Williams-Beuren syndrome (WBS). WBS, in which LIMK1 and elastin are usually deleted, is a neurocognitive disorder characterized by difficulties in visualizing objects as a sum of many parts and in constructing replicas of those objects (Frangiskakis et al. 1996).

1.6.2 Regulation of LIMK activation

The stimulation of F-actin accumulation by LIMK is activated by phosphorylation on threonine residue 508 or threonine residue 505 (Thr ^{508} and Thr $^{505)}$ in the activation loop, of LIMK1 or LIMK2, respectively(Figure 5). The activating phosphorylation is mediated by the effectors of the small Rho-GTPases: Rho-associated protein kinases (Rho-kinase/ROCKs), p21-activated kinases (PAKs), and the myotonic dystrophy related Cdc42-binding kinases (MRCKs) (Edwards et al. 1999, Sumi et al. 2001, Ohashi et al. 2000) (Figure 6). PAKs are serine/threonine kinases that are activated by binding to GTP-loaded RhoGTPases (p21), Rac or Cdc42, via a p21-binding domain (PBD) in the N-terminal regulatory region, which causes a conformational change that relieves inhibition of the C-terminal kinase domain (Bokoch 2003).

Similar to PAKs, MRCKs are serine/threonine kinases containing a PBD that binds to GTP-loaded Cdc42. Regions in the coiled-coil domains of MRCKs mediate intramolecular inhibition of the kinase domain, whereas N-terminal domain dimerization promotes transphosphorylation of MRCK monomers, which enhances kinase activity (Leung et al. 1998, Bush et al. 2000, Tan et al. 2001). The binding of MRCKs to Cdc42 may not be sufficient to relieve the autoinhibition of the kinase domain, but rather it may serve to localize MRCKs to their substrates, and activation may involve DAG binding to the cysteine-rich domain (Zhao and Manser 2005). ROCKs are also serine/threonine kinases that are activated by the binding of GTP-bound Rho to a C-terminal region of the coiled-coil domain called the Rho-binding domain (RBD), which releases inhibitory constraints on the kinase domain (Narumiya, Ishizaki, and Watanabe 1997, Amano, Nakayama, and Kaibuchi 2010). Interestingly, like LIMKs, PAKs, ROCKs, and MRCKs appear to form autoinhibited structures, which inhibits their catalytic activity under basal conditions, and upon activation autoinhibition is released enabling formation of dimers or oligomers that promote transphosphorylation, which is critical for kinase activity (Zhao and Manser 2005). Other activating phosphorylation sites on LIMK1 have been reported (Kobayashi et al. 2006). MAPK-activated protein kinase-2 (MK2), downstream of VEGF-A-induced activation of MAPK p38, has been shown to mediate phosphorylation of LIMK1 on serine 323, which activates the kinase function of LIMK1 (Kobayashi et al. 2006). P38 also phosphorylates LIMK1 on serine 310 but this does not activate LIMK1 (Kobayashi et al. 2006). Interestingly, VEGF-induced activation of LIMK1 does not require phosphorylation of Thr^{508}, however, it does require serine 323 phosphorylation. PKA has been shown to phosphorylate serines 323 and 596 which activates LIMK1 leading to cofilin phosphorylation, and this PKA-mediated LIMK1 phosphorylation may be independent of RhoGTPase signaling (Nadella et al.

2009). In contrast to the MAPK-dependent LIMK1 activation, the PKA phosphorylation sites alone appear insufficient to activate the catalytic activity of LIMK1 in the absence of Thr^{508} phosphorylation (Nadella et al. 2009). There are currently no reliable commercially available antibodies that recognize these alternative MAPK and PKA phosphorylation sites, which limits investigation into how all three phosphorylation sites contribute to LIMK function within the context of different signaling pathways and/or cellular responses.

LIMK1 has been demonstrated to autophosphorylate on serine and tyrosines. Autophosphorylation occurs independent of threonine 508 phosphorylation in the activation loop, but requires the catalytic Asp 460 in the kinase domain. LIMK1 autophosphorylation or transphosphorylation appears to be dependent on dimerization of LIMK monomers. Dimerization of LIMK1 is mediated by the binding of heat shock protein 90 (Hsp90) to proline 394 on LIMK1 (Li, Soosairajah, et al. 2006). Hsp90-dependent LIMK1 dimerization and subsequent transphosphorylation appears to play an important role in increasing LIMK1 protein stability, and also appears to enhance the ability of LIMK1 to phosphorylate cofilin. Accordingly, a mutant LIMK1 with defective dimerization, due to mutation at the critical Pro 394, has reduced capacity to transphosphorylate, diminished phosphorylation of cofilin, and also displays a shorter half-life (Li, Soosairajah, et al. 2006). Thus, LIMK1 dimerization and subsequent transphosphorylation of LIMK1 monomers may be important for achieving maximum kinase activity and maintaining protein stability.

The ability of LIMK to regulate actin dynamics is dependent on its phosphorylation of the N-terminal serine 3 of cofilin, which inactivates the F-actin-binding and depolymerization (or polymerization) activity of cofilin (Arber et al. 1998) . LIMK-dependent phosphorylation of cofilin on serine 3 leads to accumulation of actin filaments. Consequently, mutation of serine 3 to

alanine on cofilin abolishes LIMK-dependent actin reorganization. Cofilin proteins have been shown to depolymerize actin in vitro; whereas in cells they still function to sever F-actin filaments, but these severed actin filaments then generate new barbed ends that can increase actin polymerization (Funk and Bamburg 2007). The regulation of cofilin phosphorylation status is dependent on slingshot, a phosphatase, and LIMK1 (Figures 5,6). Both LIMK1 and cofilin are dephosphorylated by slingshot. The phosphatase activity of slingshot, toward cofilin, is stimulated by binding F-actin. PAK-4 mediated phosphorylation of slingshot inhibits its phosphatase activity. 14-3-3 ζ binds phosphorylated forms of cofilin and slingshot sequestering them in the inactive state (Soosairajah et al. 2005). In addition to slingshot (SSH), chronophin is also a cofilin phosphatase (Gohla, Birkenfeld, and Bokoch 2005).

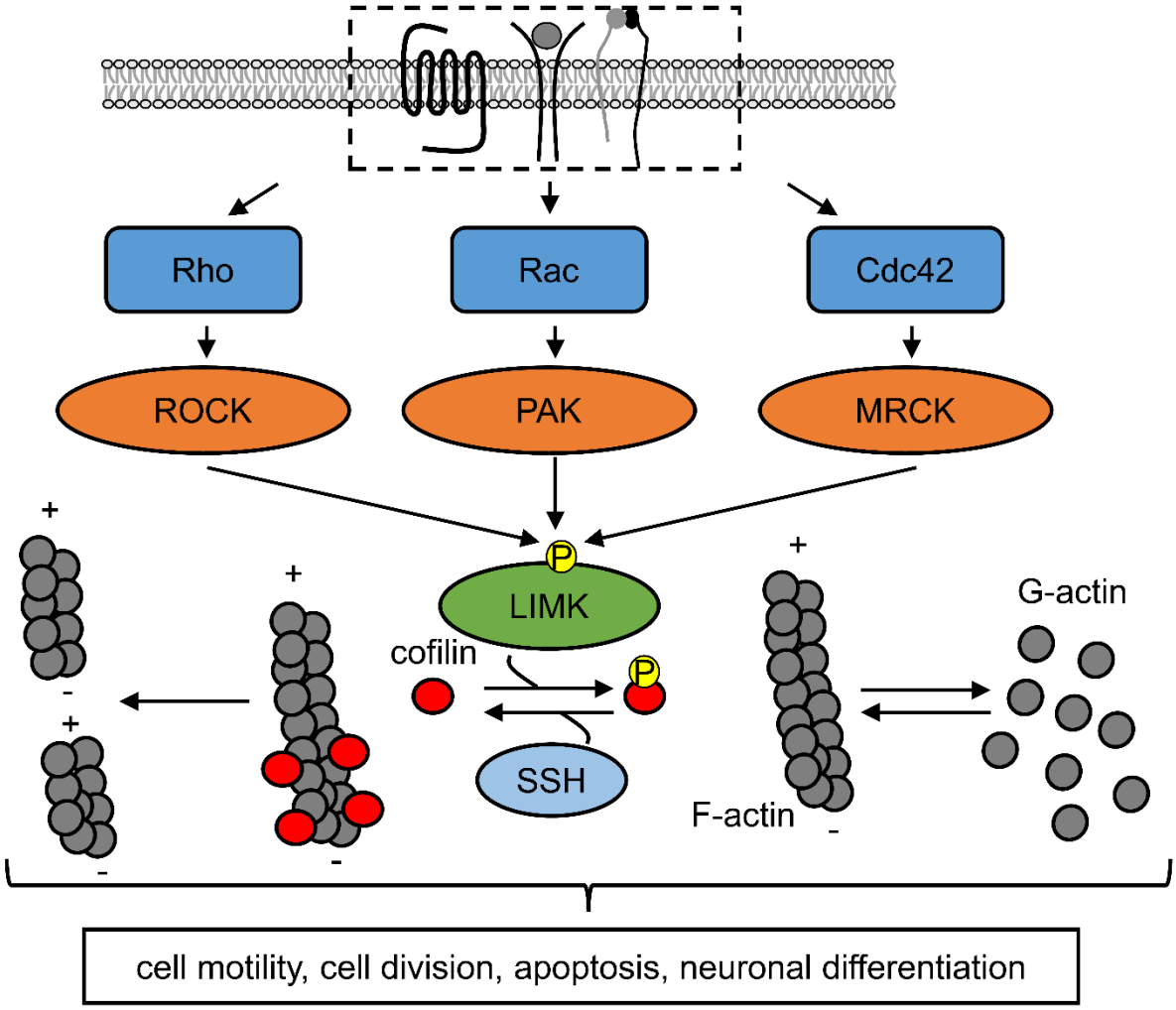


Figure 6. LIMKs are activated downstream of small RhoGTPases. A schematic showing how RhoGTPase signaling leads to activation of LIMK and actin reorganization, which is thought to account for the important role of LIMK in several cellular processes (e.g., cell motility). The receptors are shown to illustrate some of the signaling pathways in which LIMK has been implicated, such as LPA, thrombin (GPCRs), VEGF (receptor tyrosine kinase), and integrins. Dashed box around receptors indicates that not each receptor utilizes the same RhoGTPase pathway. ROCK, PAK, MRCK can each phosphorylate and activate LIMK. LIMK phosphorylates and inhibits cofilin binding to F-actin, and thereby enables F-actin to accumulate. However, cofilin is dephosphorylated by phosphatases(e.g, slingshot, SSH) enabling it to bind and sever F-actin filaments exposing new barbed ends. +, growing ends or barbed ends; -, pointed ends. For clarity SSH is not shown dephosphorylating LIMK, but LIMK is also a substrate for SSH.

1.6.2 LIMK1 in platelet activation

While LIMK1 is well studied in other cells, relatively little is known about the role of LIMK1 in platelets. In platelets, LIMK1, but not LIMK2 is expressed. LIMK1 has been shown to be activated, in thrombin and lysophosphatidic acid (LPA) stimulated platelets, during platelet shape change and aggregation (Pandey, Goyal, and Siess 2007). Using pharmacological methods it was shown, thrombin induced LIMK1 phosphorylation is mediated by ROCK (Pandey et al. 2006) but not Rac1 (Pandey et al. 2009). At a low dose of thrombin that induced only platelet shape change without aggregation or secretion, LIMK1 phosphorylation was found to increase temporally and plateaued at 60 seconds after stimulation. By contrast, under the same conditions, cofilin phosphorylation did not change significantly from baseline levels, wherein cofilin is basally phosphorylated. As expected, in the presence of ROCK inhibitor, Y-27632, platelet shape change was abolished. Moreover, low dose thrombin-induced LIMK1 phosphorylation was also abolished, and cofilin phosphorylation went below baseline levels seen in controls, particularly at 120 seconds, suggesting that cofilin may be dephosphorylated by a phosphatase during platelet shape change, which would mask a LIMK1-dependent increase in cofilin phosphorylation. At doses of thrombin that induced aggregation and secretion, LIMK1 phosphorylation increased to a plateau at 120 seconds, but by contrast, cofilin phosphorylation was dynamic: thrombin-induced dephosphorylation occurred at 30 seconds followed by rephosphorylation to baseline levels at 120 seconds. Similarly to its effect during platelet shape change, Y-27632 treatment not only abolished LIMK1 phosphorylation but unmasked the presence of a possible cofilin phosphatase antagonizing LIMK1 activity, because cofilin phosphorylation again dropped below baseline levels at 120 seconds. Thus,

based on the data with Y-27632 it appears that ROCK mediated LIMK1 activation promotes thrombin-induced platelet aggregation, dense granule secretion, and F-actin increase. With the exception of resting platelets treated with Y-27632 where it was shown that cofilin binding to F-actin was increased, these investigators observed no correlation between inhibiting LIMK1 activation with Y-27632, and thereby inhibiting cofilin phosphorylation, and the ability of cofilin to associate with F-actin in thrombin stimulated platelets (Pandey et al. 2006). Specifically, cofilin associated with F-actin upon stimulation of platelets with thrombin (at a dose that induces aggregation and secretion) even in the presence of Y-27632, which increased the levels of active (i.e., unphosphorylated) cofilin. It is still unclear how cofilin rephosphorylation can be inhibited by Y-27632 while its association with F-actin remains unchanged rather than increased, as would be expected. Moreover, the identity of the cofilin phosphatase responsible for antagonizing LIMK1-dependent cofilin phosphorylation is unknown, but a clue to its characteristics were revealed from another study by the same group using the bioactive lipid agonist lysophosphatidic acid (LPA). Lysophosphatidic acid (LPA) is a lipid that acts as a platelet agonist, which can be formed by the action of phospholipase A_2 and phospholipase D on phosphoglycerides. LPA has been shown to be enriched in oxidized LDL and atherosclerotic plaques (Schober and Siess 2012). LPA binds the LPA receptors which are G-protein coupled receptors, and platelets express 3 LPA receptors (Schober and Siess 2012). Activated LPA receptors induce Rho activation. Similar to thrombin-induced activation of LIMK, LPA stimulation of platelets at low doses induced shape change and a steady increase in cofilin and LIMK1 phosphorylation (Pandey, Goyal, and Siess 2007). Y-27632-pretreatment abolished LPA-induced LIMK1 phosphorylation and cofilin phosphorylation went below baseline unstimulated levels. Although cofilin net phosphorylation level was unchanged by low

dose LPA stimulation after 120 seconds in controls, cofilin was found to rapidly associate with actin. Stimulation of platelets with a higher dose of LPA induced a similar cofilin dephopshorylation, which was maximal at 60 seconds, followed by rephosphorylation to baseline unstimulated levels at 120 seconds. In contrast to high dose thrombin stimulation, high dose LPA did not induce secretion or platelet aggregation, indicating that cofilin dephosphorylation may be independent of integrin outside-in signaling and granule secretion (Pandey, Goyal, and Siess 2007). The rephosphorylation of cofilin was inhibited by Y-27632, suggesting it was dependent on ROCK/LIMK1-dependent phosphorylation. Interestingly, high dose LPA-induced cofilin dephosphorylation could be inhibited by chelating intracellular Ca^{2+} with BAPTA-AM, indicating the supposed cofilin phosphatase was dependent on intracellular Ca^{2+} (Pandey, Goyal, and Siess 2007). Subsequently, the same group extended these studies to show that calcineurin played a role in cofilin dephosphorylation. Using a peptide composed of the calcineurin autoinhibitory domain (CAID) fused to a cell permeable polyarginine sequence thrombin-induced cofilin dephosphorylaiton was inhibited. Moreover, they show that in thrombin stimulated platelets Rac1 and Pak 4/5/6 were upstream of cofilin dephosphorylation leading to secretion, and that this pathway was regulated by calcineurin (Pandey et al. 2009). Interestingly, they also reported no effect of a Rac inhibitor NSC23766 on LIMK1 phosphorylation, suggesting that Rac1 does not regulate LIMK1 during thrombin signaling (Pandey et al. 2009). Prior to the discovery of a Ca^{2+}-dependent phosphatase, earlier investigations had suggested that integrin αIIbβ3-dependent outside-in signaling promotes cofilin dephosphorylation. Consistent with that notion, it was reported that in the thrombin stimulated human platelets pretreatment with RGDS, an integrin antagonist, inhibited cofilin dephosphorylation (Falet et al. 2005). Similarly, platelets from a Glanzmann thrombasthenia

patient, expressing 2-3% of normal levels of αIIbβ3, exhibited enhanced cofilin dephosphorylation upon thrombin stimulation (Falet et al. 2005). Moreover, thrombin-induced changes in cofilin phosphorylation were reportedly affected by stirring conditions, also suggestive of aggregation and thus integrin-dependent regulation of cofilin phosphorylation (Falet et al. 2005). However, another group reported that neither stirring (or non-stirring) conditions nor RGDS pretreatment affected cofilin dephosphorylation during thrombin stimulation (Pandey et al. 2006). As mentioned above, it was later reported that a Ca^{2+-} dependent phosphatase, calcineurin, regulates cofilin dephosphorylation, but whether cofilin plays a role downstream of integrin αIIbβ3 ligation is still unclear (Falet et al. 2005, Pandey et al. 2006).

Platelets express ADF and non-muscle cofilin (n-cofilin). N-cofilin appears to be the dominant cofilin in platelets since megakaryocyte/platelet-specific deletion of n-cofilin, but not ADF, causes significant thrombocytopenia and increase in platelet size (i.e., macrothrombocytopenia) (Bender et al. 2010). In addition, n-cofilin null platelets do not show the typical discoid shape of normal resting platelets. Low dose thrombin-stimulated n-cofilin null platelets also appear to have a partial delay in spreading on immobilized fibrinogen (Bender et al. 2010), which would be consistent with a role for cofilin downstream of αIIbβ3. Remarkably, deletion of both ADF and n-cofilin (ADF/n-cofilin) in mice resulted in a more severe thrombocytopenia than in n-cofilin null mice, and their platelets also show highly variable morphology (Bender et al. 2010). Platelet spreading on fibrinogen was also further inhibited in ADF/n-cofilin null platelets. ADF/cofilin family members appear to compensate for one another since in ADF/n-cofilin null platelets agonist-induced F-actin polymerization is abolished, whereas in the absence of either cofilin or ADF alone only a partial defect is

present. Finally, the megakaryocytes from ADF/n-cofilin null mice were found to have defective proplatelet formation, which would explain the severe thrombocytopenia in these mice (Bender et al. 2010). In fact, whereas n-cofilin deletion is lethal in mice (Gurniak and Witke 2005), deletion of ADF is not (Ikeda et al. 2003), suggesting some non-overlapping functions in early mammalian development. Taken together, under basal/resting conditions LIMK1 is not phosphorylated, but upon stimulation by thrombin or LPA it is phosphorylated on Thr^{508}, and thus activated, and its phosphorylation increases, in general, irreversibly upon stimulation by thrombin or LPA. On the other hand, cofilin is already phosphorylated on serine 3 under basal conditions, and high doses of agonist induce transient dephosphorylation followed by rephosphorylation of cofilin, which may play an important role in regulating actin polymerization, platelet morphology, and thrombopoiesis (Bender et al. 2010).

2. PURPOSE OF STUDY

The platelet receptor for VWF, the glycoprotein Ib-IX-V complex (GPIb-IX), mediates the initial platelet adhesion to subendothelial-bound VWF at sites of vascular injury and transduces signals leading to platelet activation, stable platelet adhesion, and thrombus formation (Ruggeri and Mendolicchio 2007, Du 2007). Although, GPIb-IX is important under high shear rate flow conditions seen in arteries and arterioles, it has also been shown to be important under low shear rate conditions seen in veins (Brill et al. 2011, Joglekar et al. 2012). Previous studies indicate that GPIb-IX-induced platelet activation requires the sequential activation of Src family kinases Lyn (Liu et al. 2005, Yin, Liu, et al. 2008), Rac1 (Delaney et al. 2012), PI3K/Akt (Kasirer-Friede et al. 2004, Yin, Stojanovic, et al. 2008), cGMP-dependent protein kinase (Li et al. 2003), and the mitogen-activated protein kinases (MAPKs) (Li, Zhang, et al. 2006). However, full platelet responses to VWF require amplification signaling mediated by the ITAM signaling pathway (Liu et al. 2005, Li et al. 2010, Liu et al. 2006, Liu et al. 2008), and by TXA_2 and TXA_2-dependent granule secretion of ADP (Kroll et al. 1991, Yin, Stojanovic, et al. 2008, Liu et al. 2004). MAPKs are known to regulate $cPLA_2$, a rate-limiting enzyme in TXA_2 production (Lin et al. 1993, Kramer et al. 1996), and they are also important for mediating GPIb-IX-dependent signaling leading to TXA_2 production (Li, Xi, and Du 2001, Canobbio et al. 2004, Garcia et al. 2005). In addition, TXA_2 production may also be regulated by the actin cytoskeleton and αIIbβ3-dependent signaling (Pravost et al. 2009, Siess, Lapetina, and Cuatrecasas 1982, Aoki et al. 1998). Thus, it remains unclear how GPIb-IX signaling leads to TXA_2 generation. We hypothesized that LIMK1 may play an important role in VWF/GPIb-IX-mediated platelet activation because a well-known upstream regulator of LIMK1 activity, Rac1, had been previously shown to play an important role in VWF-induced platelet spreading and

arterial thrombosis (McCarty et al. 2005, McCarty et al. 2006). In addition, our lab recently identified an important role for Rac1 in mediating VWF-induced signaling leading to TXA_2 production (Delaney et al. 2012). In order to determine the role of LIMK1 in platelet activation we isolated platelets from LIMK1 null mice ($LIMK1^{-/-}$). We then investigated the response of wildtype (WT) and $LIMK1^{-/-}$ platelets upon stimulation with VWF as well as several other platelet agonists that selectively activate distinct platelet activation pathways. To determine the role of LIMK1 in vivo, we also assessed the ability $LIMK1^{-/-}$ mice to form occlusive thrombi upon arterial injury and measured their hemostatic response via tail bleeding time. These in vivo and in vitro assays allowed us to determine the role of LIMK1 in platelet function.

Another aim of our study was to investigate the mechanisms responsible for the thrombin receptor function of the platelet GPIb-IX-V receptor complex. The role of GPIb-IX-V in the response of platelets to thrombin has been a long-standing controversy in platelet biology. Currently, a widely accepted view is that the GPIb-IX-V receptor complex serves only as a dock for thrombin to cleave PARs. Approximately forty years ago GPIbα was shown to bind directly to thrombin with high affinity, however the physiological significance of this finding was unclear. It was also found that Bernard-Soulier syndrome patients' platelets displayed defective aggregation response to thrombin stimulation, but only at low doses. Using indirect approaches it had been suggested that thrombin binding to GPIbα served to facilitate cleavage and activation of the protease-activated receptors, which would then transduce intracellular signals leading to platelet activation. These findings were supported by genetic evidence obtained using platelets from PAR4 knockout mice which did not respond to thrombin stimulation. Nonetheless, others reported that platelets respond to catalytically inactive thrombin, suggesting PAR-independent signaling. Due to the lack of response at low doses of

thrombin in BSS patients' platelets and the finding that GPIbβ knockout mouse platelets display defective thrombin response we hypothesized that GPIb-IX played a role in promoting platelet response to thrombin. In addition, there were issues with the studies concluding PAR-independent signaling, such as the possibility of incomplete inactivation of thrombin. Moreover, secondary effects due to granule release upon thrombin stimulation could trigger activation of other receptors. In order to isolate thrombin signaling we reconstituted platelet thrombin receptor signaling in Chinese hamster ovary (CHO) cells that express endogenous PAR1 by stably expressing the human platelet GPIb-IX receptor complex. This model allowed us to directly assess the relative contributions of PARs and GPIb-IX to thrombin-induced cellular responses, such as calcium mobilization, in the absence of secondary platelet activation pathways that are also activated when platelets are stimulated with thrombin (e.g., ADP/P2Y-signaling). Using the CHO cell model, we investigated the contribution of PARs to GPIb-IX-facilitated thrombin response in the presence and absence of a PAR1-selective antagonist, SCH79797, and also by stimulating platelets or cells with catalytically inactive S195A mutant thrombin or PPACK-inactivated thrombin. To evaluate the role of GPIb-IX in platelet thrombin response, we utilized a cell-permeable peptide inhibitor, MPαC, previously developed by our lab that mimics the C-terminal 14-3-3 binding site on GPIbα, which has been previously shown to inhibit VWF-binding to GPIb and VWF-induced platelet aggregation. We also investigated the role of LIMK1 and Rac1 in GPIb-IX-dependent thrombin-induced platelet activation response using isolated platelets from Rac1 or LIMK1 null mice. These experiments allowed us to determine how GPIb-IX plays a role in thrombin-induced platelet activation.

3. METHODS

3.1 Preparation of platelets

Human blood was drawn by venipuncture from healthy volunteers. Institutional Review Board approval was obtained from the University of Illinois at Chicago, and informed consent from volunteers was obtained in accordance with the Declaration of Helsinki. ACD was used as an anticoagulant and the platelets were prepared as previously described (Marjanovic et al. 2005, Yin, Liu, et al. 2008). The platelets were then allowed to rest in a modified Tyrode's buffer for at least 1 hour at 22°C prior to use.

The generation of LIMK1-knockout mice was previously described (Meng et al. 2002) and these mice were kept on a mixed 129R1(50%)/C57BL (50%) background. Wild-type control mice and LIMK1-knockout mice used in this study were 15 to 20-week-old littermates generated from heterozygous breeding. The generation of megakaryocyte lineage-specific Rac1-knockout mice ($Rac1^{-/-}$) has been previously described(Akbar et al. 2007, Delaney et al. 2012). Washed platelets were prepared from blood drawn from the mouse inferior vena cava as previously described (Marjanovic et al. 2005). Final concentrations of 1 U/mL apyrase and 0.1 µg/mL PGE1 were added to the freshly drawn whole blood. After washing, platelets were resuspended in Tyrode's buffer. In some experiments, platelets were washed in the presence of apyrase and 5 mM EDTA in modified Tyrode's buffer (Liu et al. 2005). Hematological parameters were routinely measured using a Hemavet 950FS (Drew Scientific, Dallas, TX).

3.2 Platelet aggregation and secretion

Before the assessment of aggregation and secretion responses, washed platelets ($3x10^8$/mL)

were allowed to rest for at least 1 hour at 22°C before use. Platelet aggregation and secretion of granule ATP were determined simultaneously in a Chronolog lumiaggregometer at 37°C with stirring (1000 rpm). For some experiments, washed human platelets were preincubated with either SB203580 (20μM), U0126 (3μM), Cytochalasin D (10μM), Latrunculin A (5μM) (all from Calbiochem), or vehicle control (0.1%DMSO) for 5 minutes at 37°C. For some experiments, washed human platelets were preincubated with either MPαC (10μM) or MCsC(10μM) for 3 minutes at 37°C. Total ATP release was measured by adding LUME luciferase reagent (Chronolog) after 6 or 8 minutes of thrombin stimulation. A standard curve of ATP concentrations was used to determine the concentration of ATP. Data are from at least 3 experiments (mean ±SEM, Student's t-test).

3.3 Immunoblot detection of LIMK, cofilin, cPLA2, and P38 MAPK

Washed platelets (3 x10^8/mL) were stirred (1000 rpm) in a platelet aggregometer for various lengths of time after adding ristocetin (0.25 mg/mL) alone, or ristocetin and VWF (10 µg/mL) (human), or after adding botrocetin (2 µg/mL) with or without VWF (10 µg/mL) (mouse) or for 2 minutes with thrombin (in mouse or human platelets). In experiments using cells, CHO cells were grown to 90% confluence, serum-starved overnight, counted, and then resuspended in PBS (containing 1.5 mM Ca^{2+} and 1 mM Mg^{2+}) to 1.5x10^6 cells. Platelets or CHO cells (in suspension) were then stimulated with thrombin for 2 minutes. The reactions were stopped by addition of an equal volume of 2x sodium dodecyl sulfate (SDS) sample buffer, containing 0.2 mM E64, 2 mM PMSF and 34 µg/mL aprotinin. As previously(Li, Xi, and Du 2001, Yin, Stojanovic, et al. 2008), proteins were separated by SDS-polyacrylamide gel electrophoresis

(PAGE), transferred to polyvinylidene difluoride membranes and immunoblotted with the antibodies recognizing LIMK1/2 phosphorylated at threonine 508/505, phospho-cofilin at Ser^3, total cofilin, total LIMK1, p38 MAPK phosphorylated at threonine 180/ Tyrosine 182 (Cell Signaling, Danvers, MA), cPLA2, or cPLA2 phosphorylated at Serine 505 (Abcam and Santa Cruz). Representative data from at least 3 independent experiments are shown.

3.4 Immunoblot detection of activated Rac1

3 $x10^8$ platelets were stirred (1000 rpm) in a platelet aggregometer for 2 minutes after thrombin stimulation. In experiments using cells, CHO cells were grown to 90% confluence, serum-starved overnight, counted, and then resuspended in PBS (containing 1.5 mM Ca^{2+} and 1 mM Mg^{2+}) to $1.5x10^6$ cells. Platelets or CHO cells (in suspension) were then stimulated with thrombin for 2 minutes. Rac1 activation assays were performed, as described previously(Akbar et al. 2007). For experiments in CHO cells, samples were stimulated and collected for Rac-activity while in suspension. Anti-Rac1 antibody (No. 61051; BD Biosciences) was used for Western Blot.

3.5 Platelet adhesion under shear stress

The analysis of mepacrine (10 µM)-labeled platelet adhesion to VWF-coated surfaces under shear stress was performed essentially as previously described (Yin, Liu, et al. 2008). A cone-plate rheometer (Rheostress 1, Thermo-HAAKE, Paramus, NY) was used to introduce shear stress (800 s^{-1}) to the platelets for 5 minutes. After washing, slides were viewed with a Leica DMI RB fluorescence microscope (Leica) using an N PLAN L lens at 40/0.55 NA objective with 1.5X magnification. Stably adherent platelets were counted in ≥ 20 randomly selected fields (mean±standard error). A One-way ANOVA was used for statistical analysis

and the significance between individual treatments was determined by using Bonferroni's Multiple Comparison Test.

3.6 VWF binding assay

Washed mouse platelets (1 x10^8/mL) in a modified Tyrode's buffer containing 1% BSA and 5 mM EDTA were incubated with VWF (10 µg/mL) alone as control or VWF plus botrocetin (2 µg/mL) at 22°C for 30 minutes. After washing once with PBS, VWF binding to platelets was detected using fluorescein isothiocyanate-labeled anti-VWF antibody, SZ-29, and flow cytometry (Dai et al. 2005). Representative data from at least 3 independent experiments are shown.

3.7 Platelet adhesion and spreading on immobilized VWF or fibrinogen

Washed platelets (2x10^7/mL) were allowed to adhere and spread at 37°C for 1.5 hours on cover slides coated with fibrinogen (100 µg/mL) or VWF (30 µg/mL) (Gong et al. 2010). For adhesion on VWF, 2.0 µg/mL botrocetin was added to the wells. Slides were rinsed, fixed with 4% paraformaldehyde, and then permeabilized. Adherent platelets were stained with Alexa Fluor 546-conjugated phalloidin (Invitrogen), and observed with a Leica DM IRB fluorescence microscope using a 100X/1.30 NA oil objective as previously described (Yin, Liu, et al. 2008). Images were acquired using a Cool SNAP HQ CCD camera and processed with µManager software. To quantitate platelet adhesion, platelets were incubated in VWF-coated microtiter wells at 37°C for 1 hour. Adherent cells were quantified using a colorimetric acid phosphatase assay as previously described (Gu et al. 1999).

3.8 TXB_2 generation assay

Washed platelets ($3X10^8$/mL) were stimulated with agonists in an aggregometer at 37°C with stirring (1000 rpm). The reaction was stopped by adding 3 mM aspirin and 10 mM EDTA at 8 minutes on ice, centrifuged at 6000 g for 1 minute in a microfuge, and the supernatant stored at -70°C until analysis. A TXB_2 EIA Kit (assay designs, Ann Arbor, Michigan) was used to determine the level of TXB_2 in each sample. Experiments were repeated at least three times. All data are expressed as mean ± standard error. Statistical significance was determined using the Student's t-test.

3.9 F-actin measurement in platelets

Washed platelets ($3x10^8$/mL), either resting or stimulated with VWF/botrocetin, were fixed in 4% paraformaldehyde solution for 30 minutes at 37°C, permeabilized in a 0.2% Triton X-100 solution, and incubated with 10 U Alexa Fluor 488 phalloidin (Molecular Probes) for 1 hour at 37°C as previously described (Kovacsovics and Hartwig 1996). Samples were analyzed by flow cytometry (FACSCalibur, BD). The data shown are fluorescence intensity of single platelets as gated by the forward and side scatter.

3.10 In vivo thrombosis

Mice were anesthetized with isoflurane. The left carotid artery was isolated, and a MA-0.5PSB nanoprobe (Tran-sonic Systems, Ithaca, NY) was hooked to the artery to monitor blood flow using a TS420 flowmeter (Transonic Systems) as previously described (O'Brien et al. 2011). A filter paper disc (2-mm diameter) soaked with 1.2 μL of 10%(0.460M) ferric chloride ($FeCl_3$) (Sigma) was placed on top of the artery for 3 min. Blood flow was then monitored until 5 minutes after occlusion. Statistical analysis was performed using a

parametric, unpaired Student t-test assuming equal variances between treatments (F-test P=0.81, two-tailed t test P=0.0086, n=10 per group).

3.11 Mouse tail bleeding time

Mice were anesthetized with isoflurane and their tails were then immersed in 0.15M NaCl immediately after cutting 0.5 cm-long tail tips. Bleeding was followed visually, and time to stable cessation of bleeding (no rebleeding within 1 minute) was recorded. A nonparametric, unpaired t test was used to compare medians between treatments (Mann-Whitney test: P=0.41, n=17 per group).

3.12 PPACK-treatment of thrombin

Wild type human α-thrombin (Enzyme Research Labs) or recombinant human S195A thrombin (Kerafast) was reacted with 15 molar excess of PPACK for 1 hour at room temperature, as previously described (Greco et al. 1990). Wild type or S195A thrombin was reacted with 15 molar excess of PPACK for 1 hour at room temperature, as previously described (Greco et al. 1990). PPACK-treated thrombin was then dialyzed to remove unbound PPACK, using four sequential changes of dialysis buffer, including two overnight exchanges at 4°C to a final buffer containing 0.1% PEG-8000, 0.15 M NaCl and 10 mM HEPES pH 7.4.

3.13 Thrombin activity Assay

Thrombin activity analysis was conducted by measuring the change in fluorescence upon cleavage of a fluorogenic thrombin substrate SN-17a (Haematologic technologies Inc.). Briefly, wild type thrombin, mutant S195A thrombin, and wild type and S195A PPACK-treated thrombins were incubated with 100 μM chromogenic thrombin substrate in a buffer containing:

25 mM HEPES, 0.15 M NaCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 0.1% BSA and 0.2% PEG-8000, pH 7.4. Following automated pipetting of thrombin into plates containing thrombin substrate, changes in fluorescence were recorded using a FlexStation II microplate reader (Molecular Devices) every 30 seconds for 10 minutes at 37°C. Excitation, emission and cutoff settings were set according to manufacturer's suggestions. Except in cases where otherwise is specified, human α-thrombin (also referred to as thrombin or wild type thrombin) purified from human plasma was used (Enzyme Research Labs). Specific activity of α-thrombin is 3078 NIH units/mg. Representative data combined from 3 independent experiments are shown.

3.14 Thrombin binding assay

As previously described (Harmon and Jamieson 1985), $1x10^9$ human platelets resuspended in a thrombin binding buffer (25 mM Tris, 0.6% PEG-8000, 1% BSA, 136 mM NaCl, pH 7.4) were pretreated with MCsC or MPαC for 2 minutes and then incubated with 3 nM S195A thrombin for 10 minutes, fixed with 4% ice-cold PFA for 10 minutes, washed and then stained with 2 μg/mL goat anti-thrombin antibody (R&D system) for 1 hour at 22°C. Thrombin binding was detected using an Alexafluor 488-labeled anti-goat IgG antibody using flow cytometry. In some experiments, human platelets were preincubated with 70 μg/mL IgG or SZ2 to determine specific binding. Representative data from 3 independent experiments are shown.

3.15 Calcium mobilization assay

Thrombin-induced calcium mobilization was measured using a fluorescent calcium-sensitive dye, FLIPR Calcium 5 reagent (Molecular Devices, R8142, Explorer kit). CHO cells in suspension ($5x10^5$/mL, treated with 2.5 mM probenecid (Molecular Probes P364000, Life sciences) or platelets ($1.5x10^8$/mL) were loaded with the dye for 1 hour at 37°C, according to the manufacturer's protocol. During incubation, another 96-well plate containing serial diluted thrombin was prepared. After 1 hour of loading with the fluorescent intracellular probe, 200 μL aliquots of cells were transferred into 96-well plates, stimulated with 50μL diluted agonist and analyzed using an automated pipetting system FlexStation II microplate reader (Molecular Devices). In some experiments, CHO cells were treated with 100 μM NSC23766 or 20 μM SCH 79797 or 0.1% DMSO vehicle control just prior to fluorometric detection. Fluorescence was detected at an excitation of 485 nm, emission of 525 nm, and cut-off of 515 nm, as recommended by the manufacturer. Data from at least 3 independent experiments are shown. Statistical significance was determined using a one-way ANOVA and Bonferroni's posttest.

3.16 Reconstitution of human GPIb-IX in CHO cells

Chinese Hamster Ovary cells stably expressing wild-type GPIb-IX and mutant GPIb-IX that contains a truncation of the 5 C-terminal amino acids in the α-chain of GPIb (Δ605 cells) were previously established(Du, Fox, and Pei 1996). Similarly, CHO cell lines expressing GPIbα with a triple tyrosine to phenylalanine mutation (FFF cells), known to abolish the high affinity binding of thrombin to GPIb(De Marco et al. 1994, Marchese et al. 1995, Zarpellon et al. 2011), was stably expressed by co-transfection with GPIbβ and GPIX cDNA into CHO cells with a pLenti6/V5-Dest vector encoding wild type human GPIbα or mutant GPIbα containing

$Y^{276,278,279}F$ mutations. Levels of surface GPIb-IX expression were normalized between cell lines to similar levels by fluorescence-activated cell sorting using a Beckman Coulter MoFlo flow cytometer operated at the UIC flow cytometry core facility. In order to sort GPIb-IX expressing cells and verify GPIb-IX expression levels, cells were incubated with 5 ug/mL SZ1 or SZ2 mouse monoclonal antibodies and then fluorescently labeled with 2 μg/mL of an Alexa Fluor 488-labeled goat-anti-mouse IgG (Life technologies), as previously described(Gu et al. 1999). Equivalent GPIbα and GPIb-IX complex expression in SZ1-sorted cells was further verified on a BD Accuri™ C6 flow cytometer. In order to sort GPIb-IX expressing cells and verify GPIb-IX expression levels, cells were incubated with 5 ug/mL SZ1 or SZ2 mouse monoclonal antibodies and then fluorescently labeled with 2 μg/mL of an Alexa Fluor 488-labeled goat-anti-mouse IgG (Life technologies), as previously described(Gu et al. 1999). Equivalent GPIbα and GPIb-IX complex expression in SZ1-sorted cells was further verified on a BD Accuri C6 flow cytometer. Graphs were made using FCS express 4 (De Novo ™).

3.17 Primers used for creating mutant GPIb-IX-expressing cells

Human GPIbα was cloned into a pLENTI6/V5-DEST vector after digestion with EcoRI and XhoI restriction enzymes. The upstream and downstream sequences are (native human GPIbα DNA sequence is indicated by lowercase lettering, and mutated codons are underlined): (1) EcoRI FL-hGPIbalpha fwd:5'-GCGGAATTCGCCat gcctctcctc ctcttgctgc tcctg-3' (2) XhoI FL-hGPIbalpha rev:5' GCGCTCGAGtcagaggctgtggccagagtacctaat-3' (3) hGPIbalpha-Y276/278/279F fwd: 5'- gaa ggt gac aca gac cta ttc gat ttc ttc cca gaa gag gac act-3' (4) hGPIbalpha rev- Y276/278/279F rev: 5'- agt gtc ctc ttc tgg gaa gaa atc gaa tag gtc tgt gtc acc ttc-3'.

3.18 Fluorescence intravital microscopy and thrombin generation in vivo

Mice were anesthetized by intraperitoneal injection of ketamine and xylazine, and then the cremaster muscle was exposed. Platelet accumulation was visualized by infusion of Dylight 649- conjugated anti-CD42c antibodies (0.05 µg/g body weight (BW)). Real-time images were obtained in cremaster arterioles with a diameter of 35-45 µm in each mouse, and recorded using an Olympus BX61W microscope with a 60 x 1.0 NA water immersion objective and a Hamamatsu C9300 high-speed camera through an intensifier (Video Scope International) as previously(Delaney et al. 2014). Thrombin monoclonal antibody was labeled using an Alexa Fluor® 488 (AF 488) Protein labeling kit according to the manufacturer's instructions. Mouse IgG_1 control antibody was also labeled in parallel and verified to have identical fluorophore to antibody ratio as the thrombin monoclonal antibody. After labeling, antibodies were dialyzed against a PBS solution, protected from light and stored at 4°C. Thrombin generation was observed by infusion of Alexa Fluor 488-conjugated thrombin-specific or mouse IgG antibodies (2 µg/g BW) into WT mice, respectively. In some experiments, 0.3 µg/g BW of Dabigatran or vehicle control was infused into WT mice. The kinetics of platelet accumulation and thrombin generation were analyzed by median fluorescence values of the antibodies as a function of time in 30 thrombi in 3-4 mice per group. Under equivalent microscope settings, standard curves were generated by intravital imaging and recording of fluorescence intensities for known concentrations of AF 488 conjugated thrombin monoclonal antibody dropped onto glass slides. To quantify thrombin concentration based on these AF 488 conjugated thrombin monoclonal antibody standards, fluorescence intensity values recorded from laser injury-induced thrombin generation experiments were then plotted onto standard curves.

4. LIM KINASE-1 SELECTIVELY PROMOTES GLYCOPROTEN IB-IX-MEDIATED TXA2 SYNTHESIS, PLATELET ACTIVATION, AND THROMBOSIS

I acknowledge that this research (Figures 7 through 14; text verbatim from pages 89 to 110) was originally published in Blood. Estevez B, Stojanovic-Terpo, A, Delaney MK, O'Brien KA, Berndt MC, Ruan C, Du X. LIM kinase-1 selectively promotes glycoprotein Ib-IX–mediated TXA_2 synthesis, platelet activation, and thrombosis. Blood. 2013 May 30; 121(22): 4586–4594. © The American Society of Hematology. No permission letter is needed for reuse (See APPENDIX).

4.1 LIMK1 promotes *in vivo* thrombosis but appears dispensable for hemostasis

To determine the effect of LIMK1 deficiency on tail bleeding time and $FeCl_3$-induced carotid artery thrombosis, respectively. The time from $FeCl_3$-induced arterial injury to occlusive thrombosis was significantly prolonged in LIMK1$^{-/-}$ mice as compared with wild-type mice (Fig. 7A, 7B), indicating that LIMK1 plays an important role in arterial thrombosis *in vivo*. The inhibitory effect shown in LIMK1$^{-/-}$ mice is in the period between $FeCl_3$-induced injury and the onset of a rapid occlusive thrombosis phase (Fig 7A). Once entering the rapid occlusive thrombosis phase, there is no longer a difference between wild type and LIMK1$^{-/-}$ mice in the rate of occlusive thrombus formation (WT: $-\Delta V/\Delta t=0.295\pm0.025$; LIMK1$^{-/-}$: $-\Delta V/\Delta t=0.281\pm0.038$ (ml/min^2), $p=0.763$). Interestingly, we observed no statistically significant differences in tail bleeding time between wild-type and LIMK1$^{-/-}$ mice (Fig. 7C). These data suggest that LIMK1 is important in promoting arterial thrombosis, but appears dispensable in hemostasis.

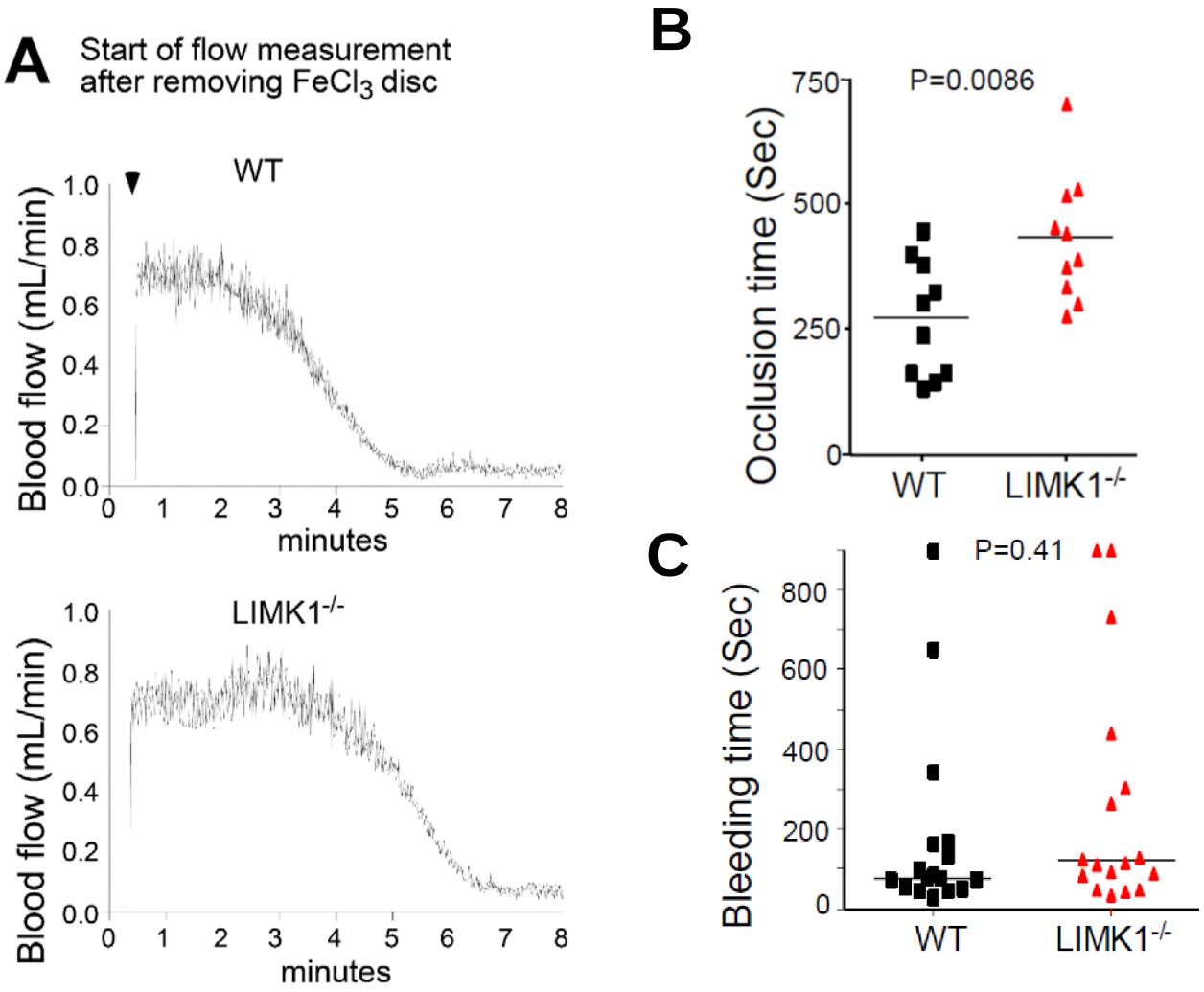


Figure 7. The effects of LIMK1 knockout on *in vivo* thrombosis and on hemostasis. (A, B) $FeCl_3$-induced occlusive thrombosis in carotid arteries of LIMK1$^{-/-}$ and wild-type (WT) mice. (A) Typical charts of $FeCl_3$-induced occlusive thrombosis in WT and LIMK1$^{-/-}$ mice as indicated by carotid artery blood flow. (B) The occlusion times for each mouse are shown as squares (wild-type, n=10) or triangles (LIMK1$^{-/-}$, n=10). The horizontal bars represent the mean occlusion time (p=0.0086, Student's t-test). (C) Tail bleeding times of each mouse are shown as squares (wild-type, n=17) or triangles (LIMK1$^{-/-}$, n=17). The horizontal bars represent the median bleeding time (p=0.41, Mann-Whitney test).

4.2 LIMK1 is important in platelet secretion and the second wave of platelet aggregation induced by VWF

Consistent with a previous report (Pandey et al. 2006), LIMK1 was detected by Western blot in wild-type mouse platelets, but undetectable in platelets from LIMK1$^{-/-}$ mice (Fig. 8A). There were no obvious differences between wild-type and LIMK1$^{-/-}$ mice in major hematological parameters including platelet count and platelet volume (data not shown). To study the role of LIMK1 in the GPIb-IX-dependent platelet response, wild-type and LIMK1$^{-/-}$ platelets were stimulated with VWF in the presence of botrocetin, a VWF-binding snake venom protein used *in vitro* to mimic the effect of the VWF-collagen interaction in increasing the affinity of VWF for GPIb-IX (Andrews et al. 1989). As expected, at a low concentration (0.5 µg/ml), botrocetin induced two waves of platelet agglutination/aggregation in wild-type platelets in the presence of VWF (Fig. 8B). The first wave is comprised mainly of GPIb-IX-mediated platelet agglutination, whereas GPIb-IX-induced, integrin-dependent platelet aggregation also plays a role (Li, Xi, and Du 2001). LIMK1 knockout did not affect the first wave of platelet agglutination/aggregation (Fig 8B). The second wave of platelet aggregation requires TXA_2-dependent platelet granule secretion, secreted ADP, and the consequent amplification of integrin activation(Kroll et al. 1991, Li, Xi, and Du 2001). The second wave of platelet aggregation and secretion of dense granules were inhibited in LIMK1$^{-/-}$ platelets (Fig. 8B), indicating that LIMK1 is important for GPIb-IX-mediated signaling leading to granule secretion and the second wave of platelet aggregation. At a higher concentration of botrocetin, the initial agglutination/aggregation was very strong and the two phases of platelet agglutination/aggregation were no longer distinguishable (Fig. 8B). However, LIMK1$^{-/-}$ platelets still showed decreased granule secretion, further indicating the importance of LIMK1 in

VWF/botrocetin-induced granule secretion. The inhibitory effects observed in LIMK1$^{-/-}$ platelets were not due to a defective ligand binding function of GPIb-IX, as botrocetin-induced VWF binding to platelets was not affected in LIMK1$^{-/-}$ platelets relative to wild-type (Fig. 8C). Similarly, botrocetin-induced platelet adhesion to VWF under static conditions was not affected by knockout of LIMK1 (Fig. 8D). These data suggest that LIMK1 plays an important stimulatory role in VWF/GPIb-IX-induced intracellular signaling leading to granule secretion and the integrin-dependent second wave platelet aggregation.

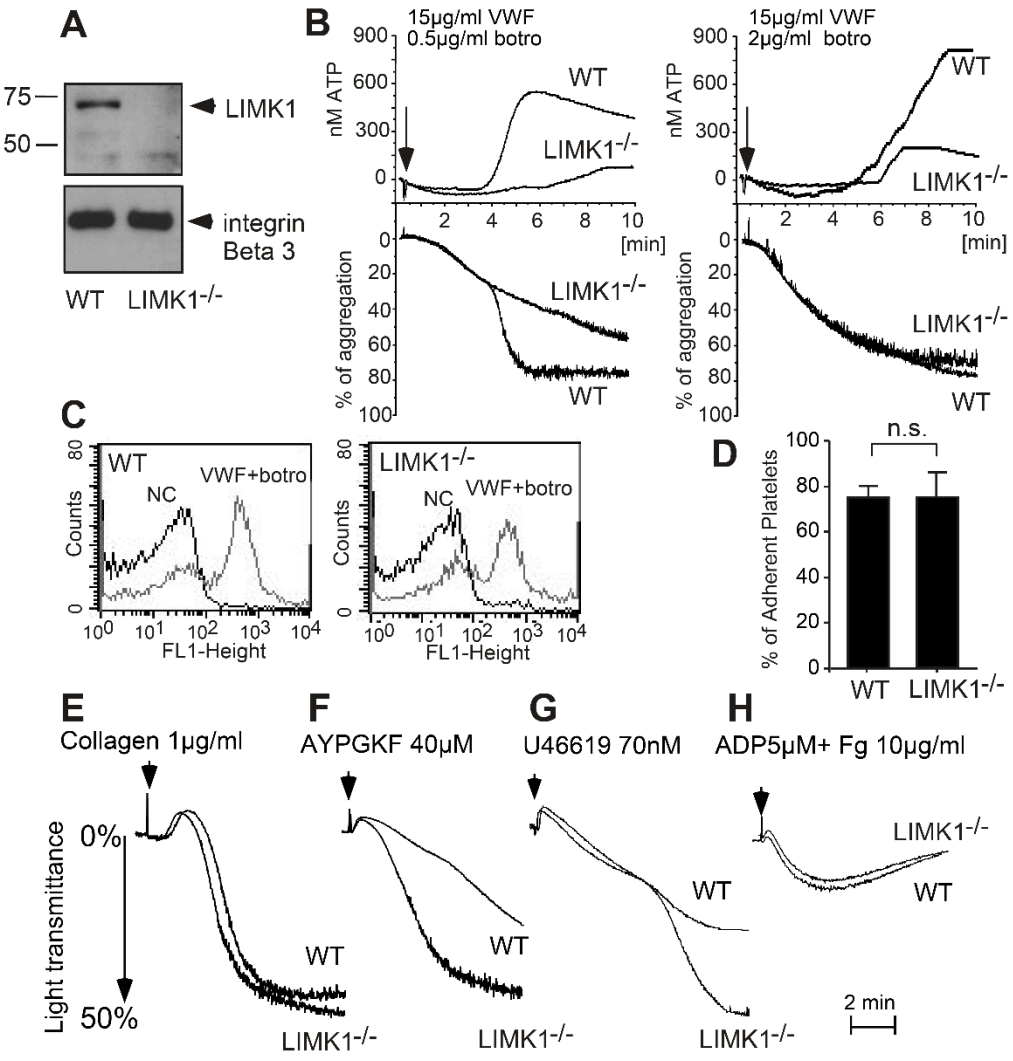


Figure 8. LIMK1 stimulates platelet aggregation and secretion induced by VWF. (A) Immunoblots of platelet lysates from wild-type and LIMK1-knockout mice probed with an anti-LIMK1 antibody and an anti-β3 integrin antibody (loading control). (B) Aggregation and ATP secretion traces of wild-type (WT) and LIMK1$^{-/-}$ platelets stimulated with VWF (15 μg/mL) and indicated doses of botrocetin. (C) Flow cytometric analysis of the binding of VWF to wild-type and LIMK1$^{-/-}$ platelets. (D) Quantitation of WT or LIMK1$^{-/-}$ platelet adhesion to VWF-coated microtiter wells in the presence of botrocetin, using an acid phosphatase assay. (E, F, G and H), Aggregation of WT and LIMK1$^{-/-}$ platelets in response to; collagen (E), PAR4AP (F), U46619 (G) or ADP and Fibrinogen (H).

4.3 LIMK1 plays differential roles in distinct platelet activation pathways

We also examined whether LIMK1 plays a role in stimulating platelet activation induced by collagen (Fig. 8E), PAR4 (thrombin receptor) agonist peptide (AYPGKF) (Fig. 8F), TXA_2 analog U46619 (Fig. 8G), and ADP (Fig 8H). Platelet aggregation responses induced by collagen or ADP were not affected by the loss of LIMK1 (Fig 8E, 7H). In contrast to the defects observed in VWF-stimulated LIMK1$^{-/-}$ platelets (Figure 8B), LIMK1$^{-/-}$ platelets stimulated by low concentrations of PAR4 agonist peptide or U46619 showed an enhanced platelet aggregation response (Fig. 8F, 8G). However, at higher concentrations of PAR4AP or U46619, no difference in aggregation was observed between wild type and LIMK1$^{-/-}$ platelets. Altogether, these results suggest that LIMK1 selectively plays a stimulatory role in VWF/GPIb-IX-dependent platelet activation, but negatively regulates some other platelet activation pathways.

4.4 The selective role of LIMK1 in VWF-induced TXA_2 production

The secretion- and integrin-dependent second wave of platelet aggregation induced by botrocetin/VWF requires the cyclooxygenase/TXA_2 signaling pathway and TXA_2-dependent granule secretion(Kroll et al. 1991, Li, Xi, and Du 2001). Thus, we further investigated whether LIMK1 is important in mediating VWF-induced stimulation of TXA_2 synthesis by measuring a stable TXA_2 metabolite, TXB_2. As expected, VWF/botrocetin induced a TXB_2 increase in wild-type platelets. In contrast, VWF/botrocetin-induced TXB_2 production was significantly reduced in LIMK1$^{-/-}$ platelets (Fig. 9A), indicating that LIMK1 is important in VWF-induced TXA_2 synthesis. We also assessed TXB_2 synthesis following stimulation with GPIb-IX-independent

platelet agonists, collagen and PAR4AP. TXB_2 was dramatically elevated by collagen in both wild-type and LIMK1$^{-/-}$ platelets with no statistically significant difference (Fig. 9B). Low dose PAR4AP induced low, but significant ($P<0.05$) amounts of TXB_2 generation both in wild-type and LIMK1$^{-/-}$ platelets (Fig. 9B), between which there was no significant difference. Thus, the stimulatory role of LIMK1 in TXA_2 generation is selective for the GPIb-IX-dependent platelet activation pathway.

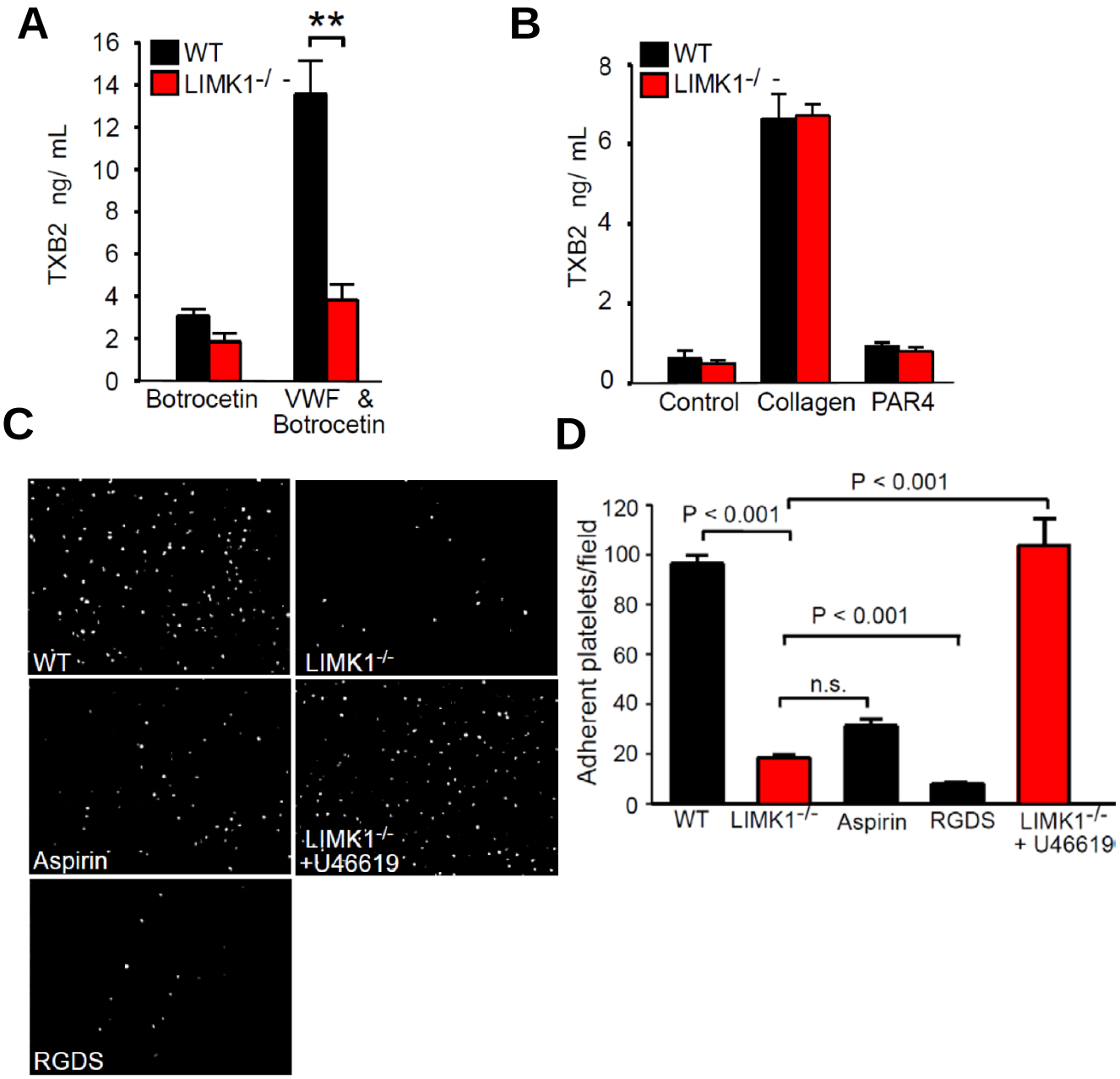


Figure 9. LIMK1 selectively promotes VWF-stimulated platelet TXB_2 production and platelet adhesion under shear stress. (A and B) TXB_2 production in wild-type and LIMK1$^{-/-}$ platelets stimulated with: (A) VWF and botrocetin or with botrocetin alone as a control; (B) collagen or PAR4AP. All data are expressed as mean ± standard error. Statistical significance was determined using the Student's t-test, ** $p<0.01$. (C) Stable adhesion of mepacrine-stained wild type and LIMK1$^{-/-}$ platelets to VWF-coated slides under a constant shear rate (800 s^{-1}). Adherent platelets are photographed under a Leica fluorescence microscope. (D) The number of adherent platelets/field in ≥ 20 randomly selected fields (4 experiments) is shown (mean ± SE). The first, third, and fourth columns are adhesion of wild-type platelets (with or without pretreatment with the integrin inhibitor RGDS or the cyclooxygenase inhibitor aspirin), and the second and fifth columns are LIMK1$^{-/-}$ platelets (with or without 40 nM U46619).

4.5 LIMK1$^{-/-}$ platelets are defective in stable platelet adhesion to VWF under shear stress, which is corrected by supplementing a TXA_2 analog

In platelets, stable adhesion to VWF under shear stress involves (1) early GPIb-IX signaling leading to integrin activation, and (2) its subsequent amplification by the second messenger TXA_2 (Savage, Saldivar, and Ruggeri 1996, Yin, Liu, et al. 2008). To differentiate in which of these two pathways LIMK1 participates, wild-type and LIMK1$^{-/-}$ platelets were stained with mepacrine and allowed to adhere to VWF-coated surfaces under flow conditions (800 s^{-1} shear rate) for 5 minutes. As expected, stable platelet adhesion to VWF under shear stress was almost completely inhibited by the integrin antagonist, RGDS peptide, and was significantly, but partially, inhibited by a saturating concentration of the cyclooxygenase inhibitor, aspirin (1 mM) (Fig. 9C-D). Similarly, stable platelet adhesion to VWF was significantly reduced, but not totally abolished in LIMK1$^{-/-}$ platelets (Fig. 9C-D), indicating LIMK1 is important in the secondary amplification of stable platelet adhesion to VWF under flow conditions. Furthermore, supplementing a TXA_2 analog, U46619, at a concentration similar to that of TXA_2 generated in platelets following VWF stimulation, corrected the adhesion defect in LIMK1$^{-/-}$ platelets, suggesting that LIMK1 promotes GPIb-IX-dependent stable platelet adhesion mainly by stimulating TXA_2 generation (Fig. 9C-D).

4.6 VWF-induced phosphorylation of LIMK1 and cofilin

To determine whether LIMK1 is activated by GPIb-IX ligation, we immunoblotted VWF/ristocetin stimulated human platelet lysates with an antibody recognizing LIMK1

phosphorylated at Thr^{508} (which indicates LIMK1 activation) (Ohashi et al. 2000). LIMK1 became phosphorylated upon VWF stimulation (Fig. 10A). The VWF/ristocetin-induced phosphorylation of LIMK1 was inhibited by a monoclonal anti-GPIbα antibody AK2 (Berndt, Du, and Booth 1988), which blocks VWF binding (Fig. 10B), indicating that VWF binding to GPIb-IX induces the activation of LIMK1.

The enzymatic activity of LIMK1 may be assessed by evaluating the phosphorylation of cofilin at Ser^3, a known LIMK1 substrate (Yang et al. 1998). Phosphorylation of cofilin at Ser^3 was induced by VWF/ristocetin in human platelets (Fig. 10C), and by VWF/botrocetin in wild-type mouse platelets, which was attenuated in $LIMK1^{-/-}$ platelets (Fig. 10D). Altogether, these data indicate that VWF binding to GPIb-IX induces activation of LIMK1 and phosphorylation of cofilin.

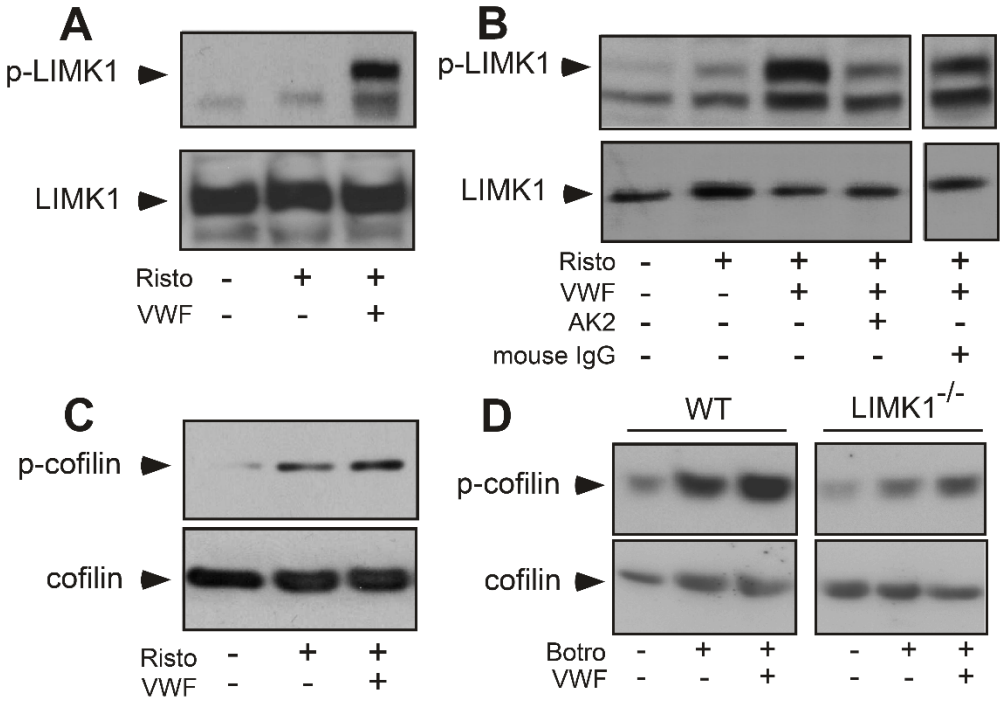


Figure 10. VWF-induced LIMK1 phosphorylation and LIMK1-dependent cofilin phosphorylation. (A, B and C) Immunoblots of washed human platelets stimulated with VWF (10 μg/mL) in the absence or presence of ristocetin (0.25 mg/mL) in a platelet aggregometer at 37°C for 6 min with (A and B) an antibody recognizing phosphorylated $Thr^{508/505}$($T^{508/505}$) in LIMK and an anti-LIMK1 antibody (loading control), (C) an antibody specific for Ser^3(S^3)-phosphorylated cofilin and an anti-cofilin antibody. In (B), platelets were preincubated with a function-blocking anti-GPIbα antibody, AK2 (10 μg/mL), or 10 μg/mL of control IgG for 6 minutes and then stimulated with VWF and ristocetin. (D) Immunoblots of wild-type and $LIMK1^{-/-}$ mouse platelets stimulated with VWF (10 μg/mL) in the presence of botrocetin (2 μg/mL) with an antibody against Ser^3-phosphorylated cofilin or an anti-cofilin antibody.

4.7 LIMK1 promotes VWF/GPIb-IX-mediated actin polymerization following platelet aggregation

A well-known function of LIMK1 is to promote actin polymerization. Thus, we investigated whether knockout of LIMK1 affects VWF/GPIb-IX-induced actin polymerization in platelets. Stimulation of wild-type platelets with VWF/botrocetin, as expected (Yuan et al. 1999), resulted in a significant increase in the amount of polymerized actin as determined by flow cytometric analysis (Fig 11A). By contrast, LIMK1$^{-/-}$ platelets were defective in GPIb-IX-induced actin polymerization following full platelet aggregation only at the 10 minute time point, but responded normally at the early time point (4 min.) during the first wave agglutination/aggregation (Fig. 11A). These data suggest that LIMK1 promotes GPIb-IX-mediated actin polymerization following full platelet aggregation, but not during the early VWF/GPIb-IX-dependent agglutination/aggregation. However, LIMK1$^{-/-}$ platelets were neither different from wild-type control platelets in adhesion and spreading on VWF or fibrinogen, nor in phalloidin staining of spread platelets (Fig. 11B). Since mouse platelets do not spread well on fibrinogen in the absence of agonist, we added a low concentration (0.0125U/mL) of thrombin to induce full platelet spreading on fibrinogen, and also measured spreading at different time points to detect possible differences in spreading kinetics in LIMK1$^{-/-}$ platelets. Surprisingly, LIMK1$^{-/-}$ platelets displayed normal actin morphology and spreading on fibrinogen regardless of thrombin stimulation or time point (Fig. 12), suggesting that integrin outside-in signaling (Coller 1980) and associated actin polymerization does not require LIMK1 during platelet spreading.

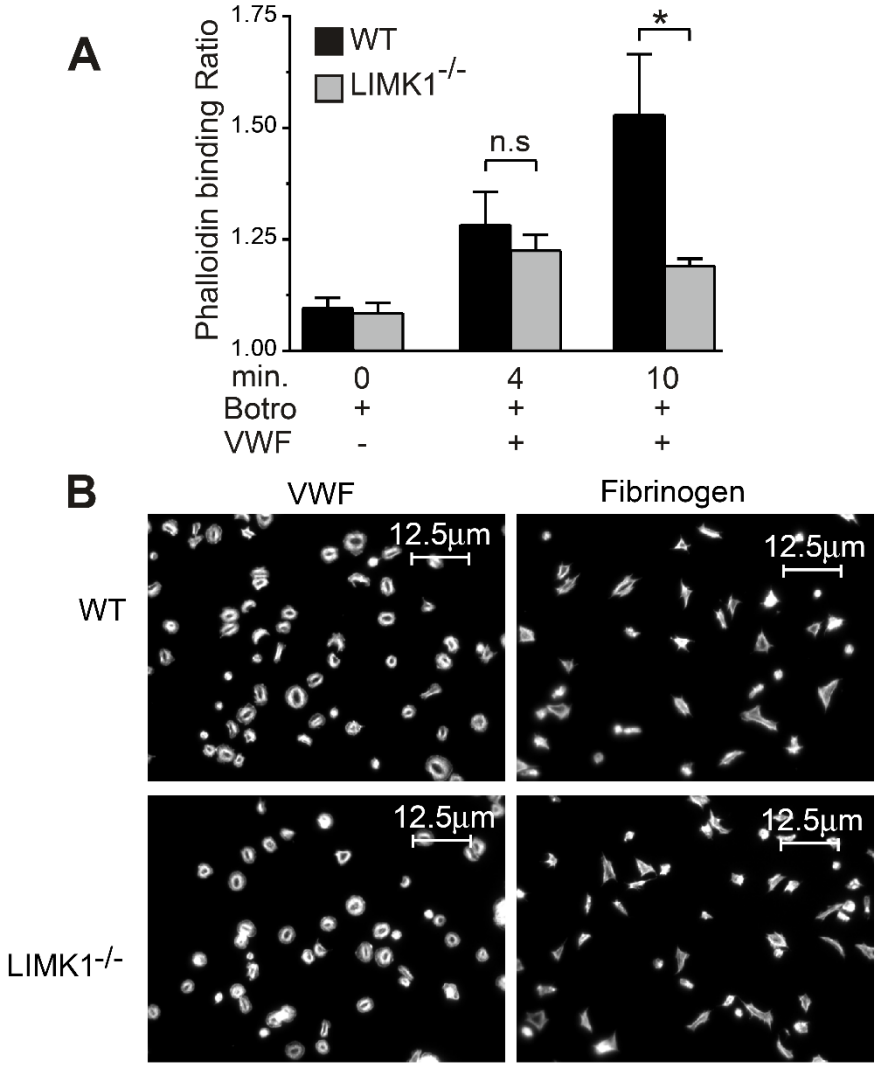


Figure 11. The role of LIMK1 in VWF/GPIb-IX-mediated actin polymerization and in platelet spreading. (A) Flow cytometric analysis of the relative amounts of polymerized actin in WT and LIMK1$^{-/-}$ platelets stimulated with 1 µg/mL botrocetin in the presence or absence of 6.4 µg/mL VWF in an aggregometer at 37˚C for 4 and 10 minutes. The ratio between the mean fluorescence intensity values of stimulated platelets versus the baseline phalloidin binding in control platelets is shown. Statistical significance was determined by one way ANOVA, * $p<0.05$. (B) Alexafluor-546-labeled phalloidin staining of polymerized actin in WT and LIMK1$^{-/-}$ platelets spread on VWF (in the presence of 2 µg/ml botrocetin) or fibrinogen-coated coverslides (90 min at 37°C). Scale bars: 12.5 µm.

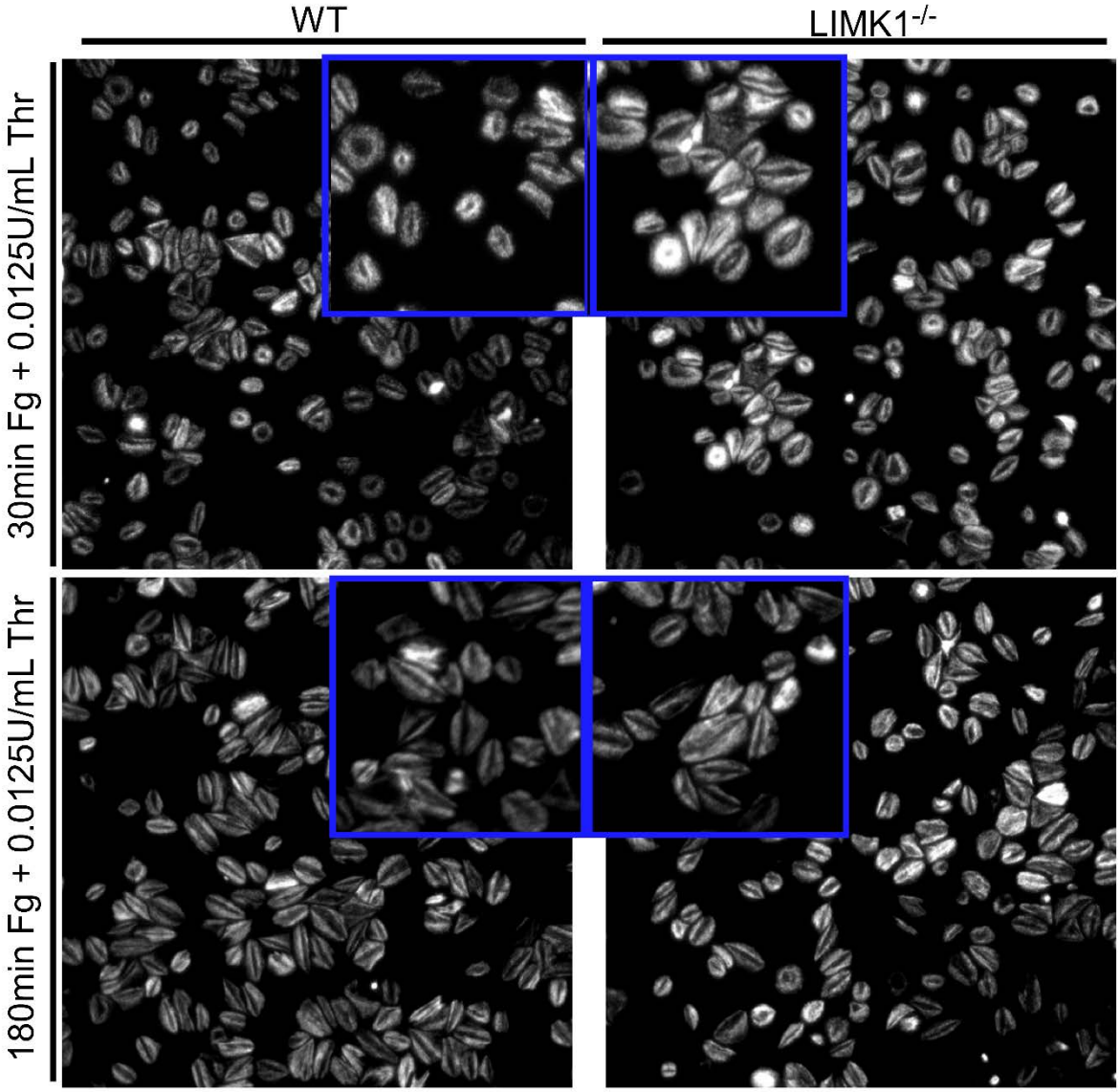


Figure 12. LIMK1$^{-/-}$ platelets display normal morphology during spreading on fibrinogen in the presence of thrombin stimulation. Alexafluor-546-labeled phalloidin staining of polymerized actin in 0.0125U/mL thrombin stimulated WT and LIMK1$^{-/-}$ platelets spread on fibrinogen (Fg)-coated coverslides 30 min (top panels) 180min (bottom panels) at 37°C. The images in the insets (blue outline) show a 3X magnified zoom of the same field. Thr, thrombin.

4.8 Actin polymerization is not required for GPIb-IX-dependent regulation of TXA_2 production

In order to determine whether the role of LIMK1 in stimulating GPIb-IX-induced TXA_2 production and platelet activation is consequential to its function in promoting actin polymerization, we tested the effect of actin polymerization inhibitors on GPIb-IX-induced TXA_2 production and platelet aggregation. Two well-known inhibitors of actin polymerization, cytochalasin D and latrunculin A, were used at concentrations known to inhibit actin polymerization. Neither had any effect on VWF/GPIb-IX-induced TXA_2 production (Fig. 13A), suggesting that GPIb-IX mediated TXA_2 production does not require actin polymerization. Interestingly, cytochalasin D and latrunculin A did not inhibit, but rather accelerated VWF-induced platelet aggregation (Fig. 13B). Therefore, LIMK1 promotes GPIb-IX-mediated TXA_2 synthesis and platelet activation via a mechanism that is independent of actin polymerization.

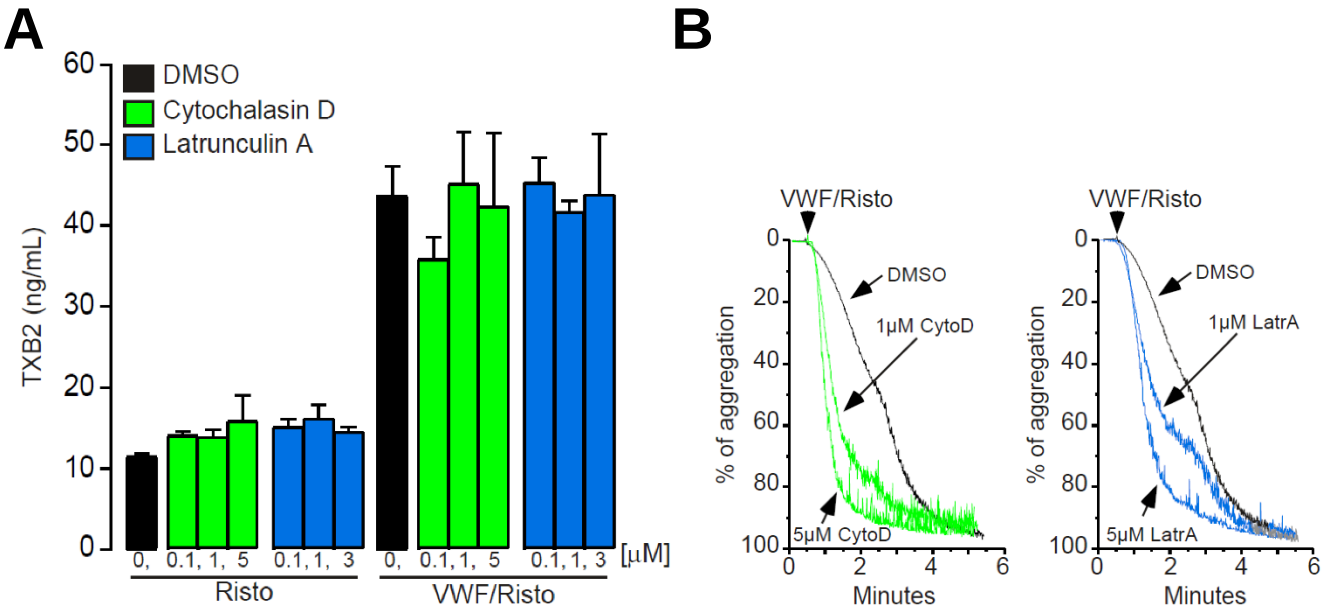


Figure 13. Actin polymerization inhibitors have no effect on VWF-induced TXB2 production, but enhance platelet aggregation response to VWF. Human platelets, preincubated with the indicated doses of Cytochalasin D or Latrunculin A, were stimulated with 10 µg/mL VWF with or without 0.35 mg/mL ristocetin or unstimulated at 37°C for 6 minutes in the aggregometer. (A) Samples were analyzed for TXB_2 and (B) aggregation traces were recorded.

4.9 LIMK1 is important for phospholipase A2 activation during GPIb-IX signaling and is regulated by GPIb-IX-induced MAPK activity

It is established that cytosolic phospholipase A_2 ($cPLA_2$) is a rate-limiting enzyme in TXA_2 synthesis and that $cPLA_2$ becomes activated upon phosphorylation at Ser^{505} (Lin et al. 1993). Thus, we assessed whether LIMK1 regulates $cPLA_2$ phosphorylation during botrocetin/VWF-induced platelet activation. $cPLA_2$ phosphorylation was markedly reduced in $LIMK1^{-/-}$ platelets compared to wild-type (Fig. 14A-B), indicating that LIMK1 plays an important role in promoting the activation of $cPLA_2$ during GPIb-dependent platelet activation.

We have previously reported the importance of the MAPKs, p38 and ERK, in GPIb-IX-induced platelet activation (Li, Xi, and Du 2001, Li, Zhang, et al. 2006). In addition, p38 and ERK are also known to stimulate $cPLA_2$ activation by phosphorylating Ser^{505} (Lin et al. 1993). To determine the relationship between MAPKs and LIMK1, we tested the effect of a p38 inhibitor, SB203580, and a MEK inhibitor, U0126, on LIMK activation. Both inhibitors abolished VWF/ristocetin-induced LIMK phoshorylation (Fig. 14-D). Conversely, GPIb-IX-induced p38 phosphorylation was not affected in $LIMK1^{-/-}$ platelets (Fig. 14A-B). These data indicate that LIMK1 is activated downstream from p38 and ERK MAPKs during GPIb-IX-dependent platelet activation and that LIMK1 likely promotes GPIb-IX-induced TXA_2 synthesis by stimulating the activation of $cPLA_2$.

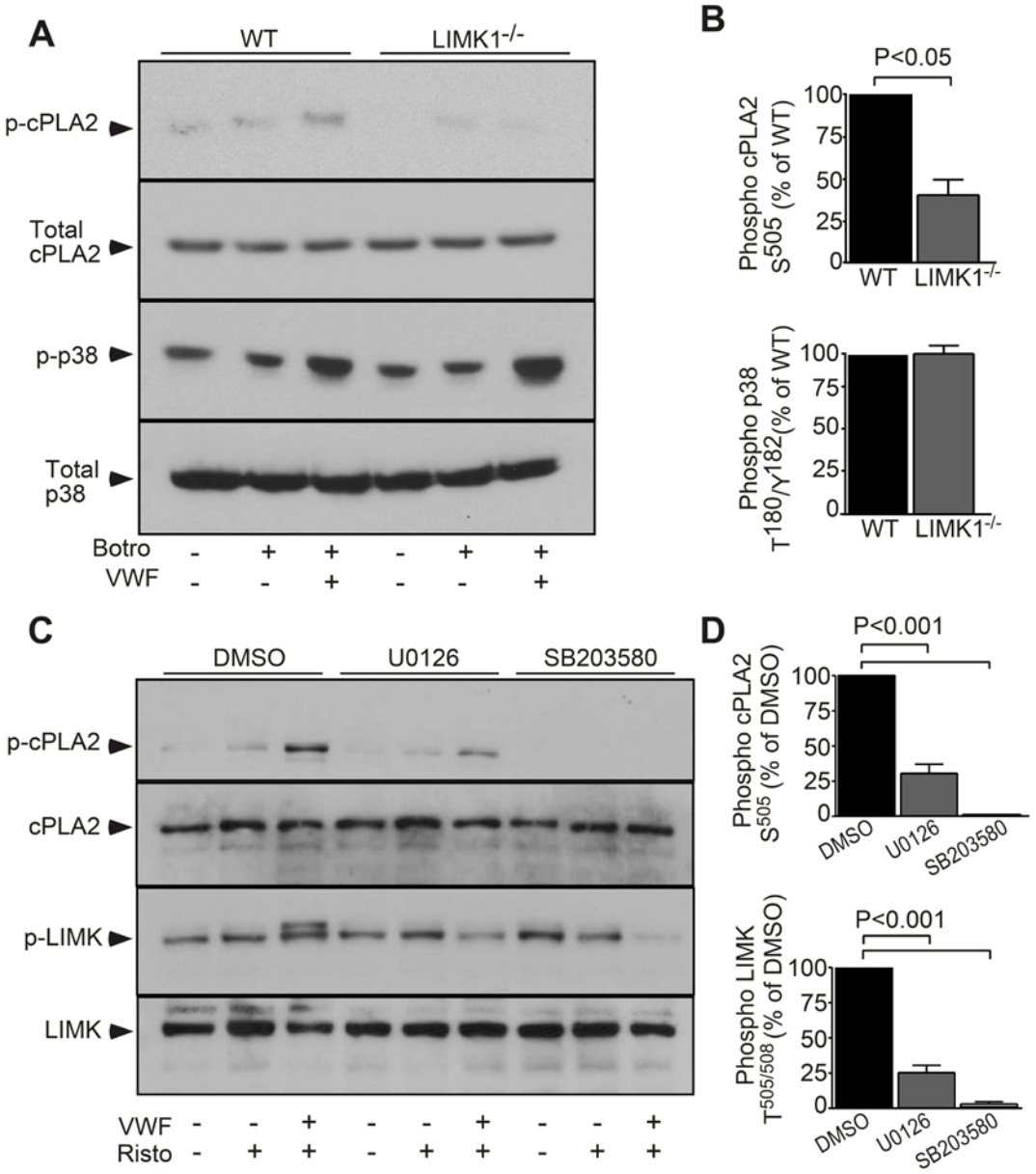


Figure 14. GPIb-IX-mediated cPLA2 activation in wild-type and LIMK1$^{-/-}$ platelets, and the effect of MAPK inhibitors on LIMK activation. (A) Immunoblot analysis of WT and LIMK1$^{-/-}$ platelets, stimulated in the presence or absence of 5 µg/mL VWF, 2 µg/mL botrocetin, or both, with antibodies recognizing phosphorylated cPLA2 Ser^{505}(S^{505}), total cPLA2, phosphorylated p38 $^{thr180/Tyr182}$(T^{180}/Y^{182}) or total p38. (B) Quantitative data from 4 experiments depicted in A using NIH Image J (uncalibrated OD, mean±SE). (C) Human platelets, preincubated with either 25 µM SB203580, 3.5 µM U0126 or DMSO for 2 minutes, were stimulated with VWF (10 µg/mL) in the absence or presence of ristocetin (0.25 mg/mL) or stirred without stimulation in a platelet aggregometer at 37°C for 6 minutes. Lysates from these platelets were immunoblotted with antibodies against phosphorylated LIMK Thr$^{505/508}$ or against phosphorylated cPLA2 Ser^{505}, and also with antibodies against LIMK1 and cPLA2 as loading controls. (D) Quantitative data for C, expressed as percentage of DMSO control.

4.10 Discussion

Anti-platelet drugs have been extensively used clinically to treat and prevent thrombosis, particularly arterial thrombosis. A major adverse effect of currently used anti-platelet drugs is bleeding. This is because the targets of current anti-platelet drugs, such as cyclooxygenase, ADP receptors, and integrins, are important for both hemostasis and thrombosis. It would be ideal if we can identify novel molecular targets for developing anti-thrombotics without significant bleeding side effects. In this study, we demonstrate that LIMK1 plays an important role in promoting arterial thrombosis, but appears to be dispensable for hemostasis as suggested by tail bleeding time. This selective role in arterial thrombosis is associated with the novel function of LIMK1 to selectively promote GPIb-IX-dependent platelet activation and stable platelet adhesion under high shear flow conditions, while having no effect on or negatively regulating GPIb-IX-independent platelet activation pathways. Furthermore, LIMK1 mediates GPIb-IX-dependent $cPLA_2$ activation and TXA_2 synthesis, which serves as a secondary amplification signaling pathway important in occlusive arterial thrombus formation, but not in the initial GPIb-IX-mediated integrin activation and platelet adhesion. Thus, while GPIb-IX-mediated initial platelet adhesion and activation is important both in thrombosis and hemostasis, LIMK1 plays a selective role in occlusive arterial thrombosis without significantly affecting bleeding. Thus, our study not only reveals a new mechanism of GPIb-IX signaling and a novel function of LIMK1, but also a potential molecular target for the development of an anti-thrombotic with minimal bleeding side effect, although it is important to further investigate the role of LIMK1 under various hemostatic and thrombotic conditions.

Our data provides the first direct evidence of an important role for LIMK1 in platelet activation. GPIb-IX-mediated platelet activation consists of two components:1.) the GPIb-IX-mediated early signaling leading to integrin activation and primary platelet response and 2.) the secondary amplification pathways, mediated mainly by TXA_2 and TXA_2-dependent ADP secretion. Data from our laboratory and others indicate that GPIb-IX-mediated early integrin activation involves the sequential activation of the Src family kinase Lyn (Liu et al. 2005, Yin, Liu, et al. 2008), Vav/Rac1 (Delaney et al. 2012), PI3K (Kasirer-Friede et al. 2004), Akt (Yin, Stojanovic, et al. 2008), cGMP-dependent protein kinase (Li et al. 2003) and MAPK pathways (Li, Xi, and Du 2001, Li, Zhang, et al. 2006). However, LIMK1 does not appear to have a role in the initial integrin activation pathway, because LIMK1$^{-/-}$ platelets show no defect in platelet spreading on VWF under static conditions, a process that requires GPIb-IX-dependent integrin activation, and show only a partial defect in platelet adhesion under flow similar to aspirin-treated platelets. We conclude that LIMK1 mediates GPIb-IX signaling mainly by stimulating GPIb-IX-dependent TXA_2 synthesis and consequent TXA_2-dependent platelet granule secretion. This conclusion is supported by data showing that GPIb-IX-dependent TXA_2 synthesis was abolished in LIMK1$^{-/-}$ platelets, and that supplementation with the TXA_2 analog, U46619, rescued the adhesion defect of LIMK1$^{-/-}$ platelets. Furthermore, LIMK1$^{-/-}$ platelets showed reduced VWF/GPIb-IX-mediated $cPLA_2$ activation, suggesting that LIMK1 stimulates VWF-induced TXA_2 synthesis by activating $cPLA_2$. It is still unclear how LIMK1 stimulates $cPLA_2$ activation. Previous studies suggest that ERK and p38 MAPKs can mediate $cPLA_2$ activation (Lin et al. 1993, Canobbio et al. 2004). Our studies showed that both p38 and ERK are activated during GPIb-IX signaling and are important in GPIb-IX-mediated platelet activation (Li, Xi, and Du 2001, Li, Zhang, et al. 2006). Interestingly, we show that LIMK is

activated downstream from the MAPK signaling pathways. Thus, it will be interesting to further investigate how MAPK-dependent activation of $cPLA_2$ is regulated by LIMK1 during GPIb-IX signaling.

To date, the major recognized function of LIMK1 is to phosphorylate and deactivate the cofilin family of actin depolymerizing factors, and thus promote actin polymerization (Yang et al. 1998, Gohla and Bokoch 2002). In agreement with this function, we observed a significant reduction in GPIb-IX-mediated actin polymerization in $LIMK1^{-/-}$ platelets following VWF-induced platelet aggregation. However, the role of LIMK1 in promoting actin polymerization is unlikely to be responsible for its role in selectively stimulating GPIb-IX-mediated TXA_2 synthesis and consequent amplification of platelet activation, as both actin depolymerizing agents and $LIMK1^{-/-}$ platelets attenuate actin polymerization, but only knockout of LIMK1 affects GPIb-induced TXA_2 synthesis. Moreover, actin polymerization inhibitors enhance VWF-induced platelet aggregation, as previously reported (Mistry et al. 2000), which is opposite to the inhibitory effect of LIMK1 knockout. Finally, cofilin phosphorylation is induced by GPIb-IX-independent and dependent agonists, yet deficiency in LIMK1 only inhibited VWF-induced platelet activation. Therefore, our results reveal a novel function of LIMK1 in stimulating GPIb-IX-mediated $cPLA_2$ activation and TXA_2 synthesis independent of its role in stimulating actin polymerization. Nevertheless, we do not exclude the possibility that LIMK1-mediated phosphorylation of cofilin and regulation of actin polymerization may also play a role in GPIb-IX-mediated platelet adhesion or in the regulation of platelet function independent of TXA_2 synthesis, since $LIMK1^{-/-}$ platelets showed a further moderate decrease in platelet adhesion relative to aspirin-treated platelets. In addition, cofilin is indeed phosphorylated during GPIb-

IX-dependent activation and it was reported previously that actin depolymerization reduced the stability of platelet adhesion under shear stress (Dopheide, Maxwell, and Jackson 2002).

Overall, our data demonstrate a novel role for LIMK1 in selectively amplifying GPIb-IX-dependent platelet adhesion and activation by mediating GPIb-IX-induced cPLA2 activation and TXA_2 synthesis, but not in other agonist receptor signaling pathways. Importantly, the selective role of LIMK1 in thrombosis, but not in hemostasis suggests a potential new strategy for anti-thrombotic development.

5. SIGNALING-MEDIATED COOPERATIVITY BETWEEN GLYCOPROTEIN IB-IX AND PROTEASE-ACTIVED RECEPTORS IN THROMBIN-INDUCED PLATELET ACTIVATION

I acknowledge that this research (Figures 15-23; text verbatim from pages 111-139) was originally published in Blood. Estevez B, Kim K., Delaney MK, Stojanovic-Terpo A, Shen B, Ruan C, Cho J, Ruggeri ZM, Du, X. Signaling-mediated cooperativity between glycoprotein Ib-IX and protease-activated receptors in thrombin-induced platelet activation. Blood. 2016 Feb 4;127(5):626-36. doi: 10.1182/blood-2015-04-638387. © The American Society of Hematology. No permission letter is needed (see APPENDIX).

5.1 The important role of GPIb-IX and intracellular interaction between GPIbα and 14-3-3 in stimulating thrombin-induced cellular signaling in a reconstituted CHO cell model

To clearly dissect the role of GPIb in thrombin signaling without complication of secondary or alternative platelet pathways, we reconstituted GPIb-enhanced thrombin signaling in Chinese Hamster Ovary (CHO) cells expressing endogenous PAR1 and the recombinant wild type human GPIb-IX complex (1b9 cells). Upon thrombin stimulation, 1b9 cells displayed enhanced calcium mobilization as compared to control CHO cells that express PAR1 but not GPIb-IX (Figure 15A-B). This result is not caused by differences in PAR1 expression as GPIb-IX-expressing CHO cells and control CHO cells expressed similar levels of PAR1 (Figure 15C). This result is similar to previous data comparing normal platelets with Bernard-Soulier syndrome platelets deficient in GPIb-IX(Jamieson and Okumura 1978), supporting the notion that GPIb-IX-expression enhances thrombin-induced cellular responses.

To verify that the stimulatory role of GPIb-IX requires GPIb-thrombin interaction, we also tested CHO cell lines expressing similar levels of GPIb-IX with GPIbα having a triple tyrosine to phenylalanine mutation ($Y^{276,278,279}$ F, (FFF)) that is known to abolish the high affinity binding of thrombin to GPIb(De Marco et al. 1994, Marchese et al. 1995, Zarpellon et al. 2011) (Figure 15D). As expected, FFF mutation also abolished the binding of an antibody, SZ2, that blocks GPIb-thrombin interaction (Figure 15E) (Ward et al. 1996), but did not affect PAR1 expression (Figure 15C). Indeed, the stimulatory role of GPIb-IX on thrombin response was diminished in FFF cells (Figure 15A-B). To investigate whether the stimulatory role of thrombin binding to GPIb requires GPIb-IX signaling, we examined whether and how deletion of the C-terminal 14-3-3 binding site (Δ605) in the cytoplasmic domain of GPIbα(Du, Fox, and Pei 1996) affects the ability of GPIb-IX to facilitate cellular response to thrombin. The stimulatory effect of GPIb-IX on thrombin-induced calcium mobilization (Figure 15A-B) was abolished in cells expressing the Δ605 mutant of GPIbα, which have comparable levels of GPIb-IX and PAR1 expression as 1b9 cells (Figure 15C- 1E). These data indicate that GPIb-IX-dependent enhancement of thrombin-induced signaling in this CHO cell model requires interaction of the C-terminal region of GPIbα with the intracellular signaling molecule 14-3-3.

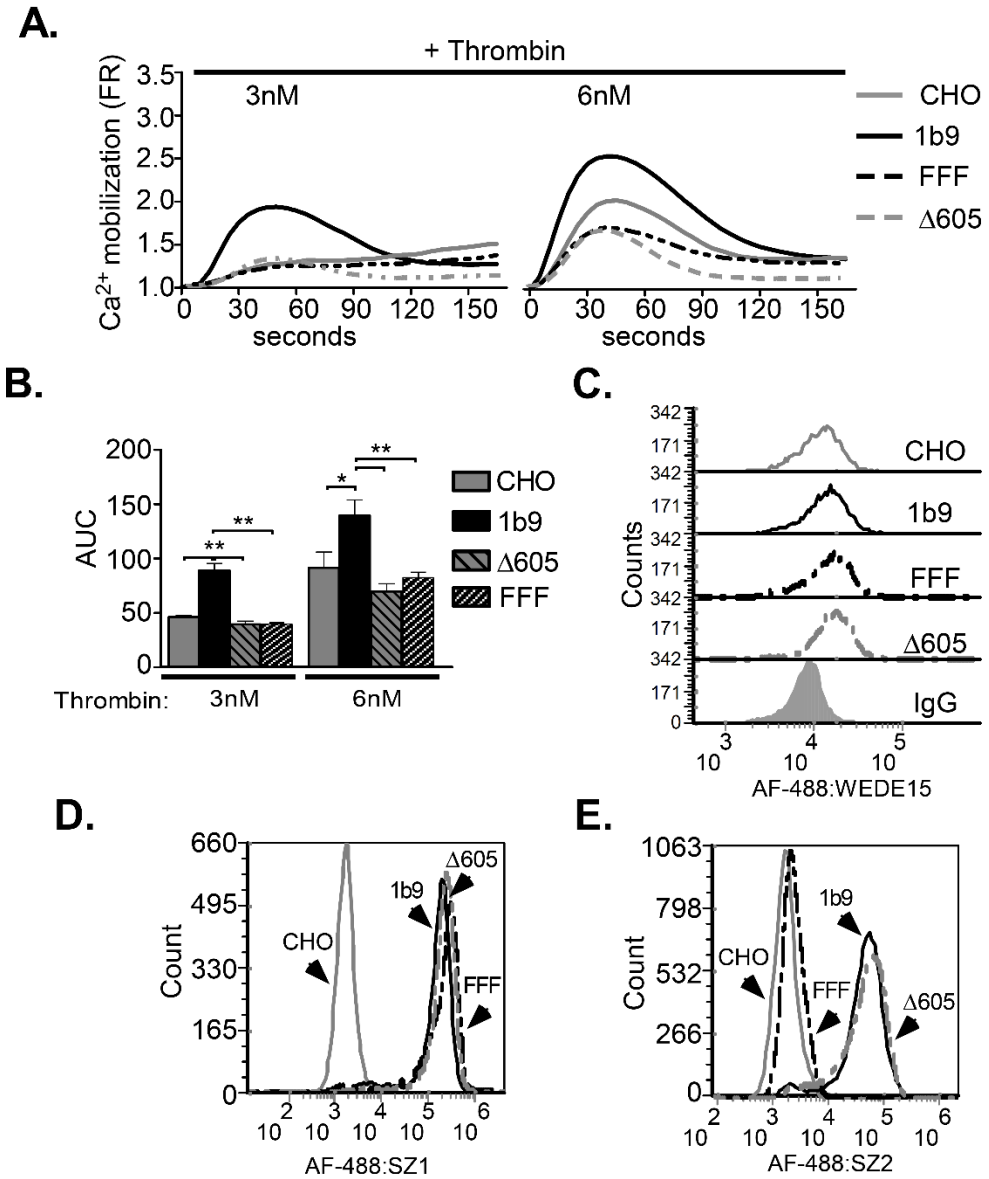
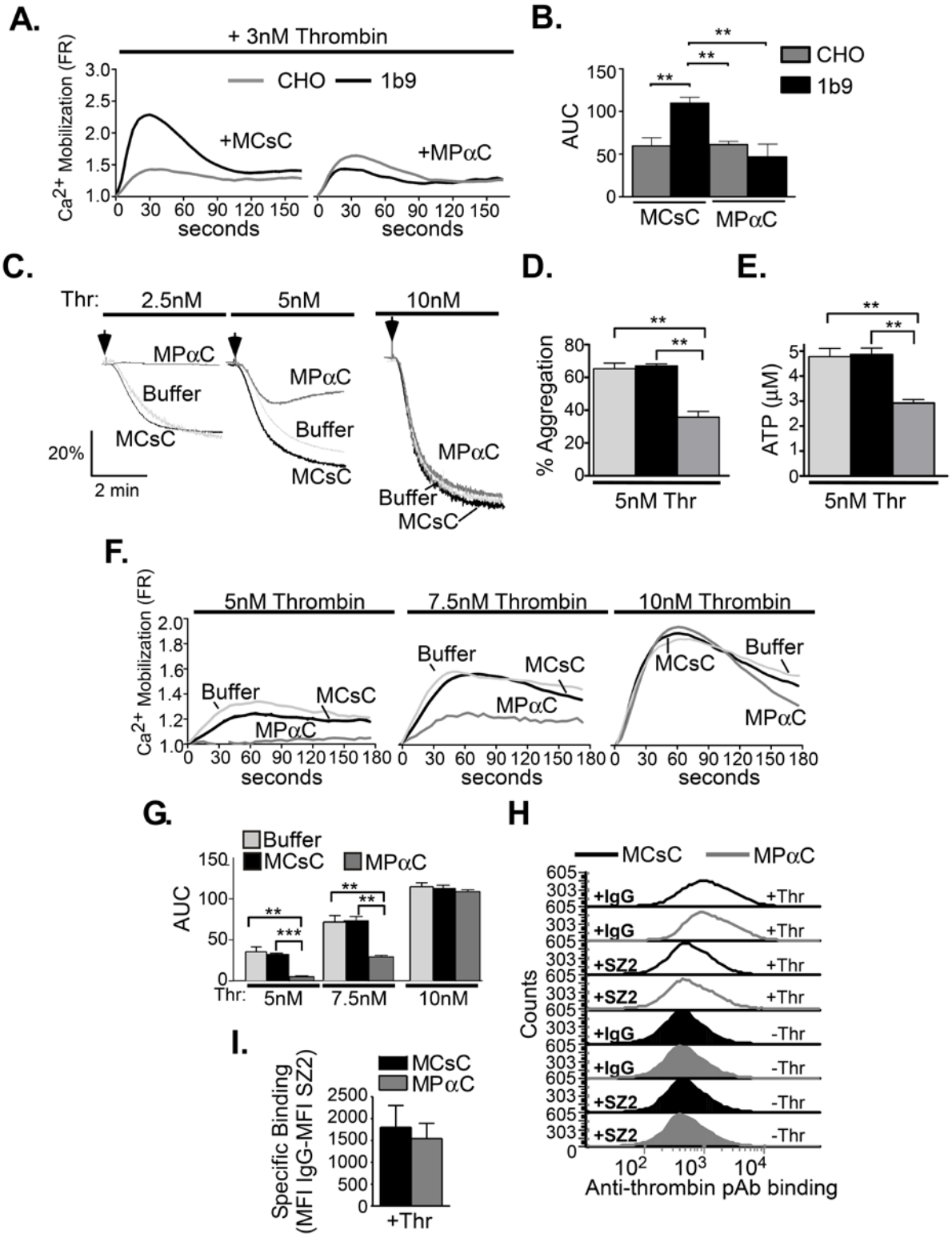


Figure 15: The importance of the C-terminal 14-3-3-binding site of GPIbα in GPIb-mediated cellular response to thrombin. (A) CHO cells expressing endogenous PAR1 and wild type human GPIb-IX (1b9), GPIb-IX with a 5 amino acid truncation in the C-terminal 14-3-3-binding site of GPIbα (Δ605), GPIb-IX with a triple tyrosine to phenylalanine mutation ($Y^{276,278,279}$ F, FFF) in the thrombin binding site of GPIbα, and control CHO cells expressing PAR1 but not GPIb-IX, were each loaded with a calcium-sensitive fluorescent dye, FLIPR Calcium 5 reagent and then stimulated with 3 or 6nM thrombin. Kinetic changes from baseline calcium fluorescence were recorded and plotted as a fluorescence ratio (FR)(Sambrano et al. 2001). (B) Quantification of the area under the curves (AUC) shown in A. from 4 independent experiments (mean±SEM) (*$p<0.05$ and **$p<0.01$, one-way ANOVA). (C-E) Flow cytometric analysis of the binding of an anti-PAR1 monoclonal antibody WEDE15 (C), an anti-GPIb-IX complex monoclonal antibody SZ1 (D), or an anti-GPIbα monoclonal antibody SZ2 (E) as detected using Alexa Fluor 488-labeled goat anti-mouse IgG to control CHO cells, 1b9, Δ605 and FFF cells.

5.2 Intracellular interaction between GPIbα and 14-3-3 is important for low dose-thrombin-induced platelet aggregation, ATP release, and calcium mobilization

We previously developed a membrane permeable inhibitor peptide, MPαC, derived from the phosphorylated C-terminal 14-3-3-binding sequence of GPIbα, which abolishes 14-3-3 binding and selectively inhibits VWF/GPIb-IX-dependent platelet response(Dai et al. 2005). MPαC selectively inhibited thrombin-induced calcium response of GPIb-expressing CHO cells but not control CHO cells, further validating its specificity for GPIb-IX-dependent cell response to thrombin (Fig. 16A-B). To investigate the role of the 14-3-3-GPIb interaction in GPIb-IX-dependent promotion of human platelet response to thrombin, we determined the effect of MPαC, or a scrambled control peptide, MCsC, on thrombin-induced human platelet aggregation. MPαC inhibited low dose thrombin-induced platelet aggregation (Figure 16C-D), suggesting that 14-3-3-GPIb interaction is important for enhancing thrombin-induced platelet aggregation. Thrombin-stimulated platelet responses include receptor-coupled calcium mobilization and granule secretion (Cattaneo et al. 1990, Brass and Joseph 1985). The observed calcium mobilization induced by low dose thrombin was not affected by integrin blocker integrilin, indicating that it is an early response independent of integrin outside-in signaling (Figure 17A-B). MPαC-treatment inhibited low dose thrombin-induced ATP-release (Figure 16E) and calcium mobilization (Figure 16F-G), which is similar to the response of GPIb-IX-deficient platelets from Bernard-Soulier syndrome patients or GPIb blocking antibody-treated platelets(Greco et al. 1996, Jamieson and Okumura 1978). These data reveal the essential function of GPIb-14-3-3 interaction in platelet response to low-doses of thrombin.


A.
+ 3nM Thrombin
CHO
1b9
+MCsC
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C.
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5nM
10nM
MPαC
Buffer
MCsC
20%
2 min
D.
% Aggregation
5nM Thr
E.
ATP (μM)
5nM Thr
F.
5nM Thrombin
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10nM Thrombin
Buffer
MCsC
MPαC
G.
Buffer
MCsC
MPαC
AUC
Thr:
5nM
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10nM
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MCsC
MPαC
+IgG
+SZ2
+Thr
-Thr
Counts
Anti-thrombin pAb binding
I.
MCsC
MPαC
Specific Binding
(MFI IgG-MFI SZ2)
+Thr

Figure 16: The effect of an inhibitor of 14-3-3-GPIb interaction on thrombin-induced cellular responses in GPIb-expressing CHO cells and platelets. (A) CHO cells or CHO cells expressing GPIb-IX were loaded with a calcium sensitive dye (FLIPR Calcium 5) and then treated with MPαC or scrambled control peptide prior to stimulation with 3 nM thrombin. Thrombin-induced calcium responses were determined as in figure 1. (B) Quantification of data shown in A (mean±SEM, 3 experiments, **p<0.01, one-way ANOVA). (C) Thrombin-stimulated aggregation of human platelets pretreated with either the MCsC (control), MPαC micellar peptide or buffer alone (no peptide treatment). (D) Quantitative data of 4 aggregation experiments as shown in C (mean±SEM). (E) Thrombin-stimulated ATP release (mean ±SEM, 4 experiments). (*p<0.05 and **p<0.01, t-test). (F) Thrombin-induced calcium mobilization in human platelets pretreated with MCsC, MPαC or buffer alone. (G) Quantification of the area under the curves (AUC) as shown in F (mean±SEM, 3 experiments, **p<0.01, and ***p<0.001, one-way ANOVA). (H) Stacked fluorescence histograms of 3nM S195A thrombin binding to washed human platelets pretreated with 10μM MPαC or control peptide MCsC as analyzed with flow cytometry using a goat anti-thrombin antibody and an Alexafluor 488-labeled anti-goat IgG antibody. Prior to thrombin addition, samples were preincubated with IgG or SZ2 to verify GPIb-dependent thrombin binding. Thr, 3nM S195A thrombin. (I) Quantification of specific binding of thrombin to MCsC or MPαC-treated platelets (mean±SEM, 3 experiments, p=0.6847). Specific binding is calculated by subtracting thrombin binding mean fluorescence intensity (MFI) of SZ-2-treated platelets from IgG-treated platelets.

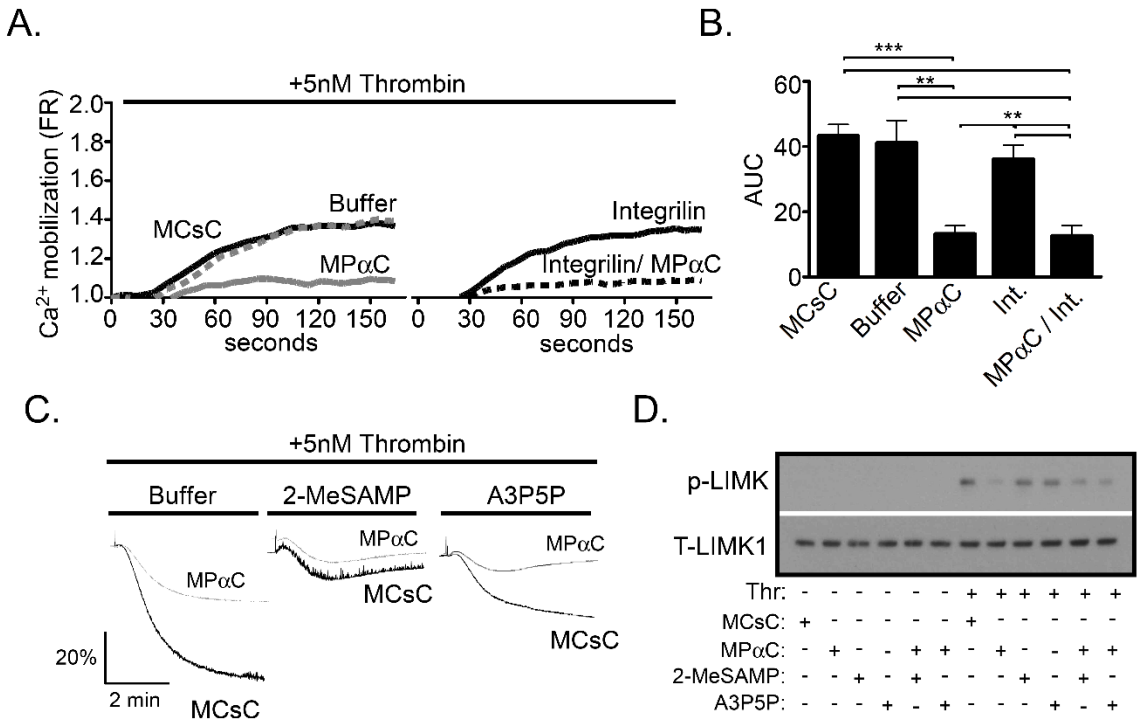

Figure 17: ADP and Integrin signaling are not required for low dose thrombin-induced GPIb signaling (A) Calcium mobilization in human platelets pretreated with 50 μM integrilin, 10 μM MCsC, 10 μM MPαC, buffer or both MPαC and integrilin and then stimulated with 5 nM thrombin. (B) Quantification of the area under the curves (AUC) as shown in A (mean±SEM, 3 experiments, **p<0.01, and ***p<0.001, one-way ANOVA). Int, denotes integrilin. (C) Aggregation of washed human platelets pretreated with 50 μM 2-MeSAMP (2-methylthioadenosine 5'-monophosphate triethylammonium, P2Y12 antagonist), 0.5 mM A3P5P (adenosine-3'-phosphate-5'phosphate, P2Y1 antagonist), in the presence of either MCsC, MPαC or vehicle buffer control (0.15 M NaCl, 10 mM HEPES pH 7.4), and then stimulated with 5 nM thrombin. (D) Western blot analysis of LIMK1 phosphorylation using an phospho-$Thr^{505/508}$-dependent anti-LIMK1 antibody in washed mouse platelets pretreated with either buffer, 50 μM 2-MeSAMP, 0.5 mM A3P5P, 10 μM MPαC, MCsC or both MPαC and A3P5P or 2-MeSAMP and then stimulated with 2.5 nM thrombin.

5.3 Intracellular interaction between GPIbα and 14-3-3 is not important for thrombin binding to GPIb

It is known that GPIb contains a high affinity thrombin binding site (De Marco et al. 1994, Gralnick et al. 1994, Marchese et al. 1995, Zarpellon et al. 2011), but whether the cytoplasmic 14-3-3 binding region is important for regulating thrombin binding to GPIb is unknown. To exclude the possibility that interfering with GPIb-14-3-3 interaction also affects thrombin binding to GPIb-IX, we measured thrombin binding to human platelets pretreated with MPαC. MPαC pretreatment did not affect low dose thrombin binding to GPIb (Figure 15H-I). In contrast, thrombin binding was inhibited by the GPIb thrombin binding site-blocking antibody SZ2 (Figure 16H). These data indicate that the 14-3-3-GPIb-IX interaction is not important for thrombin binding to GPIb-IX.

5.4 The role of Rac1 in mediating thrombin-induced GPIb-IX signaling

To further identify the thrombin-induced GPIb-IX signaling pathway involved in stimulating platelet response, we tested the hypothesis that the thrombin-induced GPIb-IX signaling pathway is similar to the VWF-induced platelet activation pathway, in which a Lyn/Rac signaling pathway plays an important role (Delaney et al. 2012). A Rac1 inhibitor, NSC23766 diminished low dose thrombin-induced calcium mobilization only in wild type GPIb-IX-expressing CHO cells (1b9), but not in control CHO cells (Figure 18A-D), indicating that

Rac1 is required for GPIb-dependent thrombin signaling leading to calcium mobilization. Importantly, upon low-dose thrombin stimulation, 1b9 cells displayed enhanced Rac1-activity, which was absent in Δ605 cells expressing the 14-3-3 binding deficient mutant GPIbα (Figure 18F-G). In human platelets, low dose thrombin similarly induced activation of Rac1, which was inhibited by the inhibitor of GPIb-14-3-3 interaction, MPαC, but not by the control peptides (Figure 18E). Furthermore, whereas thrombin responses of wild type mouse platelets, as indicated by platelet aggregation, secretion and calcium mobilization, were inhibited by MPαC, suggesting the importance of GPIb-IX-14-3-3 interaction also in mouse platelets, Rac1$^{-/-}$ mouse platelets, which already showed reduced platelet response to low dose thrombin, could not be further inhibited by MPαC (Figure 19). These data suggest that GPIb-IX-14-3-3 interaction and Rac1 mediate the same signaling pathway (Figure 19A-F). Together these data show that thrombin-induced GPIb signaling mediates Rac1-activation via a 14-3-3-dependent mechanism, and that Rac1 is required for the GPIb-IX signaling that stimulates low dose thrombin-induced cellular response.

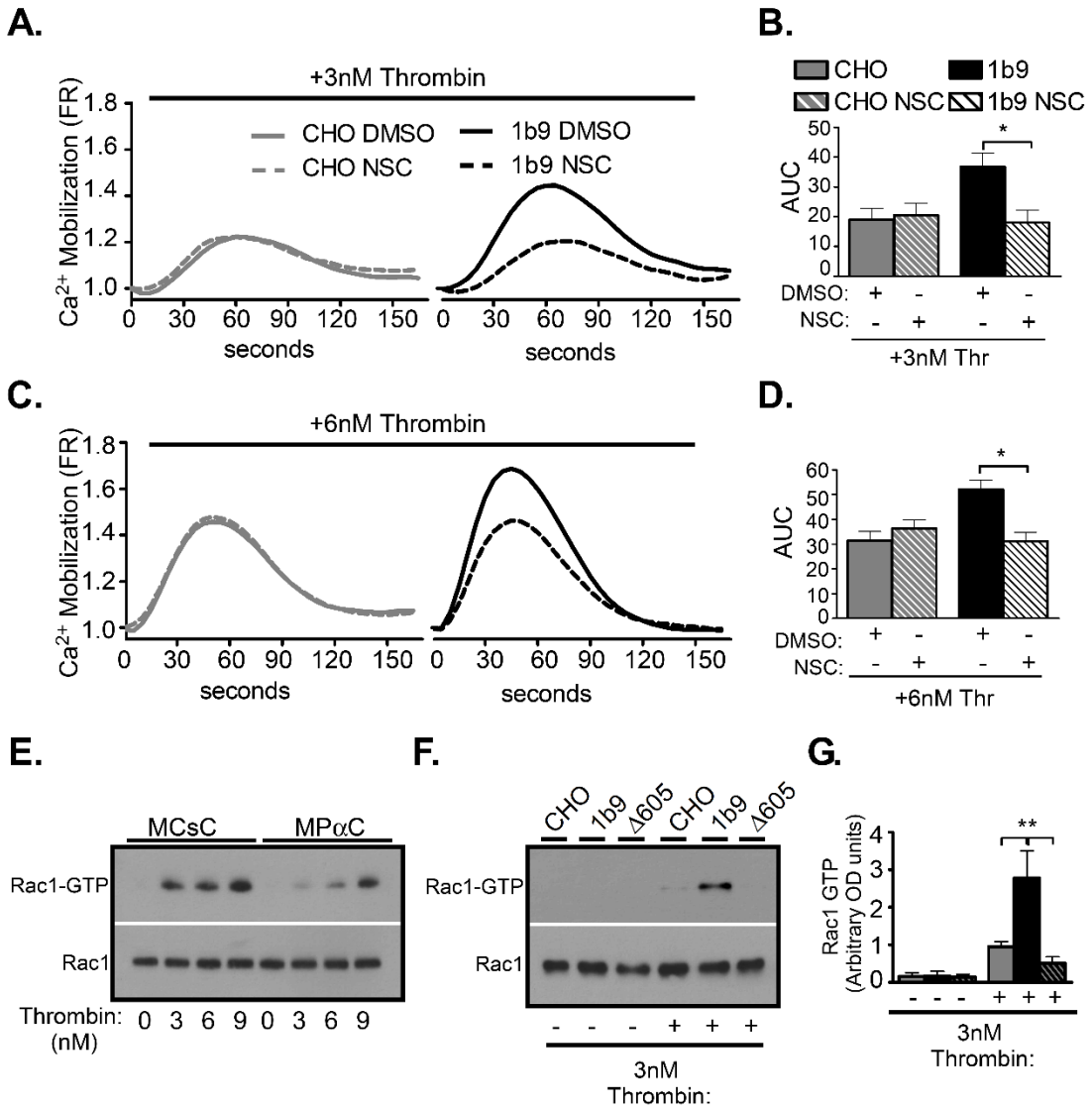

Figure 18: The GPIb-IX-dependent effect of Rac1 inhibitor NSC23766 on thrombin – induced calcium elevation. Control CHO cells and 1b9 cells were loaded with a fluorescent calcium sensitive dye, then treated with either 0.1% DMSO (DMSO) or 100μM NSC23766 (NSC). Calcium fluorescence signals of these cells were then recorded following stimulation with 3nM (A,B) or 6nM (C, D) thrombin. (A, C) Typical graphs of calcium elevation. (B,D) Quantitative data of A and C (mean ± SEM, 3 experiments, one-way ANOVA). *, $p<0.05$. (E, F) Western blot analysis of levels of GTP-bound active Rac1 in thrombin-stimulated human platelets pretreated with either MPαC or MCsC (E), or in control CHO cells or 1b9 cells (F). The amount of GTP-bound Rac1 was determined using a PAK-GST pull-down assay, followed by western blotting with an anti-Rac1 antibody. Total levels of Rac1 in platelet lysates were also determined by western blot using anti-Rac1 antibody. (G) Densitometric analysis of Western blots as shown in F (mean ±SEM, 3 experiments **,$p<0.01$, one-way ANOVA) using NIH Image J. The results were expressed as arbitrary units of uncalibrated optical density.

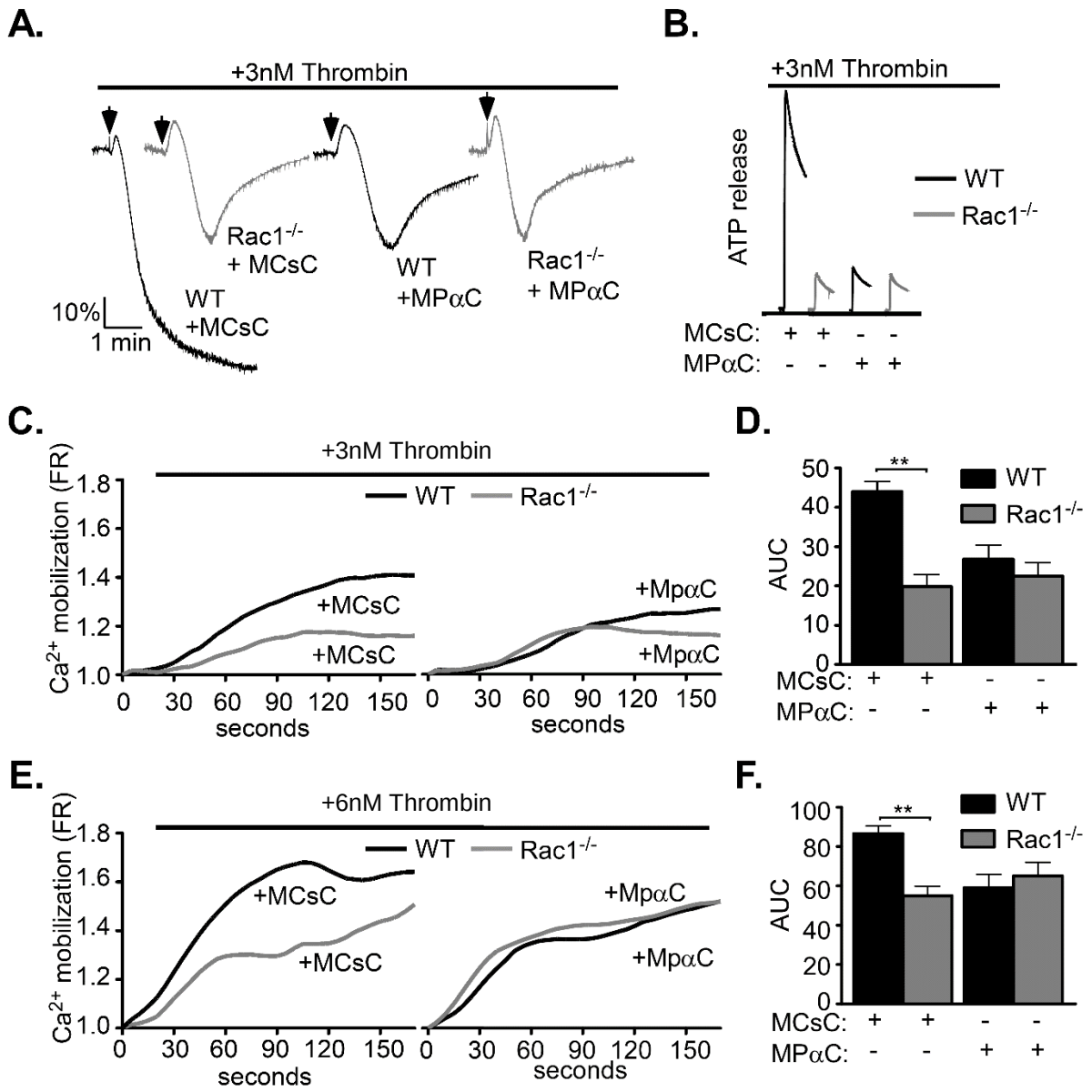

Figure 19: Comparison of thrombin-induced aggregation, ATP secretion and calcium elevation and the inhibitory effect of MPαC between wild type and Rac1$^{-/-}$ platelets. Washed wild type or Rac1$^{-/-}$ platelets were pretreated with MPαC or control peptide MCsC and the analyzed for (A) Aggregation, (B) ATP-release, and (C-F) calcium mobilization induced by thrombin. (C, E) Typical plots of calcium mobilization. (D, F) Quantitative data from 3 experiments (mean±SEM). ** indicates $p<0.01$ (one-way ANOVA).

5.5 A Rac1/LIMK1 pathway that mediates thrombin-induced GPIb-IX signaling

LIMK1 can be activated by Rac1 signaling(Yang et al. 1998), and is involved in the amplification of VWF-induced platelet activation(Estevez et al. 2013). To investigate whether LIMK1 is important in thrombin-induced GPIb-IX signaling, we determined the effects of LIMK1 knockout on platelet activation induced by thrombin. LIMK1$^{-/-}$ platelets displayed significantly reduced aggregation and ATP-release in response to low-doses of thrombin (Figure 20A-C), indicating a stimulatory role for LIMK1 in thrombin-induced platelet activation. The stimulatory role of LIMK1 in thrombin-induced platelet response is independent of PAR signaling, because LIMK1$^{-/-}$ platelets displayed significantly enhanced aggregation and ATP-release in response to PAR4-AP (Figure 20D-F). These data suggest that LIMK1 plays a stimulatory role in platelet response to thrombin but a negative regulatory role in PAR signaling. To determine whether the thrombin-induced GPIb-IX signaling activates LIMK1, we assessed LIMK1 activation as indicated by its phosphorylation in 1b9, Δ605, and control CHO cells stimulated with thrombin. Thrombin induced some degree of LIMK1 phosphorylation in CHO cells lacking GPIb-IX expression, but this was detectable only at a higher concentration of thrombin (Figure 20G). In contrast, thrombin stimulation of 1b9 cells induces much greater LIMK1 phosphorylation, indicating the role of GPIb-IX signaling in promoting LIMK1 phosphorylation. However, in Δ605 cells lacking the 14-3-3 binding site of GPIbα the enhanced LIMK1 phosphorylation was absent. These data indicate that thrombin-induced GPIb-IX signaling mediates 14-3-3-dependent LIMK1 activation. In mouse platelets, MPαC-treatment inhibited thrombin-induced LIMK1 phosphorylation suggesting that 14-3-3-GPIb-interaction is important in thrombin-

induced LIMK1 activation in platelets (Figure 20H). In contrast to the inhibitory effect of MPαC, inhibition of ADP signaling using ADP receptor antagonists did not affect thrombin-induced LIMK1 phosphorylation, suggesting that thrombin-induced GPIb-IX-dependent LIMK1 phosphorylation does not require subsequent secretion of ADP (Figure 17D). In other cell types, it is known that LIMK1 is activated downstream of Rac1(Yang et al. 1998). In platelets, however, whether thrombin signaling activates LIMK1 in a Rac1-dependent manner is unknown. Thus, to determine if LIMK1 lies downstream of Rac1, we assessed LIMK1 phosphorylation in thrombin stimulated Rac1$^{-/-}$ platelets. Thrombin-stimulated Rac1$^{-/-}$ platelets displayed defective LIMK1 phosphorylation (Figure 20I). Together, these data indicate a GPIb-IX-14-3-3-Rac1-LIMK1 signaling pathway that is important in thrombin-induced GPIb-IX signaling and GPIb-IX signaling-dependent platelet response.

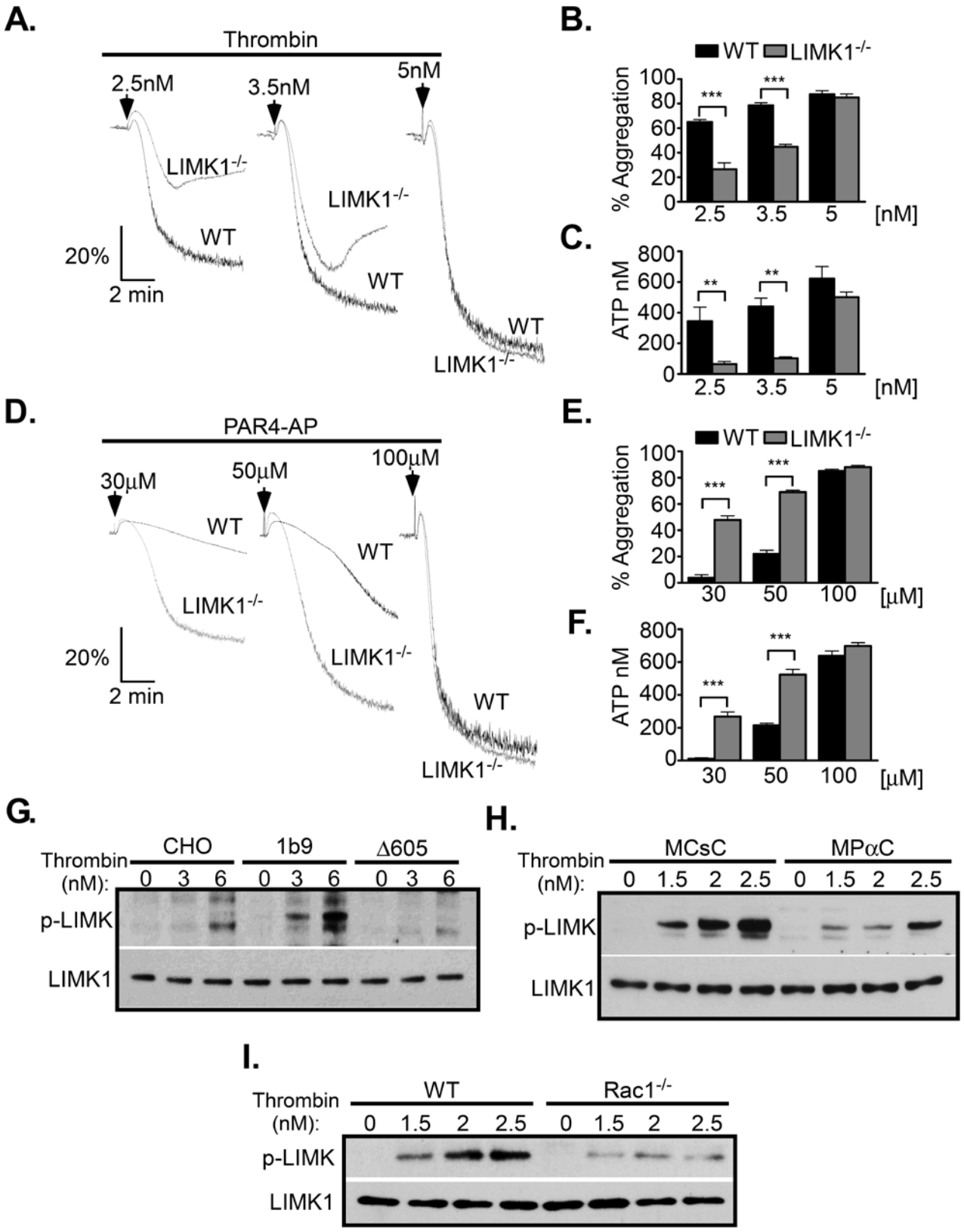
A.
Thrombin
2.5nM
3.5nM
5nM
LIMK1-/-
WT
20%
2 min
B.
WT
LIMK1-/-
% Aggregation
***
2.5
3.5
5
[nM]
C.
ATP nM
**
D.
PAR4-AP
30μM
50μM
100μM
E.
30
50
100
[μM]
F.
G.
CHO
1b9
Δ605
Thrombin (nM):
p-LIMK
LIMK1
H.
MCsC
MPαC
I.
WT
Rac1-/-

Figure 20: GPIb-IX- and Rac-1 dependent activation of LIMK1 and the roles of LIMK1 in promoting thrombin-induced platelet activation but negatively regulating PAR4-activating peptide-induced platelet activation. Aggregation and ATP release of washed wild type (WT) or LIMK1$^{-/-}$ mouse platelets in response to increasing doses of thrombin (A-C) or PAR4-activating peptide-induced (PAR4-AP) stimulation (D-F). (A, D) typical aggregation traces. (B, E) Quantification of the aggregation (mean±SEM, 4 experiments). (C, F) ATP release (mean ±SEM, 4 experiments). **$p<0.01$, and ***$p<0.001$, one-way ANOVA. Western blot analysis of LIMK1 phosphorylation using an phospho-Thr$^{505/508}$-dependent anti-LIMK1 antibody in (G) Control CHO cells, 1b9 and Δ605 cells; (H) washed mouse platelets pretreated with either MPαC or MCsC; and (I) wild type or Rac1$^{-/-}$ mouse platelets; which were stimulated with low doses of thrombin. Loading levels were determined using a phosphorylation-independent LIMK1 antibody.

5.6 Cooperativity between GPIb-IX signaling and PARs in mediating thrombin-induced cellular response

Demonstration of a role for GPIb-IX signaling in stimulating platelet response to thrombin reignites the debate with regards to whether GPIb-IX mediates PAR-independent signaling that is sufficient to initiate a cellular response. To determine whether thrombin induces a GPIb-dependent cellular response independent of PAR signaling, we treated GPIb-expressing CHO cells with a PAR1 antagonist, SCH 79797. SCH 79797-pretreatment abolished thrombin-induced calcium mobilization in 1b9 cells expressing wild type human GPIb-IX as well as in control CHO cells and Δ605 cells (Figure 21A), suggesting that thrombin-induced calcium mobilization requires PAR1 signaling in the presence or absence of GPIb-IX signaling. These data suggest that GPIb-IX signaling is not sufficient to elicit detectable calcium mobilization in response to thrombin in the absence of PAR1 signaling in this reconstituted CHO cell model. This result is consistent with a previous study showing that PAR4 knockout mouse platelets are unable to respond to thrombin (Sambrano et al. 2001). However, PAR signaling requires catalytic activity of thrombin, and a major piece of previous data suggesting PAR-independent GPIb-IX signaling was that S195A mutant thrombin that reportedly lost enzymatic activity or PPACK-inhibited thrombin can still stimulate platelet activation in the absence of GPV(Ramakrishnan et al. 2001). We did not observe cell response induced by PPACK-treated wild type thrombin (Figure 21D). As PPACK treatment carries the possibility of incomplete inhibition resulting in residual enzymatic activity that may vary

between different laboratories, we further tested whether S195A mutant thrombin is able to induce PAR-independent GPIb-IX signaling in the reconstituted CHO cell model and in platelets. Indeed, S195A mutant thrombin induced calcium mobilization, which was significantly enhanced in 1b9 cells expressing wild type GPIb-IX in a manner similar to wild type thrombin(Figure 21B). However, the S195A mutant thrombin-induced calcium mobilization was abolished by PAR1 antagonist SCH79797 (Figure 21C). These data suggest the requirement for PAR1 in the GPIb-IX-dependent cell response to either S195A or wild type thrombin in the reconstituted CHO cell model. In platelets, S195A mutant thrombin was unable to induce platelet aggregation at low doses, which is consistent with a previous report(Wu et al. 1991). However, S195A thrombin induced platelet aggregation at doses of 100 nM or higher (Figure 21D), and this effect of S195A thrombin was abolished by its treatment with PPACK (Figure 21D), suggesting that S195A thrombin contains residual enzymatic activity that is required for its activation of platelets. Indeed, the enzymatic activity of S195A thrombin at high concentrations was detectable using a fluorogenic thrombin substrate (Figure 21E), and this activity was abolished by further treatment with PPACK. Similarly, S195A thrombin induced mouse platelet aggregation at high concentrations, which was also abolished by PPACK-treatment (data not shown). These data suggest that thrombin-induced GPIb-IX-dependent cellular response requires the catalytic activity of thrombin and PAR-dependent signaling. Furthermore, these data also suggest a mutually dependent cooperativity between GPIb-IX signaling and PAR signaling that is important for platelet response to low dose thrombin.

To further investigate the mechanism of this cooperativity between GPIb-IX signaling and PAR signaling, we examined whether GPIb-IX-dependent activation of the Rac1-LIMK1 pathway requires cooperativity of PAR signaling. Pretreatment with a PAR1 antagonist in CHO

cells or PAR4 antagonist in mouse platelets abolished the thrombin-induced GPIb-IX-dependent stimulation of LIMK1 activation (Figure 21G-H). Furthermore, the PPACK-treated S195A mutant thrombin failed to induce a dose-dependent increase in LIMK1 phosphorylation in human platelets (Figure 21F). These data suggest that thrombin-induced GPIb-IX signaling requires cooperativity of PAR signaling. Taken together, our data indicate that there is a mutual cooperativity between GPIb-IX signaling and PAR signaling during thrombin-induced platelet activation (see Discussion and Conclusion). PAR-dependent signaling is important for thrombin-induced GPIb-IX signaling and thrombin-induced GPIb-IX signaling is important for stimulating PAR signaling, particularly at low thrombin concentrations. This signaling-mediated cooperativity provides a novel mechanism for the requirement of dual thrombin receptors (PARs and GPIb-IX) in mediating platelet response (Figure 21I).

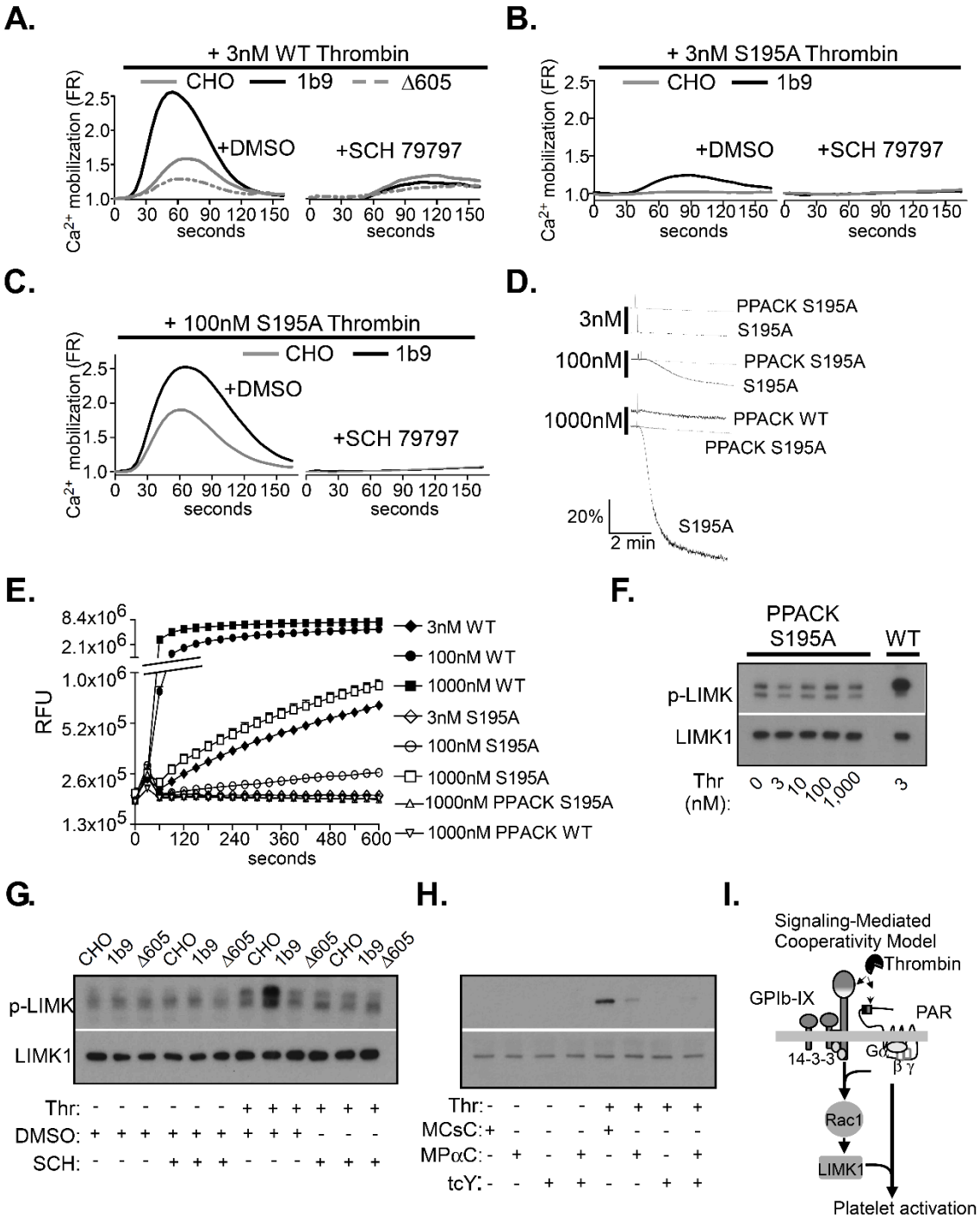
A.
+ 3nM WT Thrombin
CHO
1b9
Δ605
+DMSO
+SCH 79797
Ca2+ mobilization (FR)
seconds
B.
+ 3nM S195A Thrombin
C.
+ 100nM S195A Thrombin
D.
3nM
100nM
1000nM
PPACK S195A
S195A
PPACK WT
20%
2 min
E.
RFU
seconds
3nM WT
100nM WT
1000nM WT
3nM S195A
100nM S195A
1000nM S195A
1000nM PPACK S195A
1000nM PPACK WT
F.
PPACK S195A
WT
p-LIMK
LIMK1
Thr (nM):
G.
CHO
1b9
Δ605
Thr:
DMSO:
SCH:
H.
MCsC:
MPαC:
tcY:
I.
Signaling-Mediated Cooperativity Model
Thrombin
GPIb-IX
PAR
14-3-3
Gα
β γ
Rac1
LIMK1
Platelet activation

Figure 21: Thrombin-induced GPIb signaling requires cooperation of PARs. (A-D) Calcium mobilization in control CHO cells, 1b9 and Δ605 CHO cells pretreated with DMSO control or PAR1 inhibitor SCH79797 (20 μM). Cells were stimulated with either wild type (WT) thrombin (A) or low (B) or high (C) concentrations of S195A mutant thrombin and then recorded for calcium mobilization as described in the Methods. (D) Aggregation of human platelets stimulated with increasing doses of S195A mutant thrombin, PPACK-treated S195A thrombin and 1000 nM PPACK-treated WT thrombin, (E) In vitro enzymatic activity of WT, S195A mutant thrombin, PPACK-treated S195A mutant thrombin and PPACK-treated WT thrombin. The Y-axis is shown as a log scale of relative fluorescence units (RFU). Representative data combined from 3 independent experiments are shown. (F) Western blot analysis of LIMK1 phosphorylation in human platelets stimulated with increasing doses of PPACK-treated S195A mutant thrombin or wild type thrombin (Thr). Representative data from 3 independent experiments are shown. (G) LIMK1 phosphorylation in control CHO, 1b9 and Δ605 cells preincubated with vehicle control (0.1% DMSO) or 20μM PAR1 antagonist SCH 79797 and stimulated with WT thrombin, (H) LIMK1 phosphorylation in mouse platelets preincubated with either 10 μM MCsC (control peptide), 10 μM MPαC, and/or 2 mM PAR4 antagonist tcY-NH_2 and then stimulated with 3 nM WT thrombin. LIMK1 phosphorylation was detected using an anti-LIMK1 phospho-$Thr^{505/508}$ antibody. Loading was determined using a phosphorylation-independent anti-LIMK1 antibody. Representative data from 3 independent experiments are shown. (I) A schematic of signaling-mediated cooperativity between GPIb-IX and PARs. This cooperativity requires a unique GPIb-IX-dependent signaling pathway involving 14-3-3. Rac1 and LIMK1 and activation of this pathway requires thrombin binding to GPIb-IX and stimulation of PARs.

5.7 Low concentrations of thrombin are generated in vivo and are important for thrombus formation

To investigate whether low concentrations of thrombin are important during thrombosis in vivo, we used a laser-induced cremaster arteriolar thrombosis model that has previously been shown to require thrombin activity (Dubois et al. 2006, Dubois et al. 2007). We estimated the approximate concentration of thrombin generated after laser injury using a saturating concentration of an Alexa Fluor 488-labeled monoclonal anti-thrombin antibody, which is verified to selectively recognize both mouse and human α-thrombin (Figure 22A), but not prothrombin (as indicated by the manufacturer, also data not shown). We detected significant thrombin after laser injury by comparison to an Alexa Fluor 488-labeled IgG control antibody with identical fluorophore to antibody ratio (Figure 22B). Importantly, injection of the AF 488-labeled thrombin-specific antibody did not inhibit thrombus formation compared to IgG control (Figure 23). To quantify thrombin concentration, a standard curve of fluorescence intensity vs thrombin antibody concentration was generated under the same microscope settings. The kinetics of thrombin generation showed rapid increase after vessel injury and then plateaued around 5 minutes after injury (Figure 23). By comparing with the standard curve of known concentrations of labeled monoclonal thrombin antibody, we were able to approximate that thrombin concentrations present at the site of injury were less than 10 nM even at its plateau, and is lower than 3 nM during early platelet thrombus formation (Figure 22C-E). These findings are consistent with other methods used to estimate the concentration of thrombin generated in vivo(Zoldhelyi, Chesebro, and Owen 1993). To verify the importance of these concentrations of thrombin in this thrombosis model, we further tested the effect of a direct thrombin inhibitor, dabigatran. This thrombin inhibitor nearly abolished platelet thrombus formation after the injury

(Figure 22F). Taken together, these data suggest that low concentrations of thrombin are generated at the site of laser-induced arteriolar injury and are important for arterial thrombus formation in this mouse model of thrombosis.

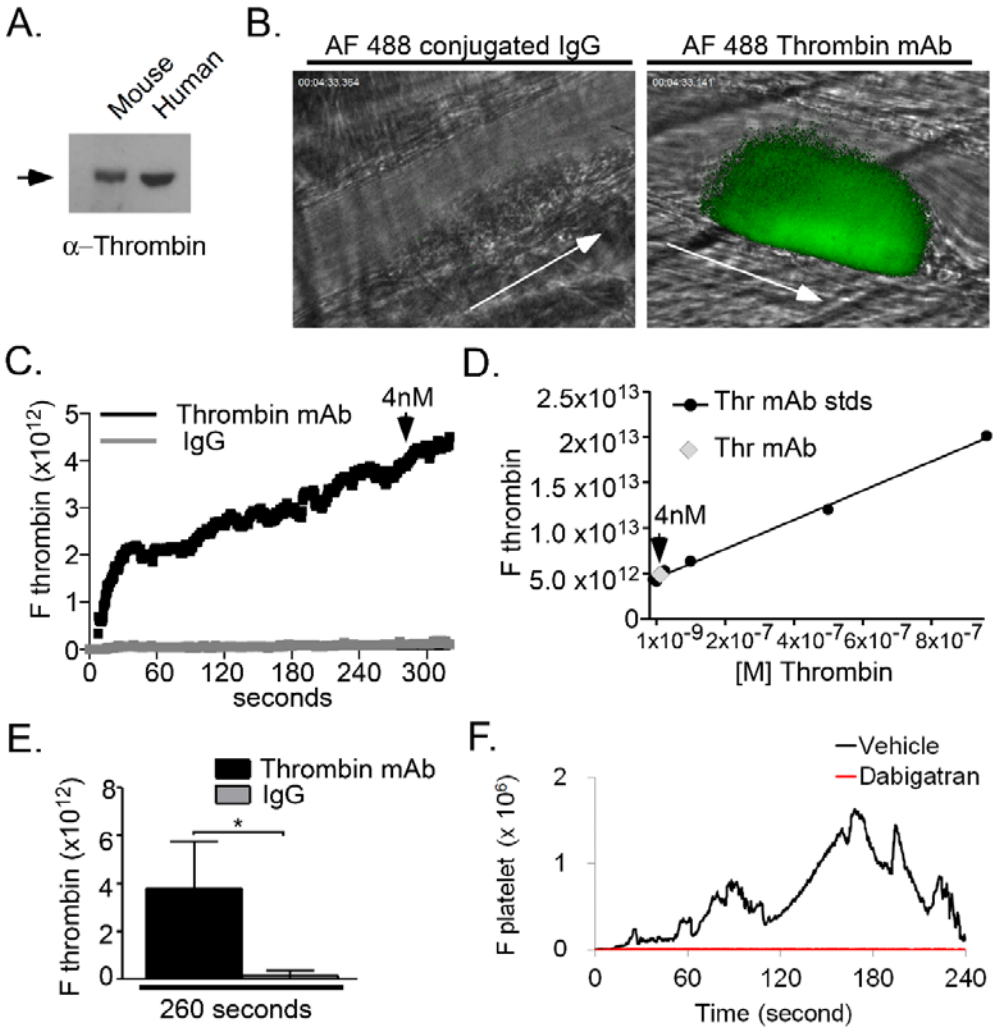

Figure 22: Low concentrations of thrombin are generated after vascular injury and are important for thrombosis in vivo. (A) Western blot detection of 0.2 mg/ml purified human and mouse α–thrombin protein using an anti-thrombin monoclonal antibody. (B) In vivo imaging of thrombin generation (green) during thrombosis captured using intravital microscopy after laser-induced cremaster arteriolar wall injury in WT mice injected with 2 mg/kg Alexa Fluor 488-conjugated anti-thrombin monoclonal antibody or an Alexa Fluor 488-conjugated control IgG. Arrows indicate the direction of blood flow. (C) Median integrated fluorescence intensity over time for Alexa Fluor 488-conjugated (AF 488) anti-thrombin antibodies and control IgGs. Based on the standard curve (D), 4 nM, denotes the approximate concentration of thrombin generated after 260 seconds of laser injury. (D) A standard curve was computed using imaging of fluorescence intensities of known concentrations of AF 488 conjugated thrombin monoclonal antibody under equivalent intravital instrument settings as in A. (E) Quantification of integrated fluorescence intensity at 260 seconds (Mann Whitney U-test,*, $p<0.05$, 30 thrombi in 4 mice per group). (F) Median integrated fluorescence intensity over time for WT mice infused with 0.3 mg/kg BW of dabigatran or vehicle control. The kinetics of platelet accumulation were plotted as the median fluorescence intensity as a function of time in 30 thrombi in 3-4 mice per group.

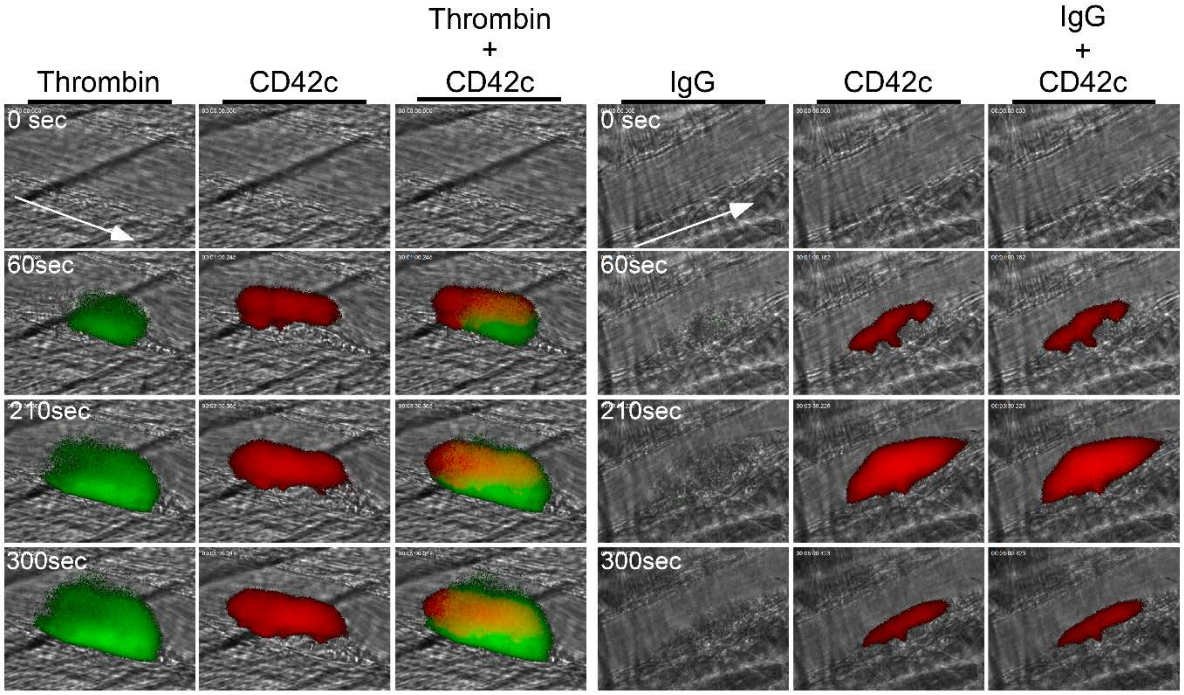

Figure 23: Thrombin generation after laser-induced injury Representative images of thrombin generation (anti-thrombin antibody, green) and platelet thrombus formation (anti-CD42c antibody, red) and merged images. Arrows indicate direction of blood flow. WT mice were injected with 2 mg/kg Alexa Fluor 488-conjugated anti-thrombin monoclonal antibody or an Alexa Fluor 488-conjugated control IgG. Arrows indicate the direction of blood flow.

5.8 Discussion

The seminal work of discovery and cloning of PARs established the knowledge that thrombin-induced cellular response requires thrombin-catalyzed cleavage and activation of PARs (Vu et al. 1991). This important advance also ignited debates about the role of the most abundant thrombin-binding membrane protein in platelets (and the “old” thrombin receptor), GPIb-IX, in thrombin-induced platelet activation. As several previous reports indicate that GPIb-IX is important in platelet response to thrombin(De Candia et al. 2001, De Marco et al. 1994, Greco et al. 1996, Jamieson and Okumura 1978), the debate is mainly about whether both GPIb-IX and PARs signal independently or GPIb-IX serves as a dock for thrombin by facilitating access and cleavage of PARs. Our data indicate that thrombin binding to GPIb-IX induces GPIb-IX-specific signaling that is important for the PAR-dependent platelet response to low dose thrombin. However, our data also indicate that the thrombin-induced GPIb-IX signaling requires cooperativity of PAR signaling, and thus is not PAR-independent. Therefore, we introduce a novel concept of signal-mediated cooperativity between GPIb-IX and PARs that drives platelet response to low dose thrombin.

Studies on platelet response to thrombin are complicated by genetic differences between humans and mice. Human platelets express both PAR1 and PAR4 whereas mouse platelets express PAR3 and PAR4 (Kahn et al. 1999). Thus, the convincing evidence for the requirement of PAR4 in mouse platelet activation cannot be applied to human platelets in which PAR1 signals play an important role. To understand the relationship between human GPIb-IX and PAR1, we successfully reconstituted GPIb-IX / PAR1 cooperativity in response to thrombin in a CHO cell line expressing recombinant human GPIb-IX and PAR1 but not PAR4, which allowed us to study the mechanisms of cooperativity between GPIb and PAR1 using

molecular biology. With this model, we were able to provide the first specific evidence of cooperativity between GPIb-IX and PAR1 in mediating calcium signaling induced by low dose thrombin. Importantly, we show that the role of GPIb-IX in stimulating thrombin response not only requires the thrombin binding site of GPIbα, but also the C-terminal 14-3-3 binding site in the cytoplasmic domain of GPIbα that is important for activating the GPIb-IX-dependent Rac1/LIMK1 signaling pathway. Deletion of the C-terminal 14-3-3 binding site in GPIbα or blocking 14-3-3 binding using a specific membrane-permeable inhibitor based on the 14-3-3 binding sequence of GPIbα diminished response to low dose thrombin. Thus, the role of GPIb-IX in stimulating cellular response to thrombin is not due to its function as a passive dock (although we do not exclude that it may also function as a dock), but requires thrombin-induced GPIb-IX signaling. Importantly, an inhibitor of GPIb-14-3-3 interaction similarly diminished platelet response to low dose thrombin both in human and mouse platelets, indicating that a similar mechanism is involved in the cooperativity of GPIb-IX with PAR1 and PAR4 in response to thrombin both in human and mouse platelets. Thus, the different PAR combinations in different species does not appear to affect GPIb cooperativity with PARs. To examine the possible in vivo relevance of this cooperativity, we detected low concentrations of thrombin (<10 nM at peak, <3 nM at earlier stages) following laser-induced arteriolar injury, and demonstrated its important role in laser-induced arteriolar thrombosis (Figure 22). Others(Croce and Libby 2007, Zoldhelyi, Chesebro, and Owen 1993, Wolberg 2007) suggested that low concentrations of thrombin were generated during systemic inflammation. However, we wish to point out that our estimation of thrombin levels is only limited to this particular model of thrombosis, and thrombin concentrations may vary in different thrombotic conditions depending on the extent of coagulation. Also, we do not claim that our estimation is

exactly accurate due to the possibility of blockage of antibody access to a population of thrombin by competing molecules. However, as thrombus formation was not affected by this high affinity antibody (Figure 23), it is unlikely to competitively interact with important functional sites of thrombin. Thus these data support the notion that the role of GPIb-IX in low dose thrombin-induced platelet activation is relevant to certain types of in vivo thrombosis such as arterial thrombosis and systemic vascular inflammation.

In elucidating the importance of GPIb-IX signaling, we have not only demonstrated the importance of 14-3-3-dependent GPIb-IX signaling function, but also for the first time identified an important role for the GPIb-IX-dependent Rac1/LIMK1-mediated signaling pathway in thrombin-induced cell response. Although Rac1 and its downstream effector kinase LIMK1 can be activated by several different platelet agonists, including PAR agonist peptides (Hartwig et al. 1995), our data indicate that low dose thrombin-induced activation of the Rac1-LIMK1 pathway is GPIb-IX-dependent and GPIb-IX-specific. First, we have shown that low dose thrombin-induced activation of Rac1 and LIMK1 is diminished in the absence of GPIb-IX expression and in cells expressing mutant GPIbα with a 14-3-3 binding site deleted. Secondly, inhibition of 14-3-3 binding to GPIbα in either human or mouse platelets resulted in diminished activation of Rac1 and LIMK1. Importantly, the LIMK1 knockout platelets showed reduced response to low dose thrombin, but showed enhanced response to PAR4 agonist peptide, excluding the stimulatory role of LIMK1 in PAR4-dependent platelet response. It is important to note that the GPIb-IX-dependent activation of Rac1 and LIMK1 can be induced by the binding of adhesive protein VWF to GPIb-IX independent of other agonist receptors (Delaney et al. 2012, Estevez et al. 2013). In contrast, thrombin is insufficient to induce significant activation of the Rac1-LIMK1 pathway without the cooperativity of PAR signaling.

The conclusion that thrombin-induced GPIb-IX signaling requires cooperativity of PAR signaling is supported by data that inhibition of either PAR1 in the reconstituted CHO cell model or PAR4 in platelets abolished thrombin-induced response, and is also consistent with previous data that PAR4 knockout platelets do not respond to thrombin(Sambrano et al. 2001). The major evidence supporting thrombin-induced PAR-independent GPIb-IX signaling comes from data that S195A thrombin or PPACK-treated thrombin induced platelet activation (Adam, Guillin, and Jandrot-Perrus 2003, Ramakrishnan et al. 2001). However, we show that whereas S195A thrombin indeed induces platelet response, this effect is dependent upon residual catalytic activity of S195A thrombin and is dependent upon PARs. Also, in a previous report(Adam, Guillin, and Jandrot-Perrus 2003), the PPACK concentration used for thrombin treatment was lower than described to be needed for complete inhibition of thrombin activity(Greco et al. 1990). In our experiments, when either wild type or S195A thrombin was treated with PPACK as previously described, platelet response was completely inhibited. Thus, our data, consistent with the data obtained with PAR4 knockout(Sambrano et al. 2001), suggests that thrombin-induced GPIb-IX-dependent platelet response requires PAR signaling. Together, our data indicate that GPIb-IX and PAR are mutually dependent to induce optimal platelet response to low concentrations of thrombin, which are important during in vivo thrombosis. This cooperativity requires a unique GPIb-IX-dependent signaling pathway involving Rac1 and LIMK1 and also requires PAR signaling. This novel finding not only provides a conceptual advance in our understanding of the mechanisms of cellular response to the important prothrombotic and proinflammatory protease, thrombin, but may also serve as the basis for developing new platelet selective anti-thrombotic and anti-inflammatory therapies targeting GPIb and PAR cooperativity.

6. CONCLUSIONS

The results of these studies provide a significant advance over the current understanding of the mechanisms by which the GPIb-IX-V complex transduces platelet activation signals, and they elucidate a previously unknown role of LIMK1 in platelet activation and thrombosis. The objective of the first study (Chapter 4) was to twofold: 1) to determine whether LIMK1 plays a role in VWF/GPIb-IX-mediated platelet activation and 2) to determine the molecular mechanisms regulating GPIb-IX-mediated thromboxane production. By isolating platelets from LIMK1$^{-/-}$ mice we were able to characterize the function of LIMK1 in various platelet activation pathways. We discovered that LIMK1 plays an important role in promoting VWF/GPIb-IX-mediated platelet activation. LIMK1$^{-/-}$ platelets displayed a defective second wave of VWF/botrocetin-induced platelet aggregation, and were defective in ATP release following VWF/botrocetin stimulation. In contrast, we learned that LIMK1 plays differential role in different agonist pathways. For example, we show that LIMK1$^{-/-}$ platelets display enhanced aggregation response upon stimulation with U46619. Using a cone plate rheometer assay to determine the ability of LIMK1$^{-/-}$ platelets to stable adhere to immobilized VWF, we found that LIMK1 was important for promoting stable platelet adhesion to VWF under shear conditions. The defective stable adhesion in LIMK1$^{-/-}$ platelets was found to be due to an absence of VWF-induced TXA_2 production, and thus, we were able to perform successful rescue of LIMK1$^{-/-}$ platelet stable adhesion by adding an analog of TXA2, U46619, at sub-threshold concentrations established in wildtype control mice. At this time, we cannot exclude the possibility that LIMK1$^{-/-}$ platelets may have enhanced response due to increased thromboxane receptor expression. We also found that LIMK1 played a selective stimulatory role in GPIb-IX-mediated TXA_2 production since absence of LIMK1 did not affect TXA_2 production by other

agonist pathways. To our surprise, we were not able to discern any detectable impact of LIMK1 deletion on actin morphology or platelet spreading, which is the most widely accepted function of LIMK1 in other cells. However, when we measured actin polymerization following VWF/botrocetin-induced platelet aggregation, at the 10 minute time point, we did see a partial reduction in actin polymerization in LIMK1$^{-/-}$ platelets. We found that actin polymerization inhibitors, latrunculin A or cytochalasin D, had no effect on TXA_2 production, and they enhanced VWF-induced platelet aggregation response, which is opposite the phenotype of LIMK1$^{-/-}$ platelets stimulated with VWF. This enhanced aggregation response could be dependent on increased cofilin-dependent actin depolymerization activity in LIMK1$^{-/-}$ platelets. However, the role of cofilin in platelet activation is unclear. We show that LIMK1 is activated by VWF, downstream of MAPKs, and that LIMK1 promotes cPLA2 phosphorylation. Finally, we found that although deletion of LIMK1 in mice does not affect hemostasis, LIMK1$^{-/-}$ mice display a prolonged time to vessel occlusion in the $FeCl_3$ carotid artery injury thrombosis model. Taken together, we have discovered a role for LIMK1 in platelet function and have identified a GPIb-IX-specific signaling pathway leading to TXA_2 production (Figure 23 PartI).

Although, the effect of cPLA2 deletion on VWF/GPIb-mediated signaling is unknown, $cPLA_2$ deletion in mouse platelets has been shown to partially inhibit collagen-induced TXA_2 production(Wong et al. 2002). Accordingly, ATP secretion and aggregation induced by collagen were also partially inhibited in $cPLA_2$ null mouse platelets. Surprisingly, $sPLA_2$/$cPLA_2$ double knockout platelets display a minor increase in severity of aggregation and secretion defects, compared to cPLA2 knockout platelets, but overall these were partial phenotypes(Wong et al. 2002). $cPLA_2$ knockout or $cPLA_2$/$sPLA_2$ double knockout did not affect TXB_2 induced by ADP or U46619, which suggests other PLA_2 isozymes are present in mouse

platelets. Tail bleeding time is also prolonged in both of these strains of PLA_2-deficient mice(Wong et al. 2002). Interestingly, no GI bleeding phenotype or other gross abnormality was reported in the $cPLA_2$/$sPLA_2$-deficient mice. In contrast, humans with $cPLA_2$ deficiency display bleeding diathesis and/or reoccurring GI ulceration(Brooke et al. 2014, Adler et al. 2008, Faioni et al. 2014). In addition, patients with severe deficiencies in $cPLA_2$ expression show reductions in ATP release in response to ADP or collagen stimulation(Brooke et al. 2014, Faioni et al. 2014), and almost abolished platelet adhesion to collagen under flow(Kirkby et al. 2015). TXB_2 released from these patients' platelets is significantly diminished, but to varying degrees depending on the type of $cPLA_2$ mutation(Kirkby et al. 2015, Brooke et al. 2014, Adler et al. 2008, Faioni et al. 2014). In our study, we did not detect gross bleeding or other anatomical abnormalities in $LIMK1^{-/-}$ mice. In addition, $LIMK1^{-/-}$ mice do not display prolonged tail bleeding time. Taken together, the selective role of LIMK1 in the VWF/GPIb-IX signaling pathway leading to TXA_2 production gives rise to an in vivo phenotype that appears distinct from deletion or inhibition of major components of the TXA_2 pathway.

A notable observation in our study of the role of LIMK1 in VWF/GPIb-IX signaling is the severe reduction in VWF-induced TXA_2 production with only partial inhibition of $cPLA_2$ Ser ^{505} phosphorylation, suggesting that in addition to phosphorylation LIMK1 may regulate $cPLA_2$ activity by another mechanism. In addition to calcium-dependent translocation of $cPLA_2$ to membranes, the catalytic activity of $cPLA_2$ is stimulated by MAPK (i.e., ERK and p38)-mediated phosphorylation at Ser ^{505} and the binding of anionic phosphoinositides to basic residues in the catalytic domain (Tucker et al. 2009, Lin et al. 1993, Kramer et al. 1996, Hirabayashi, Murayama, and Shimizu 2004). PIP_2 has been shown to promote catalytic activity of membrane bound $cPLA_2$ (Leslie 2015). Mutation of basic residues involved in PIP_2

binding to $cPLA_2$ dramatically inhibits its catalytic activity(Tucker et al. 2009). Cofilin also binds PIP_2, however, PIP_2 inhibits cofilin activity by competing with its F-actin binding site (i.e., the F-actin and PIP_2-binding sites overlap)(Van Troys et al. 2008, Van Rheenen et al. 2007, Bernstein and Bamburg 2010). Thus, another possible mechanism by which LIMK1 stimulates VWF-induced TXA_2 production is by releasing cofilin from PIP_2, and thereby increasing accessibility of PIP_2 for binding to activated $cPLA_2$. To determine if LIMK1 regulates cofilin and $cPLA_2$ localization to membranes, membrane fractionation and western blotting studies of cofilin, LIMK1, and $cPLA_2$ during VWF signaling in WT and LIMK1$^{-/-}$ platelets should be investigated.

Our studies demonstrate that LIMK1 plays differential roles in response to different platelet agonists. In GPIb-IX dependent pathways (VWF and thrombin) LIMK1 plays a stimulatory role and in GPIb-IX independent/GPCR pathways (TXA_2) LIMK1 plays a negative role. In order to better understand the differential role of LIMK1 in platelet activation, future studies should investigate the role of its primary substrate cofilin in GPIb-IX- dependent and independent pathways. Indeed, in our study we detected LIMK1-dependent phosphorylation of cofilin in response to VWF stimulation. In addition, we found that actin depolymerizing agents appear to enhance platelet response to VWF stimulation but did not affect TXA_2 production. Thus, it is likely that that cofilin activity is not responsible for the role of LIMK1 in TXA_2 regulation. In addition to cofilin, LIMK1 has been reported to phosphorylate other proteins including p25/ tubulin polymerization promoting protein (TPPP)(Acevedo et al. 2007), cAMP-responsive element binding protein (CREB)(Yang, Yoon, and Chung 2004), and possibly Nurr1 (NR4A2)(Sacchetti et al. 2006). Of these reported LIMK1 substrates, the one most likely to play a significant role in platelets is p25/TPPP. p25/TPPP is a microtubule associated protein

that appears to be widely expressed in mouse tissues. LIMK1 phosphorylation of p25/TPPP has been shown to inhibit microtubule assembly(Acevedo et al. 2007). Currently, it is unknown whether p25/TPPP is expressed in platelets.

Our discovery that LIMK1$^{-/-}$ mice are protected from thrombosis but show no abnormalities in tail bleeding time indicates that LIMK1 may be a good antithrombotic target. Aspirin use is associated with increased risk of developing GI ulcers and bleeding side effects, which are due to the roles of cyclooxygenases 1-derived metabolites PGE_2 and PGI_2 in protecting gastrointestinal mucosa and TXA_2 in promoting platelet activation(Claria 2003, Patrono et al. 2005). Aspirin is an effective inhibitor of platelet activation because it irreversibly acetylates serine in COX (in humans, Ser ^{529} in COX-1 and Ser ^{516} in COX-2) isozymes preventing arachidonic acid conversion into prostaglandins such as TXA_2. At low doses (30mg/day) aspirin is more selective for COX-1 than COX-2(Cipollone et al. 1997, Patrono et al. 2005). Platelets, unlike nucleated cells, cannot replace inactivated COX-1, and thus aspirin treatment inhibits TXA_2 production for the lifetime of the platelet. By contrast to the selective effect of LIMK1 deletion on VWF-induced TXA_2 production, aspirin blocks all platelet-derived TXA_2, which is a diffusible paracrine and autocrine agonist generated downstream of various platelet activation pathways (e.g., collagen, ADP, thrombin) and thus its regulation is important for hemostasis. Consistent with a general role for TXA_2 signaling in hemostasis, TP receptor knockout mice display prolonged bleeding time(Thomas et al. 1998). Thus, in contrast to aspirin, future development of LIMK1 targeted inhibitors may provide antithrombotic effect without adverse bleeding. However, careful drug delivery and/or design is necessary as LIMK1 is ubiquitously expressed. Selectively targeting LIMK1 inhibitors to platelet thrombi is theoretically possible(Fuentes et al. 2016), and thus deserves further attention.

We have also made an important contribution to the understanding of the mechanism by which GPIb-IX promotes thrombin-induced platelet activation. A widely accepted view in the field is that GPIb-IX only functions to facilitate thrombin-mediated cleavage and activation of PARs, and that GPIb-IX itself does not induce signal transduction. The objective of our second study (Chapter 5) was to challenge the longstanding dogma that GPIb-IX does not signal in thrombin stimulated platelets. We discovered that GPIb-IX does transduce signaling that is important for platelet activation at low doses of thrombin, but this signaling mechanism requires PAR activation. By reconstituting thrombin signaling in cells we were able to investigate the individual contributions of GPIb-IX and PAR1 in thrombin signaling. Expression of WT GPIb-IX complex enhanced cellular response to thrombin. In contrast, we found that deletion of the high affinity binding site or cytoplasmic C-terminal 14-3-3 binding motif on GPIbα abolished GPIb-IX-dependent enhancement of thrombin response. Thus, thrombin-induced GPIb-IX signaling requires a C-terminal cytoplasmic domain interaction with cytosolic adaptor protein 14-3-3, which also showed was important for activating a pathway involving Rac1 and LIMK1. Interestingly, LIMK1$^{-/-}$ platelets display defective low dose thrombin-induced platelet activation, but they show enhanced response to PAR4 activating peptide (PAR4-AP). These differential roles of LIMK1 are agreement with the notion that thrombin-induced signaling is distinct from PAR4-AP induced signaling, wherein the key difference is that in the former thrombin-GPIb-IX interaction occurs to elicit GPIb signaling. Indeed, we found that in GPIb-IX independent pathways (e.g., TXA_2/TP signaling) LIMK1 plays a negative role. On the other hand, in GPIb-IX-dependent pathways (VWF or thrombin) LIMK1 plays a positive role. Thus, the net effect in vivo may be a lack of hemostatic defect. Alternatively, since we used a a whole body LIMK1$^{-/-}$ knockout approach the in vivo effects may result from its roles in several

different cell types (e.g., endothelial cells). Furthermore, we found that GPIb-IX cooperates with activated PARs to induce optimal platelet response to thrombin. Consistent with this notion, when we investigated the effect of mutating the C-terminal 14-3-3 binding site on GPIbα or pharmacological inhibition of this site, we found that low dose thrombin-induced signaling was abolished. Moreover, we discovered that in thrombin stimulated platelets antagonism of PAR signaling using small molecule PAR-selective inhibitors or using catalytically inactivated thrombins abolished low dose thrombin signaling even in the presence of intact GPIb-IX signaling. Therefore, GPIb-IX and PAR signaling cooperate to ensure optimal platelet activation response at low concentrations of agonist (Figure 23 Part II).

In an attempt to investigate PAR-independent signaling in platelets many investigators have sought out or created mutant or chemically inactivated thrombin. Although we did not detect platelet activation with fully inactivated (see below) wildtype (WT) or mutant thrombin this does not preclude the possibility that under some conditions, such as when catalytically inactive thrombin is immobilized, they might induce platelet activation and/or some measurable signaling. Immobilized D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone (PPACK)-inactivated WT thrombin was previously shown to activate platelets in a GPIb-IX-dependent manner(Adam, Guillin, and Jandrot-Perrus 2003). In contrast, we found that PPACK-treated WT thrombin did not induce any platelet aggregation or signaling, however, we did not immobilize thrombin. Others have reported that GPV null mice display enhanced response to catalytically inactive S195A mutant thrombin(Ramakrishnan et al. 2001). We tested S195A mutant thrombin and found that this protein contains residual enzymatic activity. At doses higher than 50nM S195A thrombin induced platelet aggregation, LIMK1 phosphorylation, and induced calcium mobilization. The stimulatory effects of S195A thrombin could be abolished by

PAR1 antagonism in cells or by chemical treatment with PPACK. We measured the residual activity of S195A mutant thrombin using a fluorescent thrombin substrate assay and found S195A thrombin to be approximately 330 times less active than WT thrombin. Therefore, previous studies using catalytically inactive mutant thrombin at doses of 100nM or higher need to be carefully re-evaluated. In addition, we did not detect any response in platelets stimulated with high doses (1000nM) of PPACK-treated WT or S195A thrombin, which largely rules out PAR-independent platelet activation. However, thrombin activity may important for cleaving the GPIb-IX-V complex (e.g., GPV release) or GPIb-IX-V-mediated conformational modulation of PARs.

Our results also suggest that the GPIb-IX-mediated enhancement of thrombin signaling may have relevance to thrombosis in vivo. We found that low concentrations of thrombin are generated at the site of laser injury in cremaster arterioles in vivo. Similarly, a previous report also found that low concentrations of thrombin were generated following inflammation(Zoldhelyi, Chesebro, and Owen 1993). Consistent with an important role for thrombin activity in in vivo thrombosis, when we pretreated mice with dabigatran, a direct thrombin inhibitor, we abolished platelet accumulation following laser injury in vivo. Thus, it is conceivable that targeting thrombin-GPIb-IX interaction may be a useful antithrombotic approach. In agreement with this notion a novel allosteric direct thrombin inhibitor, sulfated β-O4 lignin (SbO4L), was developed that mimics the thrombin-binding sulfated tyrosine sequence on GPIbα (Mehta et al. 2014). SbO4L appears to exhibit both anti-platelet and anti-coagulant activity in vitro and antithrombotic activity in vivo in mice(Mehta et al. 2016, Mehta et al. 2014), suggesting that allosterically inhibiting thrombin activity while competing with thrombin-GPIbα interaction allows dual inhibition of coagulation and platelet activation. By

contrast, our 14-3-3-GPIb inhibitor peptide would not block coagulation, but it would prevent platelet activation; and previous studies in mice suggest that, genetic deletion or inhibition of the cytoplasmic C-terminal 14-3-3-binding region protects against arterial thrombosis (Yin et al. 2013, Jain et al. 2007). Thus, it would be of interest to investigate the efficacy of 14-3-3-GPIb interaction inhibition in pathological models of in vivo thrombosis.

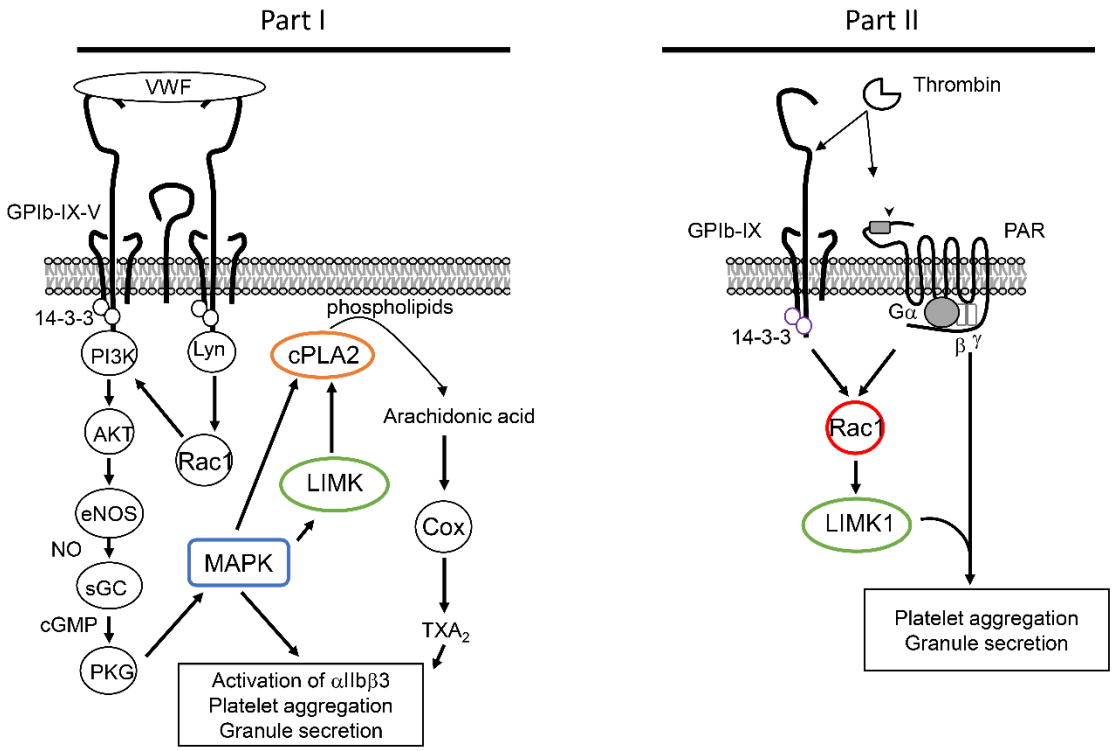

Figure 24: Signaling mechanisms of the Glycoprotein Ib-IX complex and role of Lim kinase 1 in platelet activation. A summary of GPIb-IX signaling mechanisms and role of LIMK1 in platelet activation. (Part I) LIMK1 is critical for GPIb-IX-mediated signaling leading to TXA2 production. LIMK1 is activated downstream of MAPK which are activated by the Lyn/Rac1/PI3K/PKG pathway. LIMK1 activation promotes cPLA2 activation and TXA2 production. (Part II) GPIb-IX-mediated signaling cooperates with PAR signaling to ensure optimal platelet response to low thrombin concentrations. Thrombin binding to GPIb-IX elicits a signaling pathway that is regulated by a C-terminal 14-3-3 binding site on GPIbα and involves a Rac1/LIMK1 pathway that is required for platelet response at low thrombin concentrations. PAR activation is required for thrombin signaling, but by itself is not sufficient for optimal platelet signaling response at low thrombin concentrations.

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8. APPENDIX

UNIVERSITY OF ILLINOIS
AT CHICAGO

Office for the Protection of Research Subjects (OPRS)
Office of the Vice Chancellor for Research (MC 672)
203 Administrative Office Building
1737 West Polk Street
Chicago, Illinois 60612-7227

Approval Notice
Continuing Review

December 3, 2015

Xiaoping Du, MD, PhD
Pharmacology
835 S. Wolcott Avenue
403-E M.S.B., M/C 868
Chicago, IL 60612
Phone: (312) 355-0250 / Fax: (312) 996-1225

RE: Protocol # 1999-0610
"Mechanisms of Platelet Activation"

Dear Dr. Du:

Your Continuing Review was reviewed and approved by the Expedited review process on November 24, 2015. You may now continue your research.

Please note the following information about your approved research protocol:

Protocol Approval Period:	December 4, 2015 - December 3, 2016
Approved Subject Enrollment #:	1500 (1309 enrolled)
Performance Sites:	UIC
Sponsor:	a) NIH, b) NHLBI/NIH, c) NIH-National Institutes of Health, d) NIH/NHLBI, e) NHLBI - National Heart, Lung, and Blood Institute, f) National Heart, Lung and Blood Institute, g) National Institutes of Health
PAF#:	a) 2009-05545, b) 2010-00091, c) 2011-02188, d) 2014-00591, e) 2013-00887, f) 2014-03153, g) 2014-04177
Grant/Contract No:	a) 3R01HL068819-08S1, b) R01 HL062350, c) HL080264, d) F31HL123319, e) HHSN268201400007C, f) 2R01 HL062350, g) 1R01 HL125356-01
Grant/Contract Title:	a) The cGMP-dependent protein kinase pathway in platelets, b) Signaling mechanism of platelet glycoprotein 1b-IX, c) Outside-in signaling mechanisms of platelet integrin alpha-llb-beta3,

d) Investigation into the Mechanism of the thrombin receptor function of GPIb, e) Vascular Interventions/Innovations and Therapeutic Advances (VITA): Selective Inhibitors of Intergin Outside-in Signaling as a New Generation of Anti-Thrombotics, f) Signaling Mechanisms of Platelet GPIb-IX, g) New strategies for treating septic vasculopathy, inflammation and thrombosis

Research Protocol(s):

a) Mechanisms of Platelet Activation; Version 8; 11/05/2014

Recruitment Material(s):

a) Flyer, "Volunteers Needed...," Version 2, 03/12/2002
b) Protocol #1999-0610 - Advertisement for mass mail by Internet Version 2 05/06/2004
c) Flyer "Sickle cell patients needed to volunteer for research study", Version 3, 01/06/2011
d) Advertisement for mass mail by Internet for sickle cell research group, Version 1, 01/06/2011

Informed Consent(s):

a) "Mechanisms of Platelet Activation," Version 9, 11/16/2010
b) Consent/authorization for sickle cell patients, Version 1.0, 01/07/2011

HIPAA Authorization(s):

a) "Mechanisms of Platelet Activation," Authorization, Version 2, 04/01/2004

(Please continue to use the Authorization form approved and stamped 04/06/2004)

Additional Determinations for Research Involving Minors:

These determinations have not been made for this study since it has not been approved for enrollment of minors.

Your research meets the criteria for expedited review as defined in 45 CFR 46.110(b)(1) under the following specific category:

(2) Collection of blood samples by finger stick, heel stick, ear stick, or venipuncture as follows:
(a) from healthy, nonpregnant adults who weigh at least 110 pounds. For these subjects, the amounts drawn may not exceed 550 ml in an 8 week period and collection may not occur more frequently than 2 times per week; or
(b) from other adults and children, considering the age, weight, and health of the subjects, the collection procedure, the amount of blood to be collected, and the frequency with which it will be collected. For these subjects, the amount drawn may not exceed the lesser of 50 ml or 3 ml per kg in an 8 week period and collection may not occur more frequently than 2 times per week.

Please note the Review History of this submission:

Receipt Date	Submission Type	Review Process	Review Date	Review Action
11/20/2015	Continuing Review	Expedited	11/24/2015	Approved

Please remember to:

→ Use your **research protocol number** (1999-0610) on any documents or correspondence with the IRB concerning your research protocol.

→ Review and comply with all requirements on the guidance,
"UIC Investigator Responsibilities, Protection of Human Research Subjects"
(http://tigger.uic.edu/depts/ovcr/research/protocolreview/irb/policies/0924.pdf)

Please note that the UIC IRB has the prerogative and authority to ask further questions, seek additional information, require further modifications, or monitor the conduct of your research and the consent process.

Please be aware that if the scope of work in the grant/project changes, the protocol must be amended and approved by the UIC IRB before the initiation of the change.

We wish you the best as you conduct your research. If you have any questions or need further help, please contact OPRS at (312) 996-1711 or me at (312) 413-0241. Please send any correspondence about this protocol to OPRS at 203 AOB, M/C 672.

Sincerely,

Ibraheem Oguntade
IRB Coordinator, IRB # 3
Office for the Protection of Research Subjects

Enclosure(s):

1. **Informed Consent Document(s):**
   a) "Mechanisms of Platelet Activation," Version 9, 11/16/2010
   b) Consent/authorization for sickle cell patients, Version 1.0, 01/07/2011

2. **Recruiting Material(s):**
   a) Flyer, "Volunteers Needed...," Version 2, 03/12/2002
   b) Protocol #1999-0610 - Advertisement for mass mail by Internet Version 2 05/06/2004
   c) Flyer "Sickle cell patients needed to volunteer for research study", Version 3, 01/06/2011
   d) Advertisement for mass mail by Internet for sickle cell research group, Version 1, 01/06/2011

cc: Asrar Malik, Pharmacology, M/C 868
OVCR Administration, M/C 672

UIC UNIVERSITY OF ILLINOIS AT CHICAGO

Office of Animal Care and
Institutional Biosafety Committees (MC 672)
Office of the Vice Chancellor for Research
206 Administrative Office Building
1737 West Polk Street
Chicago, Illinois 60612-7227

July 27, 2015

Xiaoping Du
Pharmacology
M/C 868

Dear Dr. Du:

The modifications requested in modification indicated below pertaining to your approved protocol indicated below have been reviewed in accordance with the Institutional Biosafety Committee Policies of the University of Illinois at Chicago and approved on 07/27/2015.

Title of Application: Signaling Mechanisms of Platelet Activation

IBC Number: 14-008

Modification Number: ***02***

Nature of Modification: ***Addition of personnel: Zheng Xu (BBP training expires on 05/11/2016), Claire Wei-Ju Chang 6BBP training expires on 06/23/2016)***

The records of the Institutional Biosafety Committee will be revised to reflect these changes. Thank you for complying with the Institutional Biosafety Committee Policies and Procedures of UIC.

Sincerely,

Randal C. Jaffe, Ph.D.
Chair, Institutional Biosafety Committee
RCJ/ *mbb*

cc: IBC File, Aleksandra Stojanovic-Terpo

UIC UNIVERSITY OF ILLINOIS AT CHICAGO

Office of Animal Care and Institutional
Biosafety Committee (M/C 672)
Office of the Vice Chancellor for Research
206 Administrative Office Building
1737 West Polk Street
Chicago, Illinois 60612

April 29, 2016

Xiaoping Du
Pharmacology
M/C 868

Dear Dr. Du:

The modifications requested in modification indicated below pertaining to your approved protocol indicated below have been reviewed and approved in accordance with the Animal Care Policies of the University of Illinois at Chicago on ***4/27/16.***

Title of Application: Signaling Mechanisms of Platelet Activation

ACC Number: 14-017

Modification Number: ***12***

Nature of Modification:

1) ***Addition of Personnel: Ying Liang***

2) ***Addition of 5/strain (Epsin1 fl/fl, Epsin1 fl/fl/Paf4 Cre , and Paf4 Cre) mice from Harvard for euthanasia and blood collection in quarantine and 15 WT controls (at UIC) to determine effect on platelet function.***

Condition of Approval: All work with mice must be in quarantine and addition modification required if further studies with new strains including breeding will be conducted.

Protocol Approved: ***3/28/2014***

Current Approval Period: ***2/18/2016*** to ***2/18/2017.***

Current Funding: ***Portions of this protocol are supported by the funding sources indicated in the table below.***
Number of funding sources: 7

Funding Agency	**Funding Title**			**Portion of Funding Matched**
NIH	*Signaling Mechanism of Platelet Glycoprotein Ib-Ix*			*All matched*
Funding Number	**Current Status**	**UIC PAF NO.**	**Performance Site**	**Funding PI**
RO1 HL062350	*Funded*	*201000091*	*UIC*	*Xiaoping Du*

Funding Agency	**Funding Title**			**Portion of Funding Matched**
NIH	*Outside-in signaling mechanisms of platelet integrin alpha-llb-beta3*			*All matched*
Funding Number	**Current Status**	**UIC PAF NO.**	**Performance Site**	**Funding PI**
HL-080264	*Funded*	*201102188*	*UIC*	*Xiaoping Du*
Funding Agency	**Funding Title**			**Portion of Funding Matched**
NIH	*Selective Inhibitors of Integrin Outside- In Signaling as a New Generations of Anti-Thrombotics - Du, Xiaoping*			*All matched*
Funding Number	**Current Status**	**UIC PAF NO.**	**Performance Site**	**Funding PI**
HHSN2682014 00007C	*Funded*	*201300887*	*UIC*	*Xiaoping Du*
Funding Agency	**Funding Title**			**Portion of Funding Matched**
NIH	*Investigation into the Mechanism of the Thrombin Receptor Function of GP1b*			*All matched*
Funding Number	**Current Status**	**UIC PAF NO.**	**Performance Site**	**Funding PI**
F31 HL123319	*Funded*	*201400591*	*UIC*	*Brian Estevez*
Funding Agency	**Funding Title**			**Portion of Funding Matched**
NIH	*Signaling Mechanisms of Platelet GPIb-IX*			*All matched*
Funding Number	**Current Status**	**UIC PAF NO.**	**Performance Site**	**Funding PI**
RO1 HL062350 (years 14-18)	*Funded*	*201403153*	*UIC*	*Xiaoping Du*
Funding Agency	**Funding Title**			**Portion of Funding Matched**
NIH	*New strategies for treating septic vasculopathy, inflammation and thrombosis*			*Portion of Grant is matched* *Only breeding- tied to ACC 14-153 for experiments*
Funding Number	**Current Status**	**UIC PAF NO.**	**Performance Site**	**Funding PI**
R01 HL 125356 (years 1-5)	*Funded*	*201404177*	*UIC*	*Xiaoping Du*
Funding Agency	**Funding Title**			**Portion of Funding Matched**
NIH	*Outside-in Signaling Mechanisms of Platelet Integrin Alpha-IIb-Beta3*			*All matched*
Funding Number	**Current Status**	**UIC PAF NO.**	**Performance Site**	**Funding PI**
R01HL080264 (years 9-13) A1 version	*Pending*	*201601556*	*UIC*	*Xiaoping Du*

This institution has Animal Welfare Assurance Number A3460.01 on file with the Office of Laboratory Animal Welfare, NIH. **This letter may only be provided as proof of IACUC approval for those specific funding sources listed above in which all portions of the grant are matched to this ACC protocol.**

Thank you for complying with the Animal Care Policies and Procedures of UIC.

Sincerely yours,

John P. O'Bryan, PhD
Chair, Animal Care Committee
JPO /*mbb*
cc: BRL, ACC File, Aleksandra Stojanovic

Title: Targeting Integrin and Integrin Signaling in Treating Thrombosis

Author: Brian Estevez, Bo Shen, Xiaoping Du

Publication: ATVB

Publisher: Wolters Kluwer Health, Inc.

Date: Jan 1, 2015

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9. VITA

NAME:	Brian Estevez
EDUCATION:	Ph. D., Cellular and Molecular Pharmacology, University of Illinois at Chicago, Illinois. 2016
	B.A., Biology, Queens College, Flushing, New York. 2009
	A.A., Liberal Arts, Queensborough Community College, Bayside, New York. 2006
TEACHING EXPERIENCE:	Guest Lecturer, PCOL 510, Molecular Pharmacology, Platelet and Thrombosis, University of Illinois at Chicago
	Teaching Assistant, GCLS 515, Receptor Pharmacology, University of Illinois at Chicago
RESEARCH EXPERIENCE:	Graduate Research Assistant, Department of Pharmacology, University of Illinois at Chicago, Illinois, 2009-2016 Laboratory of Dr. Du
	Undergraduate Research Assistant, Biology Department, Queens College, 2007-2009, New York Laboratory of Dr. Zakeri
	Undergraduate Research Training program, Center for Health Disparities and Molecular Medicine, Loma Linda University, 2008, California Laboratory of Dr. De Leon
GRANTS:	National Institutes of Health Predoctoral Fellowship (F31) 2013-2016
HONORS:	Dr. Albert and Doris Woeltjen Achievement award University of Illinois at Chicago, 2014.

Midwest Platelet Conference Young Investigator Award, 2014.

American Heart Association Young Investigator Travel Award, 2014-2016.

Chancellor's Graduate Research Fellowship, 2012.

American Heart Association Young Investigator Travel Award, 2009.

LSAMP Bridges Graduate Fellowship, 2009.

Laura and Arthur Colwin Award for Research Excellence, 2009.

Young Scientist Award, Queens College, NY, 2008.

ABRCMS "Outstanding Oral Presentation" Award in Cell Biology, 2007.

MARC U-Star Program, Queens College, 2007.

New York City Louis Stokes Alliance Participation, 2006-2007.

ORAL PRESENTATIONS:

"Differential Roles of the NADPH-Oxidase 1and 2 in Platelet activation and Thrombosis" Delaney MK, Kim K, Estevez B, Stojanovic-Terpo A, Shen B, Ushio-Fukai M, Cho J, Du X, University of Illinois at Chicago, Department of Pharmacology,Illinois, AHA meeting, Orlando, FL. 2015

"Sex Differences in Cell Death and Gene Regulation Exploring mechanisms for Sex Dimorphic Cellular Response" B. Estevez, C. Penaloza, Z. Zakeri Department of Biology, Queens College and Graduate Center of the City University of New York. ABRCMS meeting, Coronado Springs Resort, Orlando, FL. November 2008.

POSTER PRESENTATIONS:

"A Platelet Glycoprotein Ib-IX-specific 14-3-3/Rac1/LIMK1 Signaling Pathway Promotes Thrombin-Induced Platelet Activation" B Estevez, K Delaney, A Stojanovic- Terpo, X Du, 2015, ATVB, 2015;35:A598. Hilton San Francisco Union Square, San Francisco, CA. May 2015

"A Signaling Mechanism By Which Platelet Glycoprotein Ib-IX Promotes Thrombin- Induced Platelet Activation" B Estevez, MK Delaney, A Stojanovic-Terpo, X Du, 2014, Blood 124 (21), 2759-2759 McCormick Place, Chicago, IL Dec. 2015
"LIM kinase–1 selectively promotes glycoprotein Ib-IX–mediated TXA2 synthesis, platelet activation, and thrombosis" B. Estevez, A. Stojanovic-Terpo, M. K. Delaney, K.A O'Brien, M. C. Berndt, C. Ruan, and X. Du National AHA meeting, Los Angeles, Circulation. 2012;126: A14411, November 2012.

"Sex Differences in Cell Death and Gene Regulation *Exploring mechanisms for Sex Dimorphic Cellular Response*" B. Estevez, C. Penaloza, Z. Zakeri Department of Biology, Queens College and Graduate Center of the City University of New York. ABRCMS meeting, Coronado Springs Resort, Orlando FL. November 2008.

"Potential Protective Role of EFABP in NGFPC12 Cells" B. Estevez, L. Bu, M. De Leon, Center for Health Disparities in Molecular Medicine, Loma Linda University, Loma Linda, CA. August 2008.

PUBLISHED

ABSTRACTS:

"Differential Roles of the NADPH-Oxidase 1and 2 in Platelet activation and Thrombosis" Delaney MK, Kim K, Estevez B, Stojanovic-Terpo A, Shen B, Ushio-Fukai M, Cho J, Du X, University of Illinois at Chicago, Department of Pharmacology,Illinois, AHA meeting, Orlando, FL. 2015

"A Platelet Glycoprotein Ib-IX-specific 14-3-3/Rac1/LIMK1 Signaling Pathway Promotes Thrombin-Induced Platelet Activation" B Estevez, K Delaney, A Stojanovic- Terpo, X Du, 2015, ATVB, 2015;35:A598. Hilton San Francisco Union Square, San Francisco, CA. May 2015.

"A Signaling Mechanism By Which Platelet Glycoprotein Ib-IX Promotes Thrombin- Induced Platelet Activation" B Estevez, MK Delaney, A Stojanovic-Terpo, X Du, 2014, Blood 124 (21), 2759-2759 McCormick Place, Chicago, IL Dec. 2015.
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