The Role of Galpha13 in Integrin Signaling and Function

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THESIS

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Contribution of Authors

Chapter 1 is a literature review that places my dissertation question in the context of the larger field and highlights the significance of my research question. Some of the writings were previously published (Shen *et al*., Current Opinion in Cell Biology, 2012) for which I was the first author. I wrote the manuscript with the assistance of M Keegan Delaney. My advisor and the corresponding author Dr. Xiaoping Du proposed the model we described and revised the manuscript. Chapter 2 listed the detailed methods which were used in my experiments described in Chapter 3 and 4. Figure 3 and Figure 4 were published (Gong *et al*., Science, 2010) and I was the second author of the paper. I did the experiments with the first author Dr. Gong. The corresponding author Dr. Xiaoping Du conceived the idea, designed the experiments, and wrote the manuscript. I participated in the discussion. Chapter 3 represents a published manuscript (Shen, *et al*., Nature, 2013) for which I was the primary author and major driver of the research. Kelly A O'Brien and Stephen C-T Lam gave valuable suggestion for the manuscript writing. Aleksandra Stojanovic-Terpo was a major player in the project and assisted me in the experiments shown in Figure 22 and 23. Kyungho Kim and Jaehyung Cho did the *in vivo* thrombosis experiment in Figure 21B. M Keegan Delaney initially discovered the phenomenon observed in Figure 18. My research mentor, Dr. Xiaoping Du conceived the idea, mentored me through the project, and wrote the manuscript. Chapter 4 represents a published manuscript (Shen, *et al*., MBoC, 2015) for which I was the first author. I generated all the figures and played an important role in the writing of the manuscript along with the o-authors Brian Estevez, Deane Mosher, and my research mentor, Dr. Xiaoping Du. Other co-authors assisted me in technique support and

reagent preparation. Chapter 4 represents a series of my own unpublished experiments directed at answering the question (fill in). I anticipate that this line of research will be continued in the laboratory after I leave and that this work will ultimately be published as part of a co-authored manuscript. In Chapter 5 represents my synthesis of the research presented in this thesis/dissertation and my overarching conclusions. The future directions of this field and this research question are discussed.

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LIST OF ABBREVIATIONS

ACD	acid-citrate-dextrose
ADP	adenosine diphosphate
ATP	adenosine triphosphate
BSA	bovine serum albumin
cAMP	cyclic 3', 5'-adenosine monophosphate
CD	cytoplasmic domain
cGMP	cyclic guanine monophosphate
CHO	chinese hamster ovary
DAG	diacylglycerol
DMSO	dimethyl sulfoxide
EDTA	ethlenediaminetetraacetic acid eNOS
FcRγ	Fc receptor gamma
FcγRIIA	Fc gamma receptor IIA
FDA	Food and Drug Administration
FeCl3	ferric chloride
FITC	fluorescein isothiocyanate
GC	guanylyl cyclase
GPVI	glycoprotein VI
GPIbα	glycoprotein Ib alpha
GPIbβ	glycoprotein Ib beta
GPIb-IX-V	glycoprotein Ib-IX-V complex
GPIX	glycoprotein IX
GPV	glycoprotein V
GST	glutathione S-transferase
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
Ig	immunoglobulin
ITAM	immunoreceptor tyrosine kinase activation motif
IP3	inositol 1,4,5-triphosphate
MIDAS	metal ion-dependent adhesion site
MAPK	mitogen-activated protein kinase
PAGE	polyacrylamide gel electrophoresis
PAR	proteinase-activated receptor
PBS	phosphate-buffered saline
PEG	polyethelene glycol
PGE1	prostaglandin E1
PGG2	prostaglandin G2
PGH2	prostaglandin H2
PGI2	prostaglandin I2
PI3K	phosphoinositide-3 kinase
PKA	cAMP-dependent protein kinase
PKB/Akt	protein kinase B

LIST OF ABBREVIATIONS (continued)

PKC	protein kinase C
PKG	cGMP-dependent protein kinase
PLA2	phospholipase A2
PLCγ2	phospholipase Cγ2
PRP	platelet rich plasma
PTB	phosphotyrosine binding
RIAM	Rap1-GTP-interacting adaptor molecule
ROCK	Rho-dependent kinase
RGDS	Arg-Gly-Asp-Ser
SDS	sodium dodecyl sulfate
SFK	Src family protein kinase
SNARE	Soluble N-methyl maleimide-sensitive factor attachment protein
TXA2	thromboxane A2
VAMP	vessicular membrane associated protein
vWF	von Willebrand factor

Summary

Integrins are adhesion receptors which mediate many important physiological and pathological processes, such as development, immunity, inflammation, thrombosis and cancer. In particular, platelet integrin αIIbβ3 play pivotal roles in thrombosis and thus has been a therapeutic target for many anti-thrombotic drugs.

Integrins are known to transmit signaling in two directions: intracellular signaling induces the binding of key molecules, such as talin, to the cytoplasmic domain of integrins and stimulate conformational changes in the extracellular domain, leading to increased ligand affinity, which is called inside-out signaling. Conversely, ligand binding in the extracellular domain of integrins transmit signals into the cell, leading to a cascade of signaling events that stimulate cell spreading, retraction, migration, and proliferation, which is called outside-in signaling. The mechanism of integrin outside-in signaling has been unclear. We found that the heterotrimeric guanine nucleotide–binding protein (G protein) Gα13 directly bound to the integrin β3 cytoplasmic domain and that Gα13-integrin interaction was promoted by ligand binding to the integrin αIIbβ3 and by guanosine triphosphate (GTP) loading of Gα13. We therefore hypothesized that integrins could be noncanonical Gα13-coupled receptors, and Gα13 binding to integrins might be important in integrin outside-in signaling and integrin functions.

First we focused our studies on platelet integrin αIIbβ3, which has a critical role in thrombosis and haemostasis. αIIbβ3 antagonists are potent anti-thrombotic drugs, but also have the life-threatening adverse effect of causing

bleeding. It is therefore desirable to develop new anti-thrombotic agents that do not cause bleeding. Integrins transmit signals bidirectionally. Inside-out signalling activates integrins through a talin-dependent mechanism. Integrin ligation mediates thrombus formation and outside-in signaling, which requires Gα13 and greatly expands thrombi. Our studies demonstrate that Gα13 and talin bind to mutually exclusive but distinct sites within the integrin β3 cytoplasmic domain in opposing waves. The first talin-binding wave mediates inside-out signaling and also ligand-induced integrin activation, but is not required for outside-in signaling. Integrin ligation induces transient talin dissociation and Gα13 binding to an EXE motif (in which X denotes any residue), which selectively mediates outside-in signaling and platelet spreading. The second talin-binding wave is associated with clot retraction. An EXE-motif-based inhibitor of Gα13–integrin interaction selectively abolishes outside-in signaling without affecting integrin ligation, and suppresses occlusive arterial thrombosis without affecting bleeding time. Thus, we have discovered a new mechanism for the directional switch of integrin signaling and, on the basis of this mechanism, designed a new antithrombotic drug that does not cause bleeding.

Secondly, we showed that the Gα13-integrin binding is also important in integrin-dependent cell migration. Gα13 is known to transmit G protein–coupled receptor (GPCR) signals leading to activation of RhoA and plays an important role in cell migration. The mechanism underlying the role of Gα13 in cell migration, however, remains unclear. We successfully show that a direct interaction between Gα13 and the cytoplasmic domain of the integrin β1 subunit

plays a critical role in β1-dependent cell migration. Point mutation of either glutamic acid in the Gα13-binding 767EKE motif in β1 or treatment with a peptide derived from the Gα13-binding sequence of β1 abolished Gα13–β1 interaction and inhibited β1 integrin–dependent cell spreading and migration. We further showed that the Gα13-β1 interaction mediates β1 integrin–dependent Src activation and transient RhoA inhibition during initial cell adhesion, which is in contrast to the role of Gα13 in mediating GPCR-dependent RhoA activation. These data indicate that Gα13 plays dynamic roles in both stimulating RhoA via a GPCR pathway and inhibiting RhoA via an integrin signaling pathway. This dynamic regulation of RhoA activity is critical for cell migration on β1 integrin ligands.

I. LITERATURE REVIEW

(Part of the contents were previously published as Brian Estevez, Bo Shen, Xiaoping Du (2015) Targeting integrin and integrin signaling in treating thrombosis. *Arteriosclerosis, Thrombosis, and Vascular Biology*. 35,1,24-29, and Bo Shen, M Keegan Delaney, Xiaoping Du (2012) Inside-out, outside-in, and inside–outside-in: G protein signaling in integrin-mediated cell adhesion, spreading, and retraction. *Current Opinion in Cell Biology*. 245,5,600-606. Figure. 1 was previously published as Zhenyu Li, M. Keegan Delaney, Kelly A. O'Brien, Xiaoping Du (2010) Signaling During Platelet Adhesion and Activation. *Arteriosclerosis, Thrombosis, and Vascular Biology*. 30: 2341-2349)

1. Platelets

Platelets are anucleated blood cells circulating in mammals. They circulate in human blood at a concentration of 150,000,000 to 400,000,000 per milliliter(Italiano *et al.*, 2007). In non-mammalian vertebrates such as zebrafish, platelets are nucleated and are named thrombocytes(Jagadeeswaran *et al.*, 1999). The primary functions of platelets and thrombocytes are to prevent bleeding and maintain hemostasis. However, platelets/thrombocytes also contribute to other physiological and pathological functions beyond hemostasis(Ware *et al.*, 2013).

1.1 Platelet origin and morphology

Platelets and thrombocytes are possibly derived from megakaryocytes, which are differentiated from hematopoetic stem cells (HSC) which mainly reside in the bone marrow in mammals, or kidney and thymus in zebrafish(White, 1988; Hartwig, 1992; Khandekar *et al.*, 2012). Platelets are discoid shaped anucleated cells with a size of 1- 3 μm in diameter, and named by James Wright at the early 20th century when he examined blood smears using his Wright stain. The large (50–100 μm), polyploid megakaryocytes are the source of platelets, and they produce platelets through a budding process(Machlus and Italiano, 2013). Mature megakaryocytes contain multiple nuclei, massive amounts of granules and proteins(Richardson *et al.*, 2005), which are fragmented into individual platelets during platelet genesis(Italiano *et al.*, 1999). Megakaryocytes primarily emerge under the influence of thrombopoietin, a hormone produced in the kidney and liver along with IL3, IL6 and IL11(Fielder *et al.*, 1996; Quentmeier *et al.*, 1998; Tafuri *et al.*, 1999; Kaser *et al.*, 2001).

Platelets contain alpha granules (containing proteins such as P-selectin, platelet factor 4, transforming growth factor-β1, platelet-derived growth factor, fibronectin, β-thromboglobulin, vWF, fibrinogen, and coagulation factors V and XIII, etc), dense granules (containing ADP or ATP, polyphosphate, calcium, and serotonin), and lambda granules (similar to lysosomes, containing several hydrolytic enzymes). Platelets secrete their granule contents upon agonist stimulation, which are important in platelet functions in hemostasis and thrombosis(Flaumenhaft, 2003; Reed, 2004).

1.2. Platelets functions

The main platelet functions are to adhere, aggregate, secrete granule contents, and also facilitate in the coagulation. Upon vascular injury, platelets adhere to the injured site, aggregate, secrete granule contents and form negatively charged, pro-coagulate surface. Under high shear stress in the blood flow, platelet adhesion is mediated through the major platelet adhesion receptors GPIb-IX-V complex, GPVI, and integrins such as αIIbβ3. Adhered platelets aggregate to form a primary thrombus, which is further stabilized and amplified with the amplification signaling from secreted contents of platelet granules. Granular molecules are platelet agonists and enhance platelet activation and aggregation, both *in vitro* and *in vivo*. Thus, platelet granule secretion is critical for the recruitment of platelets to the injured vasculature, and stabilization and amplification phases of the thrombus formation. The secretion process is regulated by SNARE (Soluble NSF Attachment protein REceptor) proteins(Flaumenhaft, 2003). Many platelet SNARE proteins and regulators have been identified to mediate platelet granule secretion, including t-SNAREs such as syntaxin 11, v-SNAREs such as VAMP-7, STXBP5, SNAP-23, cellubrevin, etc(Feng *et al.*, 2002; Ye *et al.*, 2012; Karim *et al.*, 2013; Ye *et al.*, 2014; Zhu *et al.*, 2014; Golebiewska *et al.*, 2015; Koseoglu *et al.*, 2015). In addition, activated platelets exhibit procoagulant activity through surface expression of phosphatidylserine, and the release of procoagulant microparticles(Sambrano *et al.*, 2001; Delaney *et al.*, 2014).

1.2.1. Platelet functions in hemostasis and thrombosis

Platelets play important roles in many physiological processes such as hemostasis, wound healing, inflammation, innate immunity, angiogenesis, and are important in pathological thrombosis. The main physiological function of platelets is to maintain hemostasis. Upon vascular injury, platelets adhere to the exposed endothelium matrix and get activated. Activated platelets undergo shape change and secrete granule contents, which amplify the platelet activation cascade. Aggregated platelets then form the primary thrombus, which seals the wounded area. Platelet aggregate formation is also associated with activation of the coagulation cascade with resultant fibrin deposition, further stabilizing the clot formation. Though platelet aggregation is critical in hemostasis, formation of excessive blood clot in blood vessels leads to vessel occlusion, called thrombosis. Thrombosis is a pathological condition, including venous thrombosis and arterial thrombosis, and could lead to ischemia of downstream tissues and organs, and thereby cause diseases such as stroke and heart attack.

1.2.2. Platelet functions beyond thrombosis and hemostasis

In recent years, the roles of platelets beyond thrombosis and hemostasis become more and more appreciated, including their functions in cancer, infection, inflammation, organ regeneration, etc(Jurasz *et al.*, 2004; Ware *et al.*, 2013). The role of platelets in thrombosis and inflammation is delicately intertwined. Activated platelets secrete granule contents that are critically important in both thrombosis and inflammation(Klinger and Jelkmann, 2002). In addition, stimulated platelets are known

to be involved in heterotypic interactions with other cells in the circulation, such as neutrophils, which play important roles in the inflammation process(Harding *et al.*, 2007; Hidalgo *et al.*, 2009; Li *et al.*, 2014a; Kim *et al.*, 2015). Twenty percent of cancer patients die from thrombosis, and numerous reports confirmed that a broad range of different tumor cells induce thrombosis, including but not limited to patients suffering from lung, renal, gastric, colorectal, breast cancers, etc(Spigel and Mooney, 1977; Costantini *et al.*, 1990; Hernandez *et al.*, 1992; Pedersen and Milman, 1996; Taucher *et al.*, 2003). Thus, anti-thrombotic therapies are adopted to treat cancer patients, alleviating the thrombotic symptoms(Zacharski, 2002; Ho-Tin-Noe *et al.*, 2008; Ho-Tin-Noe *et al.*, 2009; Holmes *et al.*, 2013). Recently, studies have suggested that platelet secretion also plays important roles in post-surgery lung regeneration(Rafii *et al.*, 2015). This opens a new gate in platelet research as whether platelet secretion could be used to facilitate stem cell therapies in organ regeneration.

1.3. Platelet signaling

Under normal conditions without vascular injury, platelets circulate in blood in a resting state and the intact endothelium secretes prostacyclin (prostaglandins I_2, PGI_2). Prostacyclin inhibits platelet aggregation through the activation of adenylyl cyclase, thus increasing the intracellular level of cyclic adenosine monophosphate (cAMP) and activation of cAMP-dependent peotein kinase (PKA), which in turn phosphorylates a lot of proteins including GPIbβ, whose phosphorylation (at Ser166) leads to the inhibition of the vWF binding function of GPIb complex(Wardell *et al.*, 1989; Bodnar *et al.*, 2002).

The intact endothelium also blocks coagulation by the expression of thrombomodulin and heparin-like molecules on endothelial cell surface. Thrombomodulin functions in the anticoagulant pathway by acting as a cofactor in the thrombin-induced activation of protein C by forming a 1:1 stoichiometric complex with thrombin, thus reducing blood coagulation by converting thrombin to an anticoagulant enzyme(Salem *et al.*, 1984; Machlus *et al.*, 2009). Heparin-like molecules function by neutralization of the serine protease activity of the coagulation cascade(Rosenberg, 1985). Thus, the intact endothelium functions to maintain platelets in a resting state and inactivate coagulation factors.

Upon vascular injury, platelets are transformed from a "resting state" to an "activated state". The activated platelets are able to adhere, aggregate, secrete granule contents, and response to injury. Platelet functions depend on platelet signaling, which includes intracellular signaling cascades that lead to initial platelet adhesion and activation, and downstream signaling that regulates platelet responses including secretion and aggregation.

1.3.1. Signaling of platelet adhesion receptors

In addition to the platelet integrin receptor αIIbβ3 (detailed discussion in Chapter 2), the major platelet adhesion receptors include GPIb-IX-V complex and GPVI.

The GPIb-IX-V complex is comprised of four different transmembrane proteins: GPIbα, GPIbβ, GP IX and GP V. GPIb complex is constituted by a disulfide bond-linked GPIbα chain and GPIbβ chain (in a 1:2 ratio), which complexes with GP IX in a 1:1

ratio(Du, 2007). The GPIb-IX complex is sufficient for the ligand binding function, and GP V subunit is loosely associated with GPIb-IX complex in a 1:2 ratio(Du, 2007). GPIb is important in mediating platelet rolling and adhesion to the injury site under high shear conditions. The GPIb-IX-V complex does not interact with soluble vWF in the blood. Following vascular injury, the GPIb complex is activated through binding of the N-terminal ligand binding site of GPIb to its ligands vWF (A1 domain) or thrombin(Huizinga *et al.*, 2002; Uff *et al.*, 2002; Celikel *et al.*, 2003; Dumas *et al.*, 2003). In addition to its role as adhesion receptors, recent studies have identified GPIb as a signaling molecule that transmit cellular signals leading to platelet activation and aggregation(Ozaki *et al.*, 2005; Du, 2007).

GPIb phosphorylation is important for its signaling and functions. GPIbα phosphorylation is required for the binding of the adaptor protein 14-3-3ζ to the cytoplasmic domain of GPIb. which positively regulates the vWF binding function of GPIb complex(Dai *et al.*, 2005). Also, the phosphorylation of GPIbβ is dynamically regulated and is proposed to be the mechanism to control platelet adhesion to vWF(Fox and Berndt, 1989; Bodnar *et al.*, 2002), possibly through the dynamic regulation of GPIb interaction to the cytoskeleton(Englund *et al.*, 2001). GPIb plays a role in mediating initial platelet adhesion (and rolling when platelet activation is inhibited) under high shear flow(Cranmer *et al.*, 1999), allowing platelets to also interact with collagen through GPVI and α2β1. Previous reports also suggested that the binding of vWF to GPIb induces activation of tyrosine kinases, as demonstrated by phosphorylation of multiple proteins including Fc receptor γ-chain (FcRγ), Syk, linker for activation of T cells (LAT), Feγ receptor IIa (FcγRIIA) and phospholipase Cγ2 (PLCγ2), following GPIb-

mediated platelet activation (Razdan *et al.*, 1994; Ozaki *et al.*, 1995; Francesconi *et al.*, 1996; Sullam *et al.*, 1998; Wu *et al.*, 2001). However, the tyrosine phosphorylation of PLCγ2 is independent of integrin αIIbβ3 signaling(Wu *et al.*, 2001).

Mutations or deficiency in the GPIb could lead to various platelet diseases. One example is the rare hereditary Bernard–Soulier syndrome(Alexander and Landwehr, 1949; Gautier and Guinand-Doniol, 1952; Caen *et al.*, 1971; Nurden and Caen, 1974; Bennett and Vilaire, 1979; Ginsberg *et al.*, 1983). Patients with Bernard–Soulier syndrome have increased bleeding tendency and thrombocytopenia (reduced platelet count), and have abnormally large platelets, due to deficiency or mutations in the GPIb-IX-V complex. It was hypothesized that the enlargement of platelet size is attributed to the absence of the filamin A binding site on GPIbα that links the GPIb-IX-V complex to the platelet membrane skeleton, which is critical for the discoid shape of resting platelets(Kanaji *et al.*, 2002). In addition, a gain-of-function mutation in GPIb causes platelet-type von Willebrand disease, leading to hyperresponsive platelets and increased thrombotic risks(Othman, 2007).

A few other adhesion receptors have been identified on platelets, most notably are the collagen receptors glycoprotein VI (GPVI) and integrin α2β1. GPVI is the main adhesion receptor for collagen and also mediates collagen-dependent platelet signaling(Clemetson and Clemetson, 2001; Nieswandt *et al.*, 2001). Expression of GPVI on the platelet surface is dependent on FcRγ, which is cross-linked with GPVI through a salt bridge(Nieswandt *et al.*, 2000; Bori-Sanz *et al.*, 2003). Nieswandt and colleagues showed that GPVI is important in platelet adhesion to collagen under both static and

shear conditions(Nieswandt *et al.*, 2001). The cross-linked FcRγ ITAM motif is phosphorylated by the Src family kinases Lyn and Fyn, but not Src, following collagen binding to GPVI and receptor clustering(Ezumi *et al.*, 1998). Tyrosine phosphorylation of the ITAM motif in turn activates a cascade of downstream signaling molecules, including Syk, SLP-76, LAT, and Bruton's Tyrosine Kinase (Btk) which stimulate PI3K, PLCγ2 and activate integrins(Poole *et al.*, 1997; Quek *et al.*, 1998; Clements *et al.*, 1999; Pasquet *et al.*, 1999; Judd *et al.*, 2000; Obergfell *et al.*, 2001; Inoue *et al.*, 2003; Suzuki-Inoue *et al.*, 2003; Watson *et al.*, 2005). GPVI and integrin α2β1 play complementary roles in mediating platelet adhesion to collagen(Savage *et al.*, 1998; Kuijpers *et al.*, 2003; Siljander *et al.*, 2004). Thus, it is not surprising that integrin β1, similar to β3, mediates platelet granule secretion and hemostasis(Petzold *et al.*, 2013).

Studies of GPVI signaling heavily relies on the use of GPVI-specific ligands, including collagen-related peptides (CRPs) that contain the specific GPVI recognition motif and the snake venom toxin convulxin (CVX). The tetrameric CVX is the most potent GPVI agonist and widely adopted for GPVI signaling studies(Polgar *et al.*, 1997; Batuwangala *et al.*, 2004). However, CVX also reacts with the GPIb-IX-V complex, thus a contribution of GPIb signaling to CVX-induced GPVI signaling needs to be carefully considered and conclusions deduced using CVX alone should be carefully re-evaluated(Kanaji *et al.*, 2003).

GPIb and GPVI signaling induces integrin activation and leads to ligand binding to integrins (inside-out signaling)(Coller, 1986; Beer *et al.*, 1992; Ginsberg *et al.*, 1992; Hynes, 1992; Ginsberg *et al.*, 1995), thereby resulting in platelet aggregation and

thrombus formation (outside-in signaling)(Schwartz and Ginsberg, 2002; Shattil and Newman, 2004).

1.3.2. Signaling of platelet G-protein-coupled receptors

Many agonists stimulate platelet activation and aggregation through the signaling of G-protein coupled receptors (GPCRs). For example, a number of soluble platelet agonists are released by damaged cells and activated platelets, and produced during the coagulation or inflammation process, namely ADP, thromboxane A2, histamine, epinephrine, platelet-activating factor, etc, which all stimulate GPCR signaling. They play critical roles in platelet activation and thrombus formation(Offermanns, 2006). These soluble agonists induce platelet activation via the G protein-coupled receptors (GPCRs)(Offermanns, 2006). GPCRs are a large family of seven-transmembrane receptors that sense the environment outside the cell and activate intracellular signaling pathways and cellular responses(Kobilka, 2007). The GPCRs are coupled to various heterotrimeric G proteins, include four major families: Gq, Gi, Gs, and G12/13. Both human and mouse platelets express all four families of the G proteins, and they share a similar activation mechanism: conformational change in GPCR leads to exchange of GDP for GTP, and activation and dissociation of the G protein heterotrimer to $G\alpha$ subunit and $G\beta/\gamma$ complex. The dissociated G proteins induces a cascade of downstream signaling pathways which differ depending on G protein subtypes and cell types(Kobilka, 2007). A summary of the platelet signaling mechanism has been reviewed by our group and shown in Fig. 1(Li *et al.*, 2010).

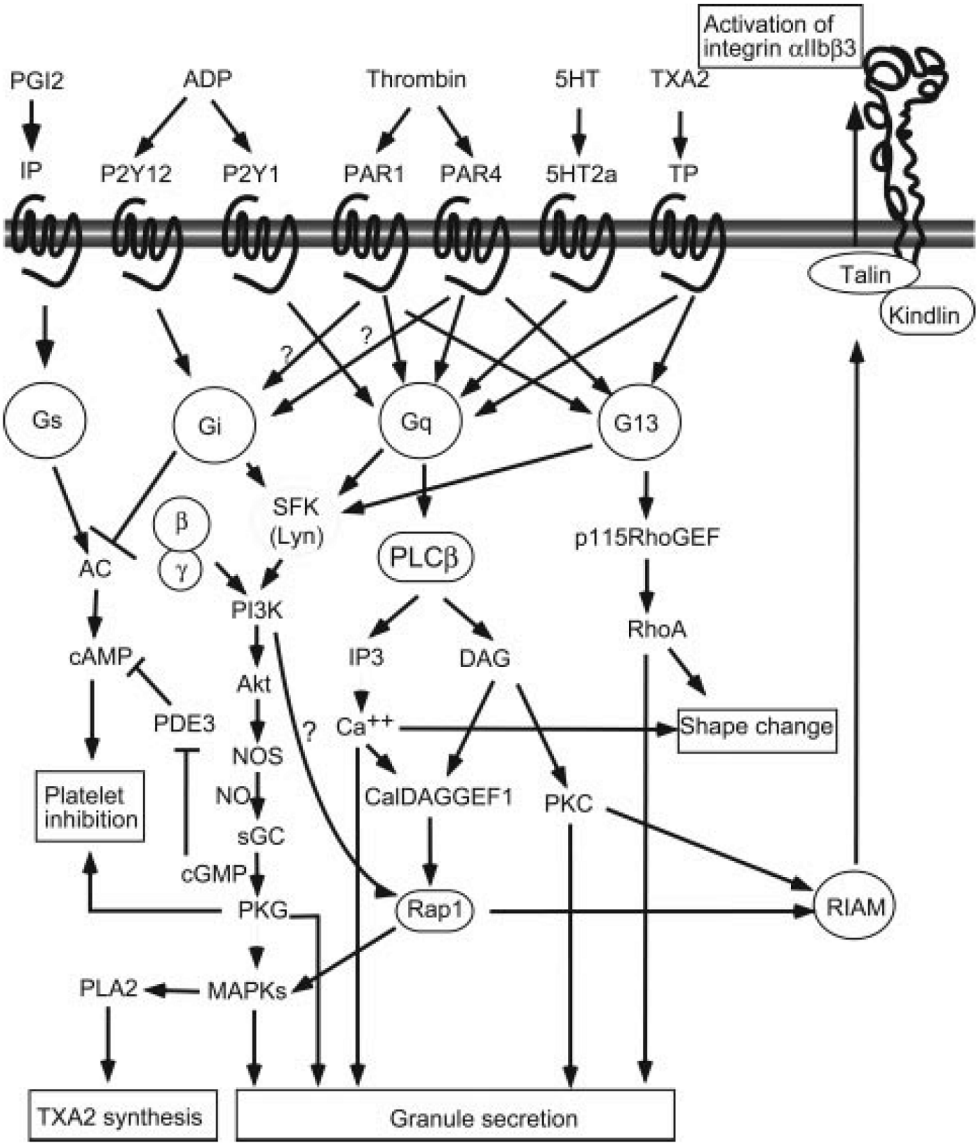

Figure. 1. GPCR-coupled platelet activation signaling, reprinted from Li, *et al*., ATVB, 2010

Various GPCR agonists stimulate platelet activation through a range of different receptors and signaling pathways. However, these signaling pathways all eventually converge into several key signaling events and activation of several pivotal protein kinases. Among these key events are platelet calcium mobilization, PLC and PI3K activation, production of thromboxane A2 and platelet granule secretion, and integrin αIIbβ3 activation.

These platelet agonists mostly lead to activation of PLC, which catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP2) to diacyl glycerol (DAG) and inositol trisphosphate (IP3), which in turn activates calcium mobilization and protein kinase C (PKC), respectively(Varga-Szabo *et al.*, 2009). Calcium is an important second messenger in many cell types. In platelets, calcium release mediates the cytoskeletal rearrangement for platelet shape change shortly after agonist stimulation(Hathaway and Adelstein, 1979). Calcium release also activates the CalDAG-GEF (calcium and diacylglycerol-regulated guanine nucleotide exchange factor) , together with DAG, which is critical in Rap1 activation and integrin activation(Shattil and Brass, 1987; Crittenden *et al.*, 2004).

1.3.2.1. Gq-mediated platelet signaling

The classic Gq-mediated signaling involves the activation of phospholipase C (PLC), which in turn hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP2) to diacyl glycerol (DAG) and inositol trisphosphate (IP3), inducing downstream signaling pathways. Platelet Gq-mediated signaling is important for various GPCR agonist

pathways which induce platelet aggregation and secretion(Offermanns *et al.*, 1997). Gq-coupled receptors include the ADP receptor P2Y1 and thrombin PAR receptors(Fabre *et al.*, 1999; Brass, 2003). It was also shown that the Gq-mediated PLCβ stimulation plays a central role in mediating platelet granule secretion, calcium release, and integrin activation following various agonist stimulation(Offermanns *et al.*, 1997).

1.3.2.2. Gi-mediated platelet signaling

Gi-coupled receptors mainly signal through the inhibition of the cAMP dependent pathway, through inhibition of adenylate cyclase. The functions of Gi in platelets is consistent with that as Gi mediates platelet activation through relieving of the cAMP-dependent inhibitory protein kinase activities. Platelet Gi-coupled receptors include the ADP receptor P2Y12, thromboxane A2 receptors (TP) and possibly the thrombin receptors PAR1/PAR4(Knezevic *et al.*, 1993; Ohlmann *et al.*, 1995; Kim *et al.*, 2002). In particular, the ADP receptor P2Y12 is required for platelet activation and aggregation induced by the GPCR agonists ADP, thromboxane A2, and low-dose thrombin(Paul *et al.*, 1999; Kim *et al.*, 2002), due to the inhibition of cAMP production(Ohlmann *et al.*, 1995; Ohlmann *et al.*, 2000). In addition, the P2Y12 receptor-coupled Gi activates the PI3K pathway in platelets, particularly through the Gβ/γ subunit-dependent PI3Kγ activation, leading to activation of Rap1b and downstream activation of integrins (as discussed above)(Li *et al.*, 2003).

1.3.2.3. Gs-mediated platelet signaling

Opposite to the Gi signaling pathway, Gs-mediated signaling is dependent on the activation of the cAMP-dependent pathway, through activation of the adenylyl cyclase. Gs is coupled to the PGI2 receptors which are responsible for the physiological inhibition of platelet activation in normal circulation(Hawiger *et al.*, 1980; Cheng *et al.*, 2002).

1.3.2.4. G12/13-mediated platelet signaling

G12/13 signal through the activation of guanine nucleotide exchange factors (GEFs), through converting GDP-bound inactive G12/13 to GTP-bound active G12/13 which binds and activates GEFs (guanine nucleotide exchange factor). G12/13 is coupled to thromboxane A2 receptors (TP) and PAR receptors in platelets(Kahn *et al.*, 1998; Djellas *et al.*, 1999). Unlike some other vascular cells, G12 seems to play a redundant role in platelet activation and aggregation(Moers *et al.*, 2003; Andreeva *et al.*, 2005). In contrast, G13 plays a pivotal role in mediating platelet activation, shape change, secretion, and aggregation induced by the thromboxane A2 analog U46619(Klages *et al.*, 1999; Moers *et al.*, 2003), presumably through its role in activating platelet GEFs such as p115RhoGEF and LARG(Huang *et al.*, 2007; Williams *et al.*, 2015).

1.4. Anti-thrombotic therapies

Heart attack and stroke are the number one killer in the U.S., representing a tremendous public health challenge. Anti-thrombotic drugs which are able to prevent and treat heart attack, stroke, and other cardiovascular diseases are thus in high demand. Thrombus formation consists of two key processes: platelet aggregation and fibrin deposition (coagulation). Thus, two anti-thrombotic strategies are widely adopted. The anti-platelet strategy inhibits platelet aggregation through targeting key signaling molecules on platelets, thus inhibiting platelet aggregation. whereas the anti-coagulation strategy inhibits the coagulation cascade and thrombin generation.

1.4.1. Anti-coagulation therapies

Anti-coagulation drugs reduce cogaulation (blood clotting), thus preventing or alleviating thrombotic symptoms such as deep vein thrombosis (DVT), myocardial infarction and stroke. There are many anti-coagulant drugs on the market now. All anti-coagulants inhibit the fibrin deposition, through (1) thrombin inhibition by antithrombin protein or direct thrombin inhibitors (hirudin, dabigatran, etc)(Di Nisio *et al.*, 2005); (2) factor Xa inhibitors, such as rivaroxaban, apixaban and edoxaban(Bruins Slot and Berge, 2013; Graff and Harder, 2013); (3) heparin or low molecular weight heparin (LMWH) and derivatives, which activate antithrombin III(Hirsh and Raschke, 2004); and (4) vitamin K antagonists such as coumarins (Warfarin)(Horton and Bushwick, 1999).

1.4.2. Anti-platelet therapies targeting GPCRs

There are generally two groups of anti-platelet drugs. One of them are those targeting directly on the platelet GPCRs, including cyclooxygenase inhibitors, thrombin inhibitors, and ADP receptor antagonists. Cyclooxygenase inhibitors aspirin and Triflusal prevent thrombosis by inhibiting cyclooxygenase and therefore thromboxane A2 production(European Stroke Organisation Executive and Committee, 2008; Anninos *et al.*, 2009; Malloy *et al.*, 2013; Alvarez-Sabin *et al.*, 2014; Cavalca *et al.*, 2014; Gouya *et al.*, 2014). The ADP receptor antagonists, including Clopidogrel, Prasugrel, Ticagrelor, are all ADP receptor P2Y12 inhibitors(Bonaca *et al.*, 2014; Hibbert *et al.*, 2014; Nanau *et al.*, 2014; Piccolo *et al.*, 2014; Schulz *et al.*, 2014; Wang *et al.*, 2014). The ubiquitous expression of the ADP receptor P2Y1 in other cells makes it an unsuitable target for anti-platelet strategy(Kunapuli *et al.*, 2003; Wijeyeratne and Heptinstall, 2011). The thrombin inhibitors includes direct thrombin inhibitor Bivalirudin and Dabigatran (also anti-coagulants)(Ndrepepa *et al.*, 2012; Shah and Feldman, 2012; Bloom *et al.*, 2014; Providencia *et al.*, 2014), and the PAR1 receptor antagonist Vorapaxar(Abdulsattar *et al.*, 2011; Morrow *et al.*, 2012; Zhang *et al.*, 2012; Alfredsson and Roe, 2015). Most of these drugs, through targeting GPCR signaling, also affect integrin αIIbβ3 activation indirectly.

1.4.3. Anti-platelet therapies directly targeting integrins

The last anti-thrombotic strategy is to inhibit integrin αIIbβ3 signaling directly. There are currently several antagonists of αIIbβ3 on the market, including Abciximab

(Reopro), Eptifibatide (Integrilin), and Tirofiban (Aggrastat)(Lang *et al.*, 2012; Bledzka *et al.*, 2013; Xu *et al.*, 2013; Ciccone *et al.*, 2014). Administration of these inhibitors are mostly through intravenous injection, which remains inconvenient for patients (e.g., patients with poor vein access or patients requiring immediate injection by non-clinical personnel). Recently, there is some progress in anti-thrombotic development: Coller's group developed a novel class of integrin αIIbβ3 inhibitors, RUC, which are potent and the most recent RUC-4 could be easily administered intramuscularly in animals(Li *et al.*, 2014b).

1.4.4. Adverse effects of anti-platelet therapies

All current anti-platelet drugs on the market have the same adverse effect of bleeding. This includes gastrointestinal bleeding and intracranial hemorrhage, which sometime could lead to lethality(Eikelboom *et al.*, 2012). This significantly limits the application of these drugs clinically.

1.4.5. Statement of Problem 1

The major disadvantage of the current anti-platelet drugs is that they all have the bleeding adverse effect, limiting the application and maximum dosage of these drugs in clinical use. Thus, it would be ideal if we could develop novel anti-thrombotic with reduced or no bleeding tendency. This issue still remains to be resolved, as all the new developments right now still possess significant bleeding effects, including the RUC

series of inhibitors(Li *et al.*, 2014b). Hypothesized therapies using selective targeting of the integrin cytoplasmic sequence that regulates talin binding to integrins would probably also lead to bleeding, although potentially milder than integrin antagonists(Stefanini *et al.*, 2014). Chapter 3 would demonstrate our design of a novel anti-thrombotic, which shows potent anti-thrombotic efficacy with no bleeding risk.

2. Integrins

Integrins are important receptors expressed on virtually all cell types, which mediate cell adhesion to the extracellular matrix proteins, or cell-cell interaction. The name "integrin" was proposed to describe this integral membrane protein complex which links the extracellular matrix to the cytoskeleton by Hyne's group in 1986(Tamkun *et al.*, 1986). Among the first identified and the most important ones include integrin αIIbβ3 (also named Glycoprotein IIb/IIIa) on platelets(Phillips and Agin, 1977; Bennett *et al.*, 1982; Marguerie *et al.*, 1984), β1 integrins on chicken and humans (also named VLA proteins)(Hemler *et al.*, 1985; Tamkun *et al.*, 1986; Hemler *et al.*, 1987), and integrin αMβ2 and αLβ2 (also named Mac-1 and LFA-1) which play pivotal roles in lymphoid and myeloid cells(Kishimoto *et al.*, 1987; Law *et al.*, 1987). Research in the last 30 years has identified a total of 8 β integrins and 18 α integrins in vertebrates. Each α integrin subunit could pair with one of the 8 β integrins to form an integrin heterodimer complex, which mediates cell adhesion to a wide array of extracellular matrix proteins, non-matrix proteins, or other cells.

Integrins not only mediate cell adhesion to the extracellular matrix proteins, but also transmit signals. Integrins are usually classified according to their respective β subunits, among them the β1, β2 and β3 subtypes are the most abundantly expressed and extensively studied. The ubiquitously expressed integrin β1 is expressed in almost all cell types. β2 integrins are expressed on leukocytes, and mediate leukocyte adhesion and migration. One of the most extensively studied integrins is the platelet-specific integrin αIIbβ3, which mediates key platelet functions such as thrombosis and hemostasis. The major ligands for integrin αIIbβ3 are fibrinogen and vWF.

The rare hereditary Glanzmann's thrombasthenia, similar to the Bernard–Soulier syndrome. is also correlated with increased bleeding tendency in patients(Alexander and Landwehr, 1949; Gautier and Guinand-Doniol, 1952; Caen *et al.*, 1971; Nurden and Caen, 1974; Bennett and Vilaire, 1979; Ginsberg *et al.*, 1983). In contrast to the Bernard–Soulier syndrome, Glanzmann's thrombasthenia patients have increased bleeding tendency but normal platelet counts. Platelets from Glanzmann's thrombasthenia patients have a normal size but abnormal integrin αIIbβ3 (deficiency or mutations), with new mutations discovered over the years(Buitrago *et al.*, 2015). Treatment of these diseases are most limited to blood transfusions, with some recent hopes for gene therapy for the future(Wilcox and White, 2003; Du *et al.*, 2013; Chen *et al.*, 2014).

2.1. Integrin structure

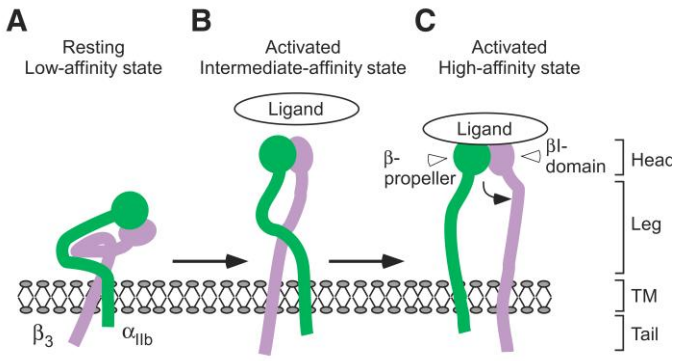

Figure 2. Conformational states during integrin activation and ligand binding. **A**, The resting (low-affinity) state is maintained by interactions between the α and β chains within the transmembrane and membrane proximal cytoplasmic domains, which constrain the ectodomain. **B**, The activated (intermediate affinity) state has an extended conformation with a closed configuration. **C**, Binding of ligand recognition sequences induces further conformational changes, resulting in an open high-affinity state. Curved black arrow indicates the swing-out motion of the β3 subunit hybrid domain on full integrin activation. TM indicates transmembrane domain. (Reprinted with permission from ATVB)

Each integrin heterodimer consists of an α and a β subunit, both of which are type I transmembrane proteins. The α/β heterodimer subunits are associated by noncovalent interactions, forming two relatively long extracellular ligand-binding domain ('head'), two single-pass transmembrane helices, and two short cytoplasmic domains ('tails') (Fig. 2). The characteristic short tails of the β integrins are critical in integrin signaling regulation, with the exception of integrin β4, which has a very long cytoplasmic tail (over 1000 amino acid residues). The crystal structures of multiple integrin extracellular subdomains are available, such as the α-I domain(Lee *et al.*, 1995a) and the β2-leg fragments(Beglova *et al.*, 2002; Xiao *et al.*, 2004; Shi *et al.*, 2005). Recently, the crystal structures of multiple integrin complexes are available, such αIIbβ3, αvβ3, and αxβ2, etc(Xiong *et al.*, 2002; Zhu *et al.*, 2008; Xie *et al.*, 2010). These crystal structures suggest that inactive integrins are in a "bent" conformation with their ligand binding sites placing in proximity to the membrane surface(Choi *et al.*, 2013). Upon integrin activation, the conformational changes in the integrin extracellular domain will lead to increased ligand affinity, ligand binding, and change from the "bent" to an upright conformation

("switchable model"(Luo *et al.*, 2007))(Choi *et al.*, 2013) (Fig. 2). However, there is debate on whether these dramatic changes are taking place during integrin activation, or if more conservative delicate changes in the bent region are more practical ("deadbolt model")(Arnaout *et al.*, 2005; Zhu *et al.*, 2007; Choi *et al.*, 2013).

2.1.1. I domains

The extracellular regions of α subunits consist of multiple regions: a seven-bladed β-propeller domain, one thigh domain, and two calf domains. β integrins and nine of the 18 α integrins also contain an I domain, which undergoes conformational changes on integrin ligand binding(Calderwood, 2004). Integrins with the α-I domain (also named as α-A domain because of its homology with the A domain found in the vWF), including α1 and α2, form complexes with integrin β1 and serve as collagen/laminin receptors. The collagen-binding α-I domains of integrins α1β1 and α2β1 recognize the specific motif of GFOGER in native collagens(Knight *et al.*, 2000).

2.1.2. Cation binding sites

The crystal structure of the integrin α-I domain suggests that integrin ligand binding requires Mg^{2+} and the metal ion-dependent adhesion site (MIDAS)(Lee *et al.*, 1995b). The cation binding sites are also identified in the integrin β-I domain in platelet αIIbβ3 integrins(Zhu *et al.*, 2008). Xiong and colleagues showed that the MIDAS is not occupied in un-ligated integrin αvβ3 crystals(Xiong *et al.*, 2001; Xiong *et al.*, 2002). However, recent data suggested that all three cation binding sites formed by the β-I domain in integrin αIIbβ3 are occupied under physiological concentrations of the

divalent cations Ca^{2+} and Mg^{2+}(Zhu *et al.*, 2008). This suggests that the MIDAS is not a ligand binding-associated cation binding site, but rather a synergistic metal ion binding site. Thus, it is believed that the SyMBS plays a positive role in mediating integrin ligand binding through synergistic cation ion binding, renamed it as SyMBS (synergistic metal ion binding site)(Zhu *et al.*, 2008).

2.2. Integrin signaling

One distinct feature of the integrin receptors is that they not only mediate cell-matrix and cell-cell adhesion, but also transmit signals in two directions(Hynes, 2002). Intracellular signals induce binding of key regulatory proteins, such as talin and kindlin, to the integrin cytoplasmic tails, leading to extracellular conformational changes, resulting in increased ligand binding affinity and consequent ligand binding ("inside-out" signaling). Conversely, ligand binding to the integrin extracellular region transmits signals into the cells, resulting in a cascade of signaling events mediating cell spreading, retraction and migration ("outside-in" signaling).

2.2.1. Platelets as a model in integrin signaling studies

Integrins are expressed in many cell types. The importance of integrin $\beta 3$ in platelets was demonstrated with a hereditary disease "Glanzmann's thrombasthenia". This disease was first described by Dr. Glanzmann in 1918, and is characterized by bleeding tendency, and defective platelet functions in patients(Braunsteiner and Pakesch, 1956; George *et al.*, 1990). The molecular mechanism of this disease remains

unclear until the 1970s, when advances in biochemistry allowed scientists to correlate this disease with an absence of platelet-specific membrane receptors, named the glycoprotein IIb (GPIIb) and the glycoprotein IIIa (GPIIIa)(Nurden and Caen, 1974). These two receptors were later found to be complexed together and also named the integrin αIIbβ3(Kunicki *et al.*, 1981; Hynes, 1987, 1992). Thus, investigation of the signaling mechanisms of integrin αIIbβ3 would have clinical implications in treating Glanzmann's thrombasthenia. Interestingly, platelets have since then become an idea tool in studying integrin signaling. There are mainly three reasons for that: (1) There are about 50,000 - 100,000 copies of integrin αIIbβ3 on each platelet surface, representing the most highly expressed integrin in all cell types(Kieffer and Phillips, 1990; Coller *et al.*, 1991); (2) The methods for assaying platelet functions were well established in the 1970s, including measurement of platelet aggregation(O'Brien, 1961; Born, 1962); (3) Platelets are anucleated and thus are not affected by cell cycle changes. These advantages are the main reasons that many major discoveries on integrin signaling and functions are first identified on integrin αIIbβ3, using platelets as the research model. However, there are also limitations using the platelet model. Anucleated platelets could not be used for over-expression or genetic manipulations. Besides, platelet secretion confounds integrin signaling pathways by amplification of the integrin signaling. The size of the platelets is also much smaller compared to other cells, thus more difficult for imaging. These were overcome by using a nucleated CHO cell model, in which over-expression of human integrin αIIbβ3 in CHO cells mimics platelets(O'Toole *et al.*, 1990). The αIIbβ3-expressing CHO cells could be genetically modified, and the integrin αIIbβ3 signaling is isolated from various secreted compounds in platelets.

2.2.2. Integrin inside-out signaling

The Ginsberg's and Shattil's laboratories had started investigating the integrin inside-out signaling more than two decades ago. In the early 1990s, they first identified that the integrin inside-out signaling is initiated with a dissociation of the non-covalent interaction between the α and β integrin cytoplasmic tails(O'Toole *et al.*, 1990; O'Toole *et al.*, 1991; Hughes *et al.*, 1996). Disruption of the integrin α-β tail interaction with point mutations or truncation mutations lead to increased ligand affinity of integrin αIIbβ3, while double mutations on both tails, which retain a salt bride maintaining this α-β tail interaction, keep integrins in their resting state(O'Toole *et al.*, 1990; O'Toole *et al.*, 1991; Hughes *et al.*, 1996). However, the molecular mechanisms inducing the separation of the α/β tails and integrin activation was unclear at that time. Later studies suggested that talin and kindlin play important roles in this process(Calderwood *et al.*, 1999; Ma *et al.*, 2008). Recently, convincing evidence also showed that the basic amino acid residues in integrin transmembrane domains (TMD) are critical in maintaining the integrin $\alpha\beta$ TMD association and keeping integrins under resting condition(Kim *et al.*, 2012).

2.2.3. Integrin outside-in signaling

Many of the studies on integrin signaling are focused on the inside-out signaling and integrin affinity modulation. There is an emerging trend on integrin outside-in signaling investigation as the cellular functions of integrin signaling are directly related to the outside-in signaling, which is responsible for intracellular responses such as

calcium mobilization, protein phosphorylation, and cytoskeletal remodeling. These intracellular events are mediated by small GTPases such as RhoA, Rac1, and Cdc42(Hynes, 2002; Hall, 2005). The early phase of integrin outside-in signaling mediates cell spreading, which is characterized by the outwards movement of cell membrane, and formation of filopodia, lamellipodia, and actin polymerization at the leading edge of a cell(Hall, 2005). During the late phase of integrin outside-in signaling, inward movement of cell membrane is observed at the trailing edge of a cell. Coordinated cell spreading at the leading edge and retraction at the trailing edge mediates directed cell migration(Huttenlocher and Horwitz, 2011). However, the molecular mechanism of this transition of early to late phase outside-in signaling is unclear. Previous studies in our lab suggested that the calpain cleavage of the RGT sequence in the C-terminus of integrin β3 could be a molecular switch regulating this process(Flevaris *et al.*, 2007). Platelet integrin αIIbβ3 outside-in signaling is responsible for platelet spreading, retraction, granule secretion, second wave of platelet aggregation, therefore playing pivotal roles in both thrombosis and hemostasis(Ginsberg *et al.*, 1995; Schwartz *et al.*, 1995; Shattil and Newman, 2004).

2.2.4. Integrin clustering

Integrin ligation leads to the lateral assembly of integrin receptors on the cell membrane, which is often referred as integrin clustering. After integrin ligation, the integrin clustering could be further promoted through transmembrane domain homo-dimerization(Schneider and Engelman, 2004; Li *et al.*, 2005; Yu *et al.*, 2011), and lateral

diffusion of integrins(Wiseman *et al.*, 2004). It was also believed that it is the multivalent ligation, but not monovalent ligation, that is responsible for inducing integrin clustering, cytoplasmic tail phosphorylation, and Src activation which are all critical in integrin outside-in signaling(Miyamoto *et al.*, 1995; Arias-Salgado *et al.*, 2003; Ginsberg *et al.*, 2005). Recent studies suggest that the separation of the integrin αIIbβ3 tails using a computationally designed peptide could induce integrin activation and clustering without integrin ligand binding, suggesting that the integrin ligation could be bypassed for clustering formation(Yin *et al.*, 2007; Shandler *et al.*, 2011).

2.3. Integrin-binding proteins

The relatively short cytoplasmic tails of integrins are critical in integrin signaling and functions. There are many proteins found to interact with the integrin tails directly or indirectly. The cytoplasmic tail of integrin αIIbβ3, particularly β3, serves as a scaffold for intracellular proteins mediating integrin signaling(Arias-Salgado *et al.*, 2003; Shattil, 2009). The αIIb tail is much shorter, and is shown to bind directly to protein phosphatase 1 (PP1c) through a conserved motif ^{989}KVGF^{992}(Vijayan *et al.*, 2004). PP1c binds to αIIb tail constitutively, and agonist activation induces PP1c phosphorylation and dissociation from the αIIb tail, leading to integrin αIIbβ3 activation(Vijayan *et al.*, 2004). The cytoplasmic domain of β integrins is longer, and a plethora of proteins bind directly or indirectly to the β integrin tails. In integrin αIIbβ3, it has been shown that talin, kindlin, filamin, VPS33B, and many PTB domain-containing proteins interact directly with the β integrin tails(Calderwood *et al.*, 1999; Buensuceso *et*

al., 2004; Arias-Salgado *et al.*, 2005a; Bennett, 2005; Kiema *et al.*, 2006; Ma *et al.*, 2008; Xiang *et al.*, 2015). Several kinases also interact with αIIbβ3, including several members of the Src family kinases (SFK), and other kinases such as Syk, integrin linked kinases (ILK), and adaptor protein such as Grb2, DOK-1 and ADAP (Jenkins *et al.*, 1998; Arias-Salgado *et al.*, 2003; Buensuceso *et al.*, 2004; Ling *et al.*, 2005; Oxley *et al.*, 2008; Kasirer-Friede *et al.*, 2014). Studies in the recent two decades have established a critical role of the integrin β3 tail in mediating the bidirectional signaling of integrin αIIbβ3(Chen *et al.*, 1992; Chen *et al.*, 1995; Wang *et al.*, 1997; Xi *et al.*, 2003). It is established now that the membrane proximal region and the two NxxY motifs in the β3 are required for inside-out signaling(Calderwood *et al.*, 1999; Vinogradova *et al.*, 2002; Tadokoro *et al.*, 2003), while the ^{759}RGT motif and the Tyr^{747}/Tyr^{759} sites are necessary for outside-in signaling(Chen *et al.*, 1994; Chen *et al.*, 1995; Xi *et al.*, 2006; Flevaris *et al.*, 2007; Su *et al.*, 2008). However, there is still many unaddressed questions in the signaling mechanisms regulating the interaction of integrins with all these intracellular proteins. It has been speculated that investigation of these proteins and the molecular mechanisms mediating their interaction with αIIbβ3 would potentially provide insights for the development of novel anti-thrombotic drugs, and drugs for other integrin-related pathological conditions such as inflammation, autoimmunity, and cancer(Ginsberg *et al.*, 2005; Shattil, 2005, 2009).

2.3.1. Talin

Talin, a FERM-domain-containing protein, was initially identified and studied as a linkage candidate molecule connecting the extracellular matrix proteins and integrins to intracellular actin filaments(Horwitz *et al.*, 1986). Later on, studies from several groups showed that the direct binding of talin head domain (THD) to integrin β subunit cytoplasmic tails is a key step in integrin inside-out signaling and integrin activation(Knezevic *et al.*, 1996; Calderwood *et al.*, 1999; Tadokoro *et al.*, 2003; Xing *et al.*, 2006). Their findings were soon confirmed by structural biology data showing the direct binding of talin to the integrin β subunit(Anthis *et al.*, 2009). Furthermore, it was shown that talin activates integrins through promotion of the integrin cytoplasmic tail separation(Vinogradova *et al.*, 2002). Qin's and Springer's laboratories showed convincing evidence that separation of the C-terminal integrin cytoplasmic tails, through either mutations on integrin β subunits or direct talin binding, activates integrins(Lu *et al.*, 2001; Takagi *et al.*, 2001). There has been also convincing evidence that the direct talin binding to the integrin cytoplasmic tails is a final common step in integrin activation(Tadokoro *et al.*, 2003). The exact molecular mechanism regulating the talin binding is still under debate. In recent years, multiple groups have suggested that talin is recruited to bind to integrins by Rap1b (a small GTPase which is a member of the RAS-like small GTP-binding protein superfamily) and RIAM (Rap1-guanosine triphosphate-interacting adaptor molecule)(Chrzanowska-Wodnicka *et al.*, 2005; Watanabe *et al.*, 2008). Rap1b-null mice showed reduced platelet aggregation, increased bleeding in a tail bleeding assay, and were protected from arterial thrombosis, explained by reduced integrin activation due to deficient talin binding(Chrzanowska-

Wodnicka *et al.*, 2005). It was previously showed that the Rap1b effector RIAM is also directly involved in this process(Watanabe *et al.*, 2008; Lee *et al.*, 2009). However, there are still some controversies about the functions of Rap1b and RIAM. In the last few years, compelling evidence shows the importance of Rap1b activation in integrin inside-out signaling and integrin activation(Bernardi *et al.*, 2006; Duchniewicz *et al.*, 2006; Carmona *et al.*, 2009). On the other hand, others suggested that Rap1b could also be activated by outside-in signaling, and this outside-in signaling-induced Rap1b activation mediates platelet spreading on fibrinogen and clot retraction(Zhang *et al.*, 2011). The most recent studies showed that RASA3 (Ras GTPase-activating protein 3) keeps circulating platelets under resting condition by restraining CalDAG-GEFI/Rap1 signaling, and P2Y12 signaling is required to inhibit RASA3 and enable sustained Rap1-dependent platelet activation and thrombus formation at sites of vascular injury(Stefanini *et al.*, 2015). Also, although RIAM has been proposed to mediate Rap1b-dependent talin binding to integrins using cell biology and biochemistry methods(Garcia *et al.*, 2007; Watanabe *et al.*, 2008; Lee *et al.*, 2009; Yang *et al.*, 2014), this mechanism does not seem to be universal in all integrin subtypes(Klapproth *et al.*, 2015; Lagarrigue *et al.*, 2015; Stritt *et al.*, 2015). The mechanisms mediating talin-dependent integrin activation thus remains to be unveiled.

Interestingly, it was also recently shown that talin mediates integrin-dependent clot retraction, during the presumed late-phase of integrin outside-in signaling(Haling *et al.*, 2011). It was clear that talin-dependent linkage of the fibrin clot to the actin cytoskeleton via integrins is important in the talin-dependent clot retraction(Haling *et al.*, 2011). However, the mechanism of the talin's role in this process remains unclear.

2.3.2. Kindlin

The FERM domain, consisting of F1, F2, and F3 subdomains, is a common protein motif found in many cytoskeletal and signaling molecules. The FERM domain is a unique motif which is often involved in the linkage of intracellular proteins to the membrane(Chishti *et al.*, 1998). Similar to talin, kindlin also contains a FERM domain and was identified as a crucial component mediating cell adhesion to the extracellular matrix(Xie *et al.*, 2010). There are three different isoforms of kindlins, and deficiency in kindlin results in severe defects in integrin signaling, cell adhesion and cytoskeletal organization. Kindlin-1 deficiency causes Kindler Syndrome, a rare disease characterized by skin blistering and congenital poikilodermas(Larjava *et al.*, 2008). Kindlin-2 or kindlin-3 is indispensable for cell-ECM adhesion, as knockout of either one leads to embryonic or postnatal lethality(Larjava *et al.*, 2008). Kindlin plays important roles in both integrin inside-out and outside-in signaling. It was hypothesized that kindlin acts as a co-activator during integrin activation, through direct binding to the NPxY motif in the β integrin tails(Ma *et al.*, 2008; Moser *et al.*, 2008; Harburger *et al.*, 2009). Kindlin regulates integrin activation by facilitating talin binding and ligand binding affinity of integrins ("co-activator" hypothesis), and/or by mediating integrin clustering and ligand binding avidity of integrins ("one-two punch" hypothesis)(Bledzka *et al.*, 2012; Ye *et al.*, 2013). Kindlin is also important in integrin outside-in signaling, although its mechanism remains unclear, and its function during outside-in signaling may not involve direct kindlin binding to integrins(Montanez *et al.*, 2008; Petzold *et al.*, 2013).

2.3.3. Src family kinases

Pro-oncogenic tyrosine protein kinase Src is a family of non-receptor tyrosine kinases, including nine different members. The Shattil's laboratory has shown that the Src family kinases c-Src, Fyn, Hck, Lyn and c-Yes interact with integrin β1, β2, and β3(Arias-Salgado *et al.*, 2003). In particular, they showed that the carboxyl terminal of integrin β3 binds constitutively to the c-Src kinase(Arias-Salgado *et al.*, 2003). It was reported that this direct interaction is mediated by the SH3 domain of the c-Src, and the C-terminal ^{759}RGT motif in integrin β3(Arias-Salgado *et al.*, 2003; Flevaris *et al.*, 2007). It is now clear that the αIIbβ3 integrin outside-in signaling requires c-Src binding to the ^{759}RGT motif, and that outside-in signaling both induces and requires the phosphorylation of the ^{747}Tyr/^{759}Tyr sites in the β3 tail(Law *et al.*, 1999; Xi *et al.*, 2006). The early phase outside-in signaling mediates RhoA inhibition and cell spreading, through c-Src activation of the RhoGAPs (Rho GTPase Activating Proteins), such as p190RhoGAP(Arthur *et al.*, 2000; Arthur and Burridge, 2001). On the other hand, initial integrin signaling would induce phosphorylation of the ^{747}Tyr/^{759}Tyr sites, leading to elevated intracellular calcium level and activation of calpain(Fox *et al.*, 1993). After that, late phase integrin outside-in signaling is initiated and calpain cleavage of the ^{759}RGT site in integrin β3 relieves c-Src activation, leading to RhoA activation and cell retraction(Xi *et al.*, 2006; Flevaris *et al.*, 2007). The coordinated retraction in the trailing edge and spreading at the leading edge of cells drive cell migration(Huttenlocher and Horwitz, 2011). Thus, this provides a molecular switch mechanism which explains how integrin outside-in signaling mediates both cell spreading, retraction, and directional cell migration(Gong *et al.*, 2010; Huttenlocher and Horwitz, 2011).

2.4. Integrin signaling and G proteins G13 and RhoA

Integrins are not only adhesion receptors, but also transmit signals bidirectionally: intracellular signals induces increased affinity of the extracellular region to ligands and integrin ligand binding ('inside-out' signaling); while extracellular signals transmit into the cells and elicit intracellular signaling cascades that lead to cellular responses such as spreading, secretion, and migration ('outside-in' signaling). Integrin signaling requires both heterotrimeric G proteins (for 'inside-out' signaling and integrin activation as previously shown, also 'outside-in' signaling as will be discussed in Chapter 3 and 4) and monomeric small G proteins ('outside-in' signaling).

2.4.1. The role of Gα13 in integrin signaling

The heterotrimeric G proteins were known to mediate integrin activation, such as Gq/ 11 and Gi/o (Ginsberg *et al.*, 1992; Li *et al.*, 2010; Kim *et al.*, 2011). Knockout of Gα13 in mouse platelets, however, did not completely abolish platelet aggregation, but rather only inhibits the integrin-dependent second wave of platelet aggregation, in response to low concentrations of GPCR agonists, suggesting that its main function is in the amplification phase of integrin-dependent platelet responses, which are mainly dependent on integrin outside-in signaling(Moers *et al.*, 2003). However, the role of Gα13 in platelet integrin αIIbβ3 outside-in signaling is unknown. In nucleated cells, Gα12/13 has been shown to mediate integrin-dependent directional cell migration(Radhika *et al.*, 2004; Bian *et al.*, 2006; Shan *et al.*, 2006; Tan *et al.*, 2006). However, the role of Gα13 in this process is also unclear.

2.4.2. The role of RhoA in integrin signaling

The binding of ECM ligands to integrins initiates outside-in signaling, leading to a cascade of intracellular responses, which are important in cellular functions such as cell spreading, retraction and cell migration on the ECM. Integrin-mediated cell spreading and subsequent retraction are common to nearly all cell types. Furthermore, coordinated spreading and retraction are critical in directed cell migration on ECM matrices(Moissoglu and Schwartz, 2006; Huttenlocher and Horwitz, 2011). The integrin-dependent cell retraction, spreading, and migration are mediated by small GTPases such as RhoA, Rac1, and Cdc42(Barry *et al.*, 1997; Hynes, 2002; Hall, 2005). The early phase of integrin outside-in signaling mediates cell spreading, which is characterized by the outwards movement of cell membrane, and formation of filopodia, lamellipodia, and actin polymerization(Hall, 2005). The late phase of integrin outside-in signaling mediates cell retraction, characterized by inward movement of cell membranes and the associated cytoskeleton. Coordinated cell spreading at the leading edge and retraction at the trailing edge mediates directed cell migration(Huttenlocher and Horwitz, 2011).

RhoA is an important mediator of cell retraction. When cells are imbedded in flexible tissues and gel-like matrices, cell retraction causes these tissues or gel-like matrices to shrink, as in the cases of wound healing and clot retraction. On rigid surfaces, retraction of actin–myosin complexes results in the formation of stress fibers. In non-muscle cells, actin–myosin-dependent retractile forces are stimulated by phosphorylation of myosin light chain (MLC), which can be mediated by MLC kinase (MLCK) and downregulated by MLC phosphatase (MLCP). GTP-bound Rho, which consists of three highly homologous isoforms RhoA, RhoB and RhoC, activates its

downstream effector Rho-kinase (ROCK), which subsequently induces phosphorylation and inhibition of MLCP(Kimura *et al.*, 1996), and culminates in the generation of a retractile force. Members of the Rho subfamily of small GTPases also interact with different isoforms of formins, which facilitate actin polymerization and formation of stress fibers(Kitzing *et al.*, 2010; Vega *et al.*, 2011). However, these different Rho isoforms may play different roles in cells, as they differ in subcellular localization, regulator and effector specificity(Wheeler and Ridley, 2004; Kitzing *et al.*, 2010; Vega *et al.*, 2011; Ridley, 2015). Integrin ligation dynamically regulates Rho activity. During the early phase of cell spreading, Rho activity is transiently downregulated probably via Src-dependent phosphorylation of p190RhoGAP(Ren *et al.*, 1999; Arthur *et al.*, 2000; Flevaris *et al.*, 2007), but is later activated(Flevaris *et al.*, 2007). A recent study shows that tensional force applied to ligated integrins induces SFK Fyn-dependent activation of leukemia- associated Rho GEF (LARG) and the mitogen activated protein kinase (MAPK)- and focal adhesion kinase (FAK)-enhanced activation of GEFH1(Guilluy *et al.*, 2011), both of which activate RhoA.

RhoA may play an inhibitory role in cell spreading, during which Cdc42 and Rac have been shown to mediate filopodia and lamellipodia formation, respectively(Ridley and Hall, 1992). RhoA may regulate Rac activity to restrict overextension of the cell(Vega *et al.*, 2011). Interestingly, cycles of membrane protrusion and retraction occur locally in the leading edge of spreading cells. A study suggests that the function of RhoA in regulating clock rhythm of protrusion waves is controlled by PKA phosphorylation of RhoA at Ser188 and consequent increase in its GDI affinity(Tkachenko *et al.*, 2011). Although RhoA could be important in normal process of

cell spreading and migration, it may not be required for cell spreading. Previous studies showed that inhibition of RhoA actually leads to increased cell spreading(Arthur and Burridge, 2001; Flevaris *et al.*, 2007; Vega *et al.*, 2011). Knockout of RhoA in megakaryocytes and platelets showed no defect in cell spreading(Pleines *et al.*, 2012). Also, it has been established that RhoA is transiently inhibited during the early phase of cell spreading, shortly after cell adhesion(Ren *et al.*, 1999; Arthur and Burridge, 2001; Flevaris *et al.*, 2007). These studies suggest that RhoA-mediated contraction may serve to limit cell spreading. Cell spreading is associated with small G protein mediated waves of actin polymerization in filopodia and lamellipodia. It has been presumed that actin polymerization is required for cell spreading; however, there has been evidence indicating that actin polymerization does not directly cause cell spreading, particularly the early phase of cell spreading. A study on the physical properties of cell spreading suggests that the dynamics of cell spreading follows a universal power-law behavior, and can be modeled as a viscous adhesive cortical shell (membrane and associated cytoskeleton) enclosing a less viscous interior (cytosol)(Cuvelier *et al.*, 2007). Inhibition of actin polymerization with cytochalasin D accelerates cell spreading, suggesting that actin polymerization may not be required for cell spreading but may serve as a constraint(Cuvelier *et al.*, 2007). Consistently, although Rac and Rho both facilitate actin polymerization, Rac and Rho play opposing roles during integrin-dependent cell spreading. In this respect, Rac induces phosphorylation of p190RhoGAP and inhibition of RhoA, directly or indirectly via a ROS-dependent mechanism, and this function is important in promoting cell spreading(Sander *et al.*, 1999; Nimnual *et al.*, 2003). Another mechanism whereby Rac suppresses Rho activity is via its effector PAK, which

phosphorylates and inactivates p115RhoGEF, a RhoGEF known to be important in stimulating GPCR-mediated RhoA activity(Rosenfeldt *et al.*, 2006).

Upon cell adhesion to integrin ligands, cells spread and retract. In polarized cell movement, cell spreading mainly occurs at the leading edge, while retraction dominates in the rear. Clearly, some molecular mechanisms must exist to allow a cell to switch its response to integrin outside-in signaling from spreading to retraction. Such a switch mechanism has been characterized for β3 integrins in our lab: following the binding of extracellular ligands to integrin αIIbβ3, G13-β3 interaction and consequent activation of β3-bound c-Src induces c-Src-dependent inhibition of RhoA(Flevaris *et al.*, 2007; Gong *et al.*, 2010). This process is required for cell spreading. c-Src also phosphorylates β3 at Y747 and Y759(Law *et al.*, 1999), protecting β3 from cleavage by calpain(Xi *et al.*, 2006), a calcium-dependent protease. Following cell spreading, de-phosphorylation of Y759 and consequent cleavage of β3 by calpain at Y759 removes the c-Src binding site thus abolishing c-Src-mediated inhibition of RhoA and inducing subsequent activation of RhoA-dependent contractile signaling and cell retraction(Flevaris *et al.*, 2007; Ablooglu *et al.*, 2009). Cell spreading mediated by other integrin subtypes, such as β1 integrin, also requires transient inhibition of RhoA via SFK-dependent activation of p190RhoGAP(Arthur *et al.*, 2000; Arthur and Burridge, 2001; Schober *et al.*, 2007). However, c-Src does not appear to directly bind to the C-terminal sequence of β1(Arias-Salgado *et al.*, 2003) but rather can be recruited to the integrin-focal adhesion complex by FAK, which is implicated in β1-dependent activation of Src(Schaller *et al.*, 1994). Thus, although calpain activity is also important in β1-dependent cell migration on

matrix proteins(Huttenlocher *et al.*, 1997), it is possible that the exact molecular mechanisms regulating the 'switch' from spreading to retraction are different amongst integrin subtypes and/or cell types.

2.4.3. Statement of problem 2

Most of the studies on the bidirectional signaling of integrins focus on the mechanisms regulation integrin activation. The most exciting discoveries on this aspect are probably the identification of the roles of talin and kindlin in mediating integrin activation(Calderwood *et al.*, 1999; Ma *et al.*, 2008; Moser *et al.*, 2008). We know now that the talin binding to integrins serves as the last common step in integrin activation(Tadokoro *et al.*, 2003). Chapter 3 shows that Gα13 is a new binding partner of most integrin β cytoplasmic domains. Furthermore, the role of Gα13-integrin binding in αIIbβ3 integrin outside-in signaling is demonstrated. Chapter 4 shows the role of Gα13 in integrin β1 outside-in signaling. Both chapters show that the direct binding of Gα13 to integrins mediates integrin-dependent c-Src activation and transient RhoA inhibition.

II. MATERIALS AND METHODS

A. Animals and Reagents

Integrin $\beta3^{-/-}$ mice were obtained from the Jackson Laboratory. Talin-$1^{(fl/fl)}$, PF4-Cre mice were kindly provided by Dr. Brian Petrich and Dr. David Critchley(Petrich *et al.*, 2007). Animal usage and protocol were approved by the institutional animal care committee of the University of Illinois at Chicago. For all animal experiments, mice with the similar age, weight, and sex ratio (1:1, except for laser-induced thrombosis) are used for control and specific treatment. The individual mice chosen for the specific treatment are decided randomly. Human integrin $\beta3$ cDNA was cloned into pcDNA3.1 vector following digestion with Hind III and Xho I, or pLenti6-V5/Dest vector following digestion with EcoR I, Mfe I, and Xho I. Truncation mutants and integrin E to A mutants were either previously reported(Xi *et al.*, 2003)[288](Xi *et al.*, 2003b)[288][288][1] or generated using PCR and cloned into pcDNA3.1 vector by Bam HI and Xho I(Xi *et al.*, 2003). Human integrin β_1 cDNA was cloned into plenti6/V5-DEST vector following digestion with EcoRI and Xho I. Integrin E to A mutants were generated using PCR and cloned into plenti6/V5-DEST vector by EcoRI and Xho I.

Human talin head domain (THD) cDNA, corresponding to N-terminal talin amino acid residues 1-433, was cloned into pcDNA3.1 vector and pMal-C2 vector between EcoR I and Xho I sites. Anti-RhoA antibody was purchased from Cytoskeleton, Inc.; anti-Gα13(sc410), anti-c-Src (sc18), anti-talin (sc7534), and anti-integrin β3 (sc6627), anti-integrin $\beta1$ K20 (sc18887) and anti-integrin $\beta1$ JB1B (sc59829) antibodies were from Santa Cruz Biotechnology, Inc.; anti-Gα13(26004) was from NewEast; anti-

phospho-Src Y^{416} antibody was obtained from Cell Signaling; anti-talin (TA205) was from Millipore; anti-talin antibody 8d4 (T3287) was obtained from Sigma; anti-GST tag antibody, Alexa Fluor® 555 conjugate was from Millipore; PAC1 antibody (340507) and anti-mouse αIIb antibody MWReg3 (14-0411) were obtained from BD Biosciences; anti-human integrin β3 antibody MAb15, LIBS6 and 8053 rabbit serum were kindly provided by Dr. Mark Ginsberg (University of California, San Diego, La Jolla, CA); Lipofectamine 2000, viraPower lentivirus expression system, Alexa Fluor 546-conjugated phalloidin, Fluor 488-conjugated anti-mouse secondary antibody, talin-1 shRNA plasmids (NM-011602), and non-specific shRNA control vector were from Invitrogen; Y-27632 is from Calbiochem; Fibrinogen from Enzyme Research Laboratories; Lipofectamine 2000, ViraPower™ Lentiviral Expression System, Alexa Fluor 546-conjugated phalloidin were from Invitrogen; Fibronectin was from BD Biosciences; Active Rho Pull-Down and Detection Kit was from Pierce, Thermo Scientific.

B. Purified Gα13 and THD binding to integrin cytoplasmic domains

GST-tagged integrin cytoplasmic domain proteins were coated onto Pierce Glutathione-coated plates (15140) overnight at 4°C. After washing twice with NP40 buffer (50 mM Tris, pH 7.4, 10 mM $MgCl_2$, 150 mM NaCl, 1% NP-40, 1 mM sodium orthovanadate, 1 mM NaF) with complete protease inhibitor cocktail tablets (1 tablet/5 ml buffer, Roche), purified THD or Gα13 proteins were added onto the plate in NP40 buffer (for Gα13 binding, buffer contained 30 μM AlF_4^-). Bound THD or Gα13 was estimated with anti-talin or anti-$G\alpha_{13}$ antibody, horse radish peroxide (HRP)-conjugated

secondary antibody, and 3,3',5,5'-Tetramethylbenzidine Substrates (Pierce, 34021). The wells were washed three times with NP40 buffer between each of these steps. The reactions were terminated with 1 M sulphuric acid and measured for OD_{450}. For the competitive inhibition assay, increasing concentrations of THD or Gα13 was added to the reactions.

C. Platelet preparation and spreading on immobilized fibrinogen

Studies using human platelets were approved by the institutional review board at the University of Illinois at Chicago, and consent for the use in research was obtained from all donors. Washed human (healthy donors with no sickness or medication within the past 2 weeks prior to donation) and mouse (8 - 12 weeks old) platelets were prepared as previously described and resuspended in modified Tyrode's buffer(Flevaris *et al.*, 2007). Washed platelets were allowed to spread on 100 µg/ml fibrinogen (Enzyme Research Laboratories)-coated coverslips for different time points, fixed, permeabilized, stained and viewed with a Leica RMI RB microscope or Zeiss LSM510 META confocal microscope, as previously described(Yin *et al.*, 2008). Quantification of the surface area of spreading platelets was performed using NIH ImageJ. Statistical significance was determined using Student's *t*-test.

D. Platelet aggregation assay

Platelet aggregation and secretion were measured in a turbidometric platelet aggregometer (Chronolog) at 37°C with stirring (1000 rpm). Washed platelets (3×10^8/ml) in modified Tyrode's buffer were stimulated with thrombin (Enzyme Research Laboratories). Aggregation traces shown are representative of at least three independent experiments.

E. Fibrinogen and PAC1 binding assay

For the fibrinogen binding assay, washed human or mouse platelets resuspended in modified Tyrode's buffer were incubated with 10 µg/ml Oregon Green-conjugated fibrinogen (Molecular Probes) and PAR4AP as described previously(Xi *et al.*, 2003). The reaction was diluted with PBS and analyzed by flow cytometry using an Accuri C6 flow cytometry (BD). PAC1 binding was measured with FITC-labelled PAC1 antibody (Molecular Probe).

F. Co-immunoprecipitation

Similar as previously described(Arias-Salgado *et al.*, 2003), platelets or CHO-1b9 cells expressing recombinant integrin αIIbβ3 were solubilized in NP40 lysis buffer (50 mM Tris, pH 7.4, 10 mM $MgCl_2$, 150 mM NaCl, 1% NP-40, 1 mM sodium orthovanadate, 1 mM NaF), with complete protease inhibitor cocktail tablets (1 tablet/5 ml buffer, Roche). Lysis debris was cleared after centrifugation at 14,000g for 10 min. Lysates

were then immunoprecipitated with rabbit anti-Gα13 IgG, anti-integrin β3 rabbit serum or an equal amount of rabbit IgG or pre-immune serum for 2 hours before Protein A/G sepharose beads were added. After incubation of Protein A/G sepharose beads for 45 min at 4 °C, beads were centrifuged down and washed for six times with NP40 lysis buffer. Immunoprecipitates were analyzed by immunoblotting.

G. RhoA activity assay

Platelets or αIIbβ3-expressing CHO cells in modified Tyrode's buffer or adherent on immobilized fibrinogen were solubilized in cold NP40 lysis buffer at 4°C, and debris-cleared lysates were incubated for 1 hour with purified GST-RBD beads, washed, and then immunoblotted with an anti-RhoA monoclonal antibody, as previously described(Ren and Schwartz, 2000).

H. Bone marrow transplantation

Bone marrow stem cells were isolated from femur and tibias of 6-8 week old integrin $\beta3^{-/-}$, or C57/BL6 mice using the MACS lineage cell depletion kit (Miltenyi Biotec). Stem cells were subsequently infected twice with concentrated lenti-virus containing shRNA or cDNA constructs, as described in Animals and Reagents section, using a Lenti-X concentrator (Clontech). The cells were then retro-orbitally injected into irradiated recipient mice (5Gy for integrin $\beta3^{-/-}$ mice and 9.6Gy for C57/BL6 mice, one million cells per recipient mice) one day after irradiation.

I. Platelet Adhesion Assay

As previously described(Xi *et al.*, 2003), washed platelets were pre-incubated with vehicle or peptides, or with either 1 mM $MnCl_2$ or 0.18 μg/ml LIBS6 prior to plating. After 1 hour incubation at 37°C, adherent platelets were estimated by measuring platelet phosphatase activity with 0.3% *p*-nitrophenyl phosphate in 1% Triton X-100, 50 mM sodium acetate, pH 5.0 for 1 hour at 37°C. The reaction was stopped with 1 M NaOH. Results were determined by reading OD_{405}. Statistic significance was determined using Student *t*-test (n=3).

J. Immunofluorescence and confocal microscopy

αIIbβ3-expressing CHO cells or platelets suspended in Tyrode's buffer were added to fibrinogen-coated cover slides and incubated at 37°C for various lengths of time. Cells were fixed, permeabilized, BSA blocked, stained with mAb15 (followed by Fluor 488-conjugated anti-mouse secondary antibody) and/or Alexa Fluor-546 conjugated phalloidin, and viewed with a Zeiss LSM510 META confocal microscope or with Leica DM IRB fluorescence microscope, Photometrics CoolSNAP HQ camera and μManager software. Cell surface area was measured by NIH ImageJ analysis of 5-10 random images. Statistical significance was determined using Student *t*-test.

K. Clot Retraction Assay

As previously described(Flevaris *et al.*, 2007), human PRP was incubated with vehicle or peptides for 5 minutes at room temperature prior to stimulation with thrombin. The two-dimensional size of retracted clots was quantified using Image J software, and statistical significance was determined using Student *t*-test (n=3).

L. Peptide inhibitors

Myristoylated peptides were synthesized and purified at the Research Resource Center at the University of Illinois at Chicago. These peptides include: mP13 (Myr-KFEEERARAKWDT), mP5 (Myr-EEERA), mP6 (Myr-FEEERA), and the corresponding control peptides mP13Scr (Myr-EEARERKDWAKFT), mP5Scr (Myr-EEARE), and mP6Scr (Myr-ERAFEE). The peptides were prepared in DMSO for use *in vitro*, and in micellar formulation for *in vivo* (and *in vitro*) use. For micellar formulation, PEG2000-DSPE, L-α-phosphatidylcholine, and peptides were mixed at a molar ratio of 45:5:2. The micelles were suspended to form micelle colloid in HEPES-saline buffer (10 mM HEPES, 150 mM NaCl, pH 7.4), peptide concentration 1 mM) as previously described(Krishnadas *et al.*, 2003).

M. *In vivo* $FeCl_3$-induced thrombosis and tail bleeding time

7-8 week-old C57/BL6 mice were anesthetized by isoflurane inhalation. Retro-orbital injection of peptide micelle or integrilin (5 μmol/kg mouse weight) were performed 15 minutes prior to experimentation. Carotid arterial thrombosis was induced with a filter paper disc (d = 2 mm) soaked with 1.2 μl of 7.5% $FeCl_3$(O'Brien *et al.*, 2011). Blood flow was monitored with a TS420 flow meter using a MA-0.5SB dopler probe (Transonic Systems, Ithaca, NY). Data were analyzed using one-way ANOVA. Tail bleeding time analysis were performed as previously described(Marjanovic *et al.*, 2005). Time to stable cessation of bleeding was defined as no evidence of rebleeding for 60 seconds. Bleeding exceeding 15 minutes was immediately stopped by applying pressure. Statistical significance was determined using the Mann-Whitney test. Similar results were also obtained with a nonparametric ANOVA. For bleeding assays measuring total blood loss, cut mouse tails were immersed in Microcentrifuge tubes with 1.5 ml of 0.15 M NaCl at 37 °C for 15 minutes. The hemoglobin concentration in the tube was determined using a HemoCue photometer. Data were analyzed using one-way ANOVA. The experiments were performed in double-blinded fashion.

N. Intravital Microscopy and Laser-induced thrombosis.

Similar to the methods described previously(Cho *et al.*, 2012)[4], Wt male mice (6-8 weeks old) were anesthetized via IP injection of ketamine and xylazine and placed on a thermo-controlled blanket (37°C). The cremaster muscle was exteriorized and superfused with thermo-controlled (37°C) bicarbonate-buffered saline for the duration of

experiments. Fluorescence and brightfield images were recorded using an Olympus BX61W microscope with a 60 x/1.0 NA water immersion objective and a high speed camera (Hamamatsu C9300) through an intensifier (Video Scope International). Fluorescence images were captured at 20 frames per second, and data were analyzed using Slidebook v5.5 (Intelligent Imaging Innovations). Arteriolar wall injury was induced with a micropoint laser ablation system (Photonics Instruments). Platelet accumulation was visualized by infusion of Dylight 649-labeled anti-mouse CD42c (Emfret, 0.05 µg/g body weight body weight) into mice. Vehicle control, Integrilin, scrambled peptide, or mP6 were infused 3 minutes prior to laser injury. Laser-induced thrombi were generated at different sites in the blood vessel, with new sites upstream of earlier thrombi. Data were collected for 5 minutes following laser injury. The kinetics of platelet accumulation was analyzed by median fluorescence values of the antibodies as a function of time in approximately 30 thrombi in 3 mice per group. Statistical difference of fluorescence intensity (mean ± SD) at selected time points was also determined using Student's *t*-test. The experiments were performed in double-blinded fashion.

O. Cell culture

CHO(Xi *et al.*, 2003), 293FT, and GD25 cells were cultured in DMEM complete medium, which comprises of the Dulbecco's Modified Eagle's Medium (Cellgro) supplemented with 10% fetal bovine serum (Corning), 2 mM L-glutamine (Corning), 100 U/ml penicillin, 100 µg/ml streptomycin (Corning), 1mM sodium pyruvate (GIBCO), 0.1 mM non-essential amino acids (GIBCO), and 10 mM HEPES buffer (GIBCO). Cells

were serum starved in DMEM medium containing every component above except the fetal bovine serum for at least 4 hours prior to experiments.

P. Lentiviral infection and integrin β1 reconstitution

Gα13 shRNA lentivirus was prepared using pLL3.7-scrambled shRNA or pLL3.7-Gα13 shRNA (#1 and #2), which were co-transfected into sub-confluent 293FT cells with pLP1, pLP2 and pLP/VSVG plasmids (Invitrogen) using Lipofectamine 2000. After 48 hours, cell culture supernatant was collected, filtered, and used to infect CHO or GD25 cells. Similarly, expression of integrin β1 in GD25 cells is achieved by infecting GD25 cells with 293FT supernatant after plenti6-V5-DEST-β1 transfection using the ViraPower™ Lentiviral Expression System. Expression of various Wt or mutant integrin β1 on GD25 cell surface was assayed by flow cytometry using anti-integrin β1 antibody K20.

Q. Cell spreading assay

Cultured cells were detached with 0.053 mM EDTA and resuspended in serum free DMEM medium(Flevaris *et al.*, 2007). Cells were allowed to spread on 10 μg/ml fibronectin-coated coverslips for different time points, fixed, permeabilized, stained with Alexa Fluor 546-conjugated phalloidin, and viewed with a Leica RMI RB microscope as previously described(O'Brien *et al.*, 2011). Quantification of the surface area of spreading cells was performed using NIH ImageJ.

R. Wound healing assay

Cells were cultured until subconfluent, and serum starved in serum-free DMEM medium for at least 6 hours prior to the experiment. After starvation, scratches were made using p200 pipet tips, and DMEM complete medium was supplied to the cells. Cell migration and wound healing were monitored by taking pictures at selected time points using a bright-field microscopy.

S. Transwell migration assay

Fibronectin was coated on the outer surface of 6.5 mm transwell inserts with 8-μm pore size (Corning) overnight at room temperature. The insert was washed with PBS, and blocked with 2% BSA in PBS for 30 min at 37°C. Serum-starved cells in DMEM with 0.5% FBS were seeded on the upper chamber of the insert and allowed to migrate for 6 hours in a 37 °C incubator. After that, cells on the outer surface were fixed with 3.7% paraformaldehyde, stained with crystal violet, and visualized in a bright-field microscopy(Huttenlocher *et al.*, 1996).

T. *In situ* RhoA immunofluorescence Assay

GD25 cells were allowed to spread on immobilized fibronectin (10 μg/ml), fixed with 3.7% paraformaldehyde, permeabilized and incubated with purified GST-RBD proteins as previously described(Flevaris *et al.*, 2007). After that, cells were stained with anti-integrin β1 antibody conjugated with Alexa Fluor 488, and anti-GST tag antibody

conjugated with Alexa Fluor 555, and viewed with a Zeiss LSM510 META confocal microscope. Images were analyzed using ImageJ.

U. Statistics

For most of the data sets, Student's *t*-tests were performed after data were confirmed to be normally distributed; otherwise Mann-Whitney test (for bleeding time analysis) was applied. Analyses were performed with GraphPad Prism 4 software. The sample size (n) with adequate power was determined by power analysis. Unless otherwise specified, an asterisk indicates $p < 0.005$.

III. A DIRECTIONAL SWITCH OF INTEGRIN SIGNALING AND A NEW ANTI-THROMBOTIC STRATEGY

(Previously published as Haixia Gong, Bo Shen, Panagiotis Flevaris, Christina Chow, Stephen C-T Lam, Tatyana A Voyno-Yasenetskaya, Tohru Kozasa, Xiaoping Du (2010) G protein subunit Gα13 binds to integrin αIIbβ3 and mediates integrin "outside-in" signaling. *Science*. 327,596,3340-343., and Bo Shen, Xiaojuan Zhao, Kelly A O'Brien, Aleksandra Stojanovic-Terpo, M Keegan Delaney, Kyungho Kim, Jaehyung Cho, Stephen C-T Lam, Xiaoping Du (2013) A directional switch of integrin signalling and a new anti-thrombotic strategy. *Nature*. 503,7474,131-135)

1. Gα13 bind directly to integrin β1 and β3 cytoplasmic domains

To determine the if there is potential interaction between integrin β3 and Gα13 in platelets, the platelet integrin β3 subunit was coimmunoprecipitated by Gα13 antibody, but not with control immunoglobulin G (IgG) antibody, from platelet lysates (Fig. 3A). Conversely, Gα13 was coimmunoprecipitated by β3 antibody, but not with control immunoglobulin G (IgG) antibody (Fig. 3B). Interestingly, coimmunoprecipitation of β3 with Gα13 was enhanced by guanosine triphosphate γS (GTP-γS) or AlF4– (Fig. 3A). Thus, β3 is present in a complex with Gα13 in platelets, preferably the active GTP-bound Gα13. To determine whether Gα13 directly binds to the integrin cytoplasmic domain, we incubated purified recombinant Gα13{Kozasa, 1998} with agarose beads conjugated with glutathione S-transferase (GST) or a GST-β3 cytoplasmic domain

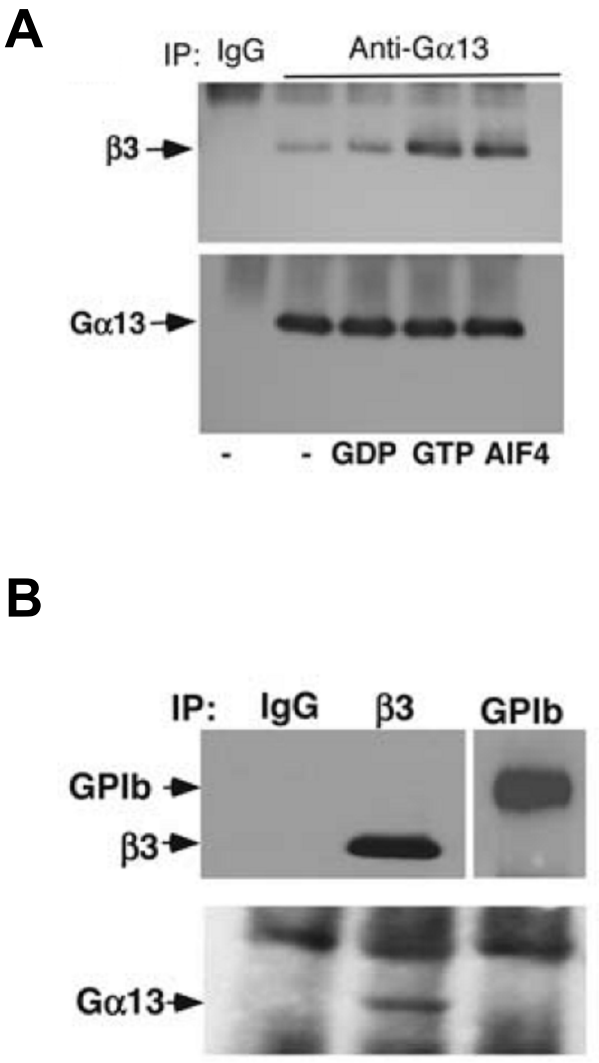

Fig. 3. Interaction of Gα13 to β3 in platelet lysates. (A) Proteins from platelet lysates were immunoprecipitated with control IgG or antibody to Gα13 with or without 1 mM GDP, 1 mM GTP-γS, or 30 mM $AlF4^-$. Immunoprecipitates were immunoblotted with antibody to Gα13 or β3 [monoclonal antibody 15 (mAb15)] (B) Proteins from platelet lysates were immunoprecipitated with control mouse IgG, antibody to αIIbβ3 [D57], or an antibody to the glycoprotein Ibα (GPIb). Immunoprecipitates were immunoblotted with antibodies to Gα13, β3, or GPIb.

fusion protein (GST-β3CD). Purified Gα13 bound to GST-β3CD, but not to GST (Fig. 4A). Purified Gα13 also bound to the β1 integrin cytoplasmic domain fused with GST (GST-β1CD) (Fig. 4B). The binding of Gα13 to GST-β3CD and GST-β1CD was detected with GDP-loaded Gα13, but enhanced by GTP-γS and $AlF4^-$ (Fig. 4A-B), indicating that the cytoplasmic domains of β3 and β1 can directly interact with G13 and that GTP enhances the interaction. The Gα13-β3 interaction was enhanced in platelets adherent to fibrinogen, and by thrombin, which stimulates GTP binding to Gα13 via GPCR (Fig. 4C). Hence, the interaction is regulated by both integrin occupancy and GPCR signaling.

2. Mutually exclusive binding of talin and Gα13 to β3

To identify the integrin β3 cytoplasmic domain sequence which is important in mediating Gα13 binding, co-immunoprecipitation experiments were performed using anti-β3 rabbit serum, using CHO cells expressing both the human αIIb and β3 integrins. of Gα13 with various β3 C-terminal truncation mutants suggests that Gα13 binding involves the β3 sequence between Lys 729 and Thr 741 (Fig. 5B), but not the kindlin- or c-Src-binding RGT sequences (Fig. 5A and 5B). The importance of the β3 sequence between Lys 729 and Thr 741 is further verified by similar co-immunoprecipitation experiments using an anti-Gα13 antibody, which immunoprecipitates both Gα13 and integrin β3 only in WT, 759 and 741 truncation mutant-expressing CHO cell lysates (Fig. 5C).

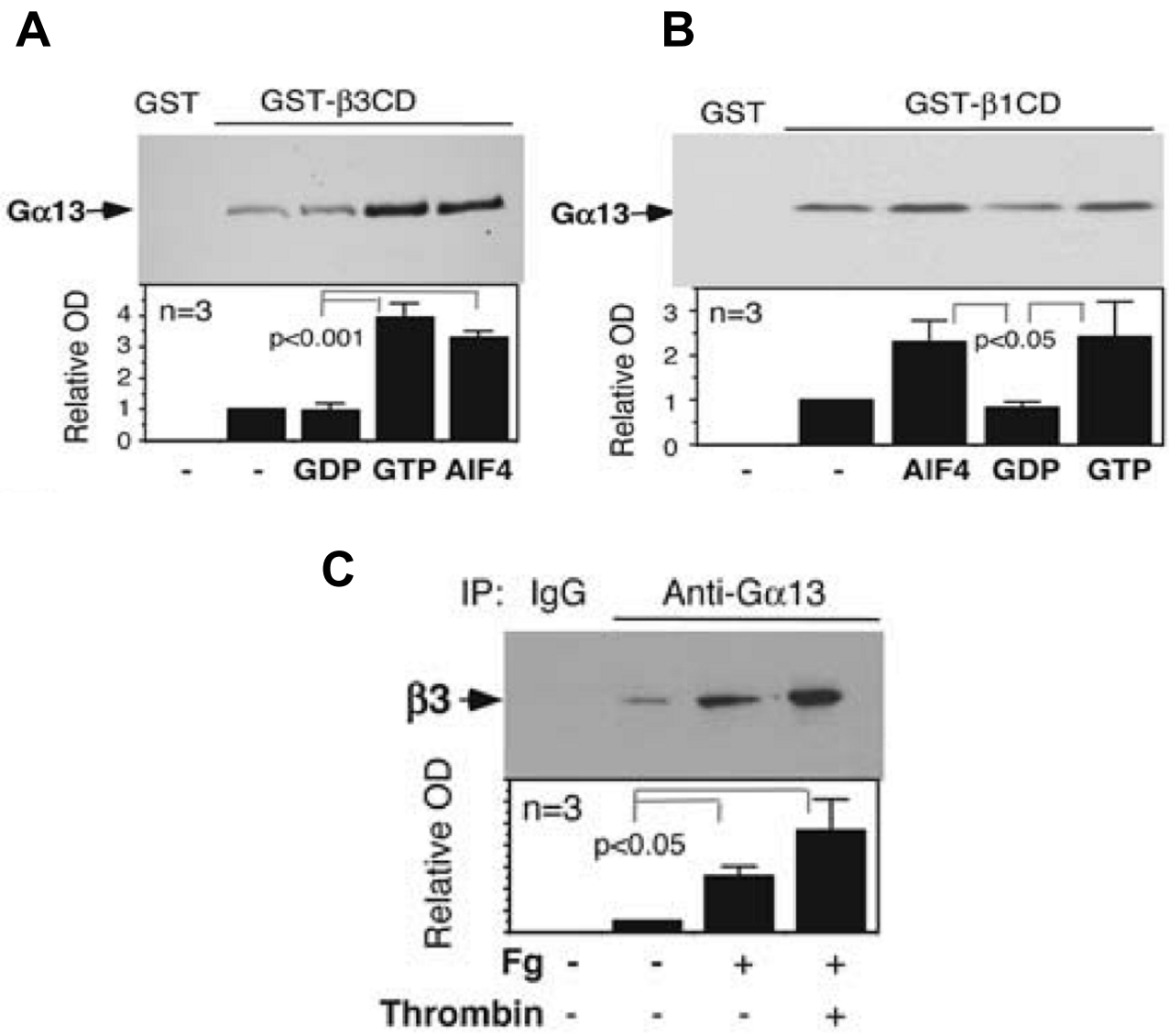

Fig. 4 Binding of Gα13 to β3 is dependent on GTP. (A and B) Purified GST-β3CD (A) or GST-β1CD (B) bound to glutathione beads was mixed with purified Gα13 with or without 1 mM GDP, 1 mM GTP-γS, or 30 mM AlF_4^-. Bound proteins were immunoblotted with antibody to Gα13. Quantitative data are shown as mean +/- SD and P value (t test). (C) Lysates of control platelets or platelets adherent to fibrinogen in the absence or presence of 0.025 U/ml thrombin were immunoprecipitated with antibody to Gα13 and then immunoblotted with mAb15. Quantitative data are shown as mean +/- SD and P value (t test).

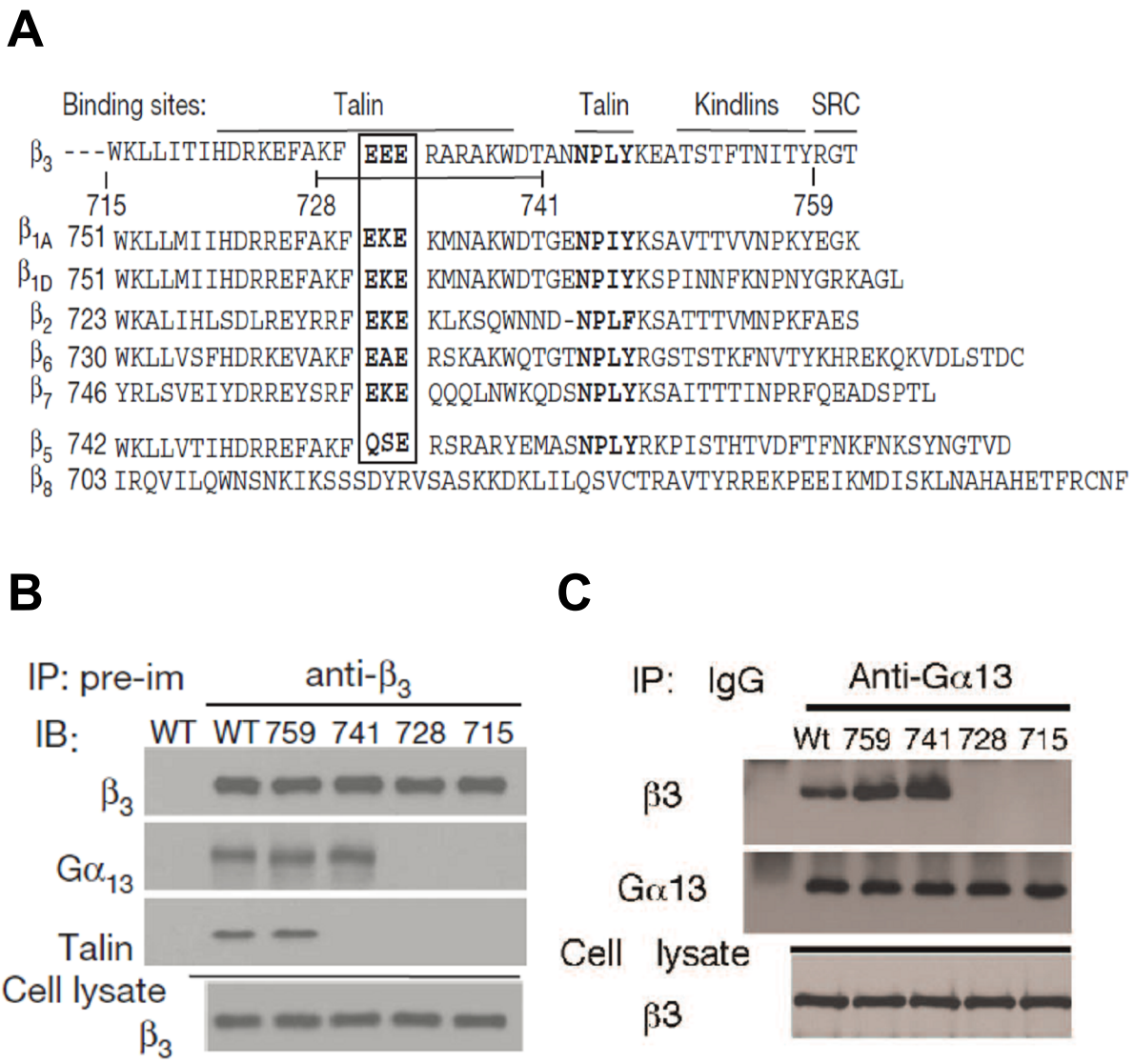

Fig. 5. Binding of Gα13 to β3 is dependent on β3 cytoplasmic domain amino acids 728 to 741. (A) The sequence of the human β3 cytoplasmic domain and its alignment with other β subunits, showing conserved EXE motifs and binding sites for talin, kindlins and SRC. (B) Coimmunoprecipitation of wild-type (WT) and truncatedmutant β3 with Gα13 and talin using anti-β3 or control pre-immune (pre-im) rabbit serum. Immunoprecipitates (IP) and CHO cell lysates (10% of that used in immunoprecipitation) were immunoblotted (IB) with indicated antibodies. (C) LCoimmunoprecipitation of wild-type (WT) and truncatedmutant β3 with Gα13 using an anti-Gα13 antibody or equal amount of control rabbit IgG. Immunoprecipitates and lysates (equivalent of 10% used for immunoprecipitation) were immunoblotted with anti-Gα13 and anti-β3 antibodies.

Alignment of different β integrin cytoplasmic domains reveals an EXE motif in this region, in which the first and third Glu residues are conserved among most β subunits, except for integrin β8 (Fig. 5A). The EXE motif- containing β1, β2 and β3 all bound Gα13, but not β8 (Fig. 6A, 6B). Thus, we hypothesized that the first and third Glu residues may be involved in the Gα13 binding. To test this hypothesis, coimmunoprecipitation experiment was performed with anti-integrin β3 serum, using CHO cells expressing wild-type and various E to A mutants of integrin β3. We show that wild-type and E732A mutant β3 coimmunoprecipitated with Gα13, but the E731A, E733A, AAA (E731–733A) (Fig. 7A, 7B), DED(E731D/E733D) and QSE (E731Q/E732S) (Fig. 7C) mutants did not, indicating that the first and third Glu residues within the EXE motif are important for Gα13 binding. Synthetic peptides containing the EEERA sequence inhibited Gα13–β3 interaction (see 3.5), verifying this EXE-motif-containing Gα13-binding site.

The cytoplasmic domain of integrin β3 is known to bind various intracellular proteins, including talin, kindlin, and Src family kinases (SFK). The EXE motif is located within a talin-binding region (Fig. 5A)(Patil *et al.*, 1999; Wegener *et al.*, 2007). Over-expression of the integrin-binding talin head domain (THD) in αIIbβ3-expressing cells inhibited Gα13 co-immunoprecipitation with β3 (Fig. 8A). Purified recombinant THD and Gα13 competed directly for binding to purified glutathione S-transferase (GST)–β3 cytoplasmic domain fusion protein (GST–β3CD) (Fig. 8B, 8C), indicating that Gα13 and talin are mutually exclusive in binding to β3.

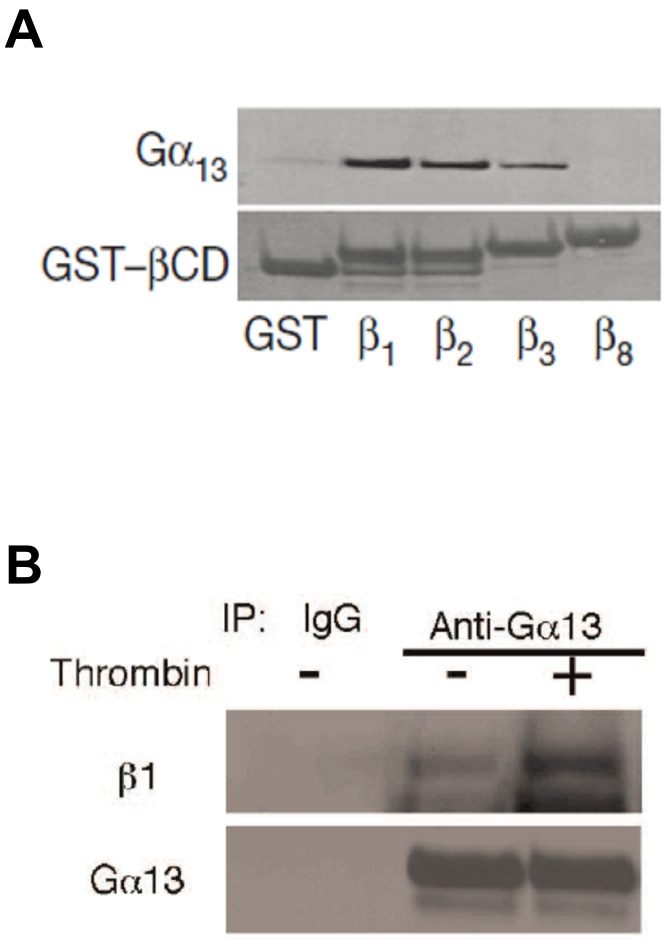

Fig. 6. Binding of Gα13 to β3 is conserved in ExE motif-containing β integrins. (A) Binding of purified recombinant Gα13 to glutathione-bead-bound GST, and cytoplasmic domain fusion proteins GST-β1CD, GST-β2CD, GST-β3CD or GST-β8CD. (B) Lysates from human platelets (with or without stimulation with 0.025U/ml thrombin) were immunoprecipitated with anti-Gα13 antibody or equal amount of control rabbit IgG. Immunoprecipitates were immunoblotted with anti-Gα13 and anti-β1 antibodies. Gα13 is associated with β1, which is increased after thrombin stimulation.

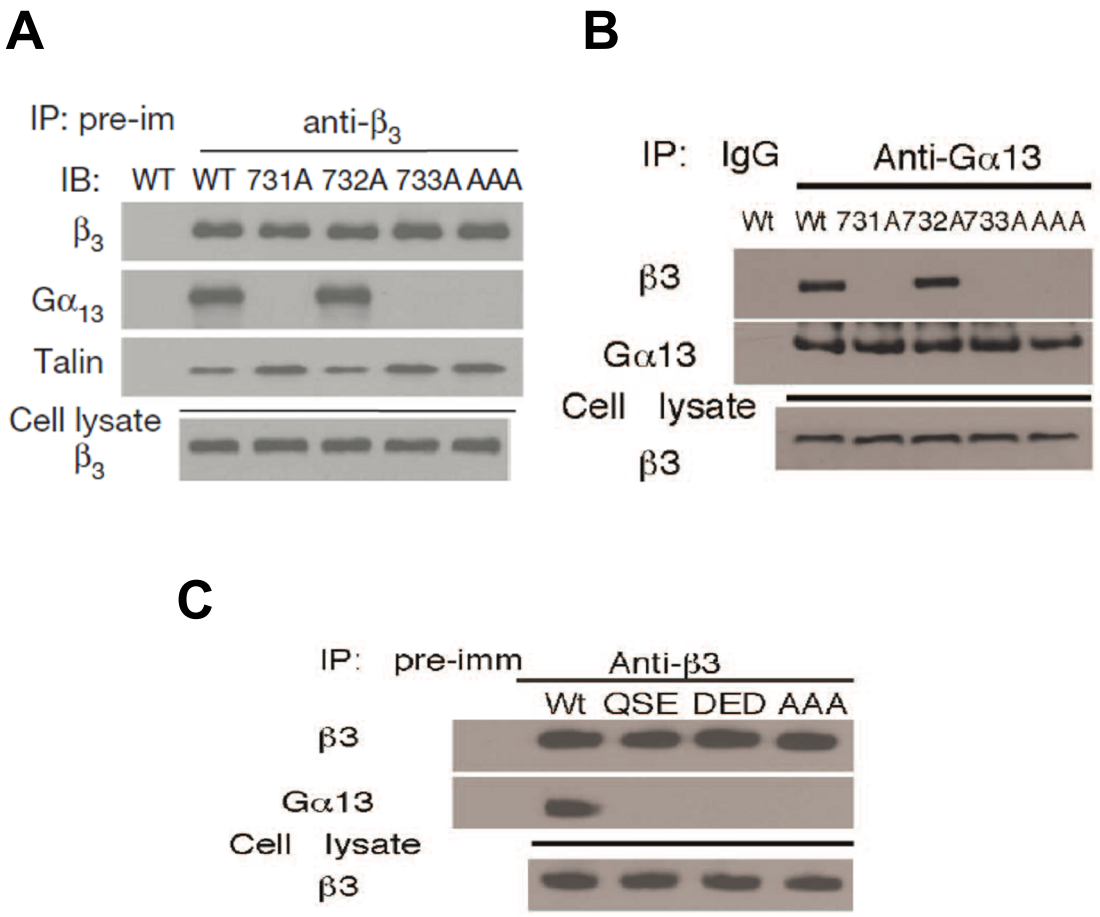

Fig. 7. Binding of Gα13 to β integrins is dependent on the conserved ExE motif. (A) Coimmunoprecipitation of CHO-cell-expressed wild type or EXE motif-mutated β3 with Gα13 and talin using anti-β3 or pre-immune rabbit serum. (B) Coimmunoprecipitation of CHO-cell-expressed wild type or EXE motif-mutated β3 with Gα13 using an anti-Ga13 antibody or equal amount of control rabbit IgG. (A and B) Immunoprecipitates and lysates (equivalent of 10% used for immunoprecipitation) were immunoblotted with anti-Gα13, anti-talin and anti-β3 antibodies. (C) In addition to the AAA mutation, conserved mutations of EEE to DED and EEE to QSE (as found in β5) were introduced to the β3 cytoplasmic domain. These mutants were co-transfected with wild-type aIIb into CHO cells, which were sorted to achieve comparable expression levels with wild-type-αIIbβ3- expressing cells. Lysates from these cells were immunoprecipitated with anti-β3 or equal amount of pre-immune rabbit serum. Lysates (10%) and immunoprecipitates were immunoblotted with anti-Gα13 or anti-β3.

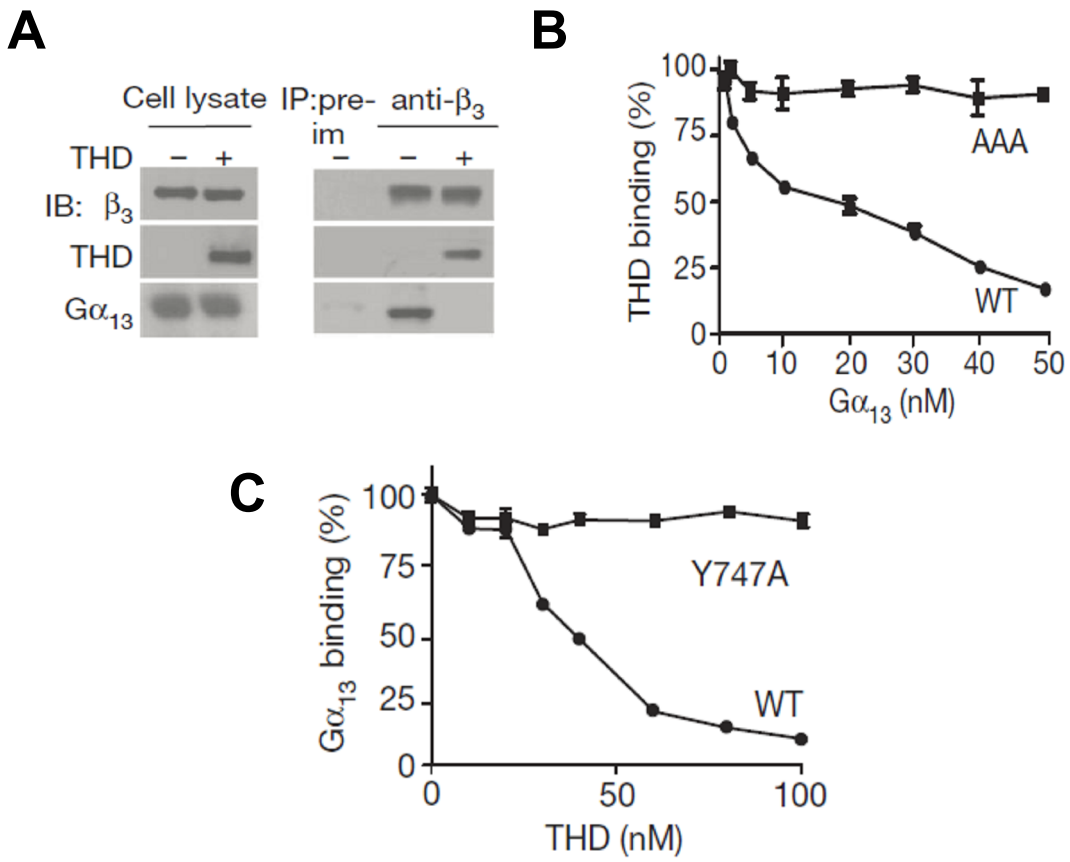

Fig. 8. Mutually exclusive binding of Gα13 and talin to β3 integrin cytoplasmic domain. (A) Coimmunoprecipitation of CHO-cell-expressed integrin αIIbβ3 with Gα13 and THD after transfection with cDNA encoding THD. (B, C) Inhibition of the binding of THD (20nM) (B) or Gα13 (40 nM) (C) to immobilized GST-β3CD proteins (wild-type and negative control mutants) by increasing concentrations of Gα13 (B) or THD (C). Bound Gα13 or THD was detected using anti-Gα13 or anti-talin. Error bars represent mean +/- s.d.

3. Dynamics of talin and Gα13 binding to β3 and the role of talin in integrin signaling.

Interestingly, the binding of talin and Gα13 is regulated temporally during integrin signaling (Figure 7). The first wave of talin association with αIIbβ3 occurred after thrombin-stimulated inside-out signaling (Fig. 9A, 9B) and before the onset of integrin ligation (as indicated by platelet aggregation (Fig. 9C). However, after integrin ligation, talin association with αIIbβ3 was diminished (Fig. 9A, 9B). The second wave of talin–β3 association occurred after full platelet aggregation (Fig. 9A-C), the timing of which correlates with clot retraction. Opposite to the waves of talin binding, the Gα13–β3 association was even lower than the basal level during inside-out signaling when the first talin binding wave occurred (Fig. 9A, 9B), but peaked after integrin ligation when the first talin-binding wave subsided, and then decreased again during the second talin-binding wave (Fig. 9A, 9B). Thus, inside-out and various phases of outside-in signaling are associated with coordinated and opposing waves of Gα13 and talin binding to β3.

Importantly, an increase in Gα13 binding to integrin can only be induced when integrin is activated in the presence of fibrinogen, but not by integrin activation alone (Fig. 10A). Conversely, the integrin inhibitors RGDS (Arg-Gly-Asp-Ser, Fig. 9A, 9B) or EDTA (ethylenediaminetetraacetic acid, Fig. 10B, 10C) prevented dissociation of talin from β3 and inhibited Gα13–β3 interaction in thrombin-stimulated platelets. Thus, the switch from a talin-bound to a Gα13-bound state of αIIbβ3 is initiated by the binding of macromolecular ligands.

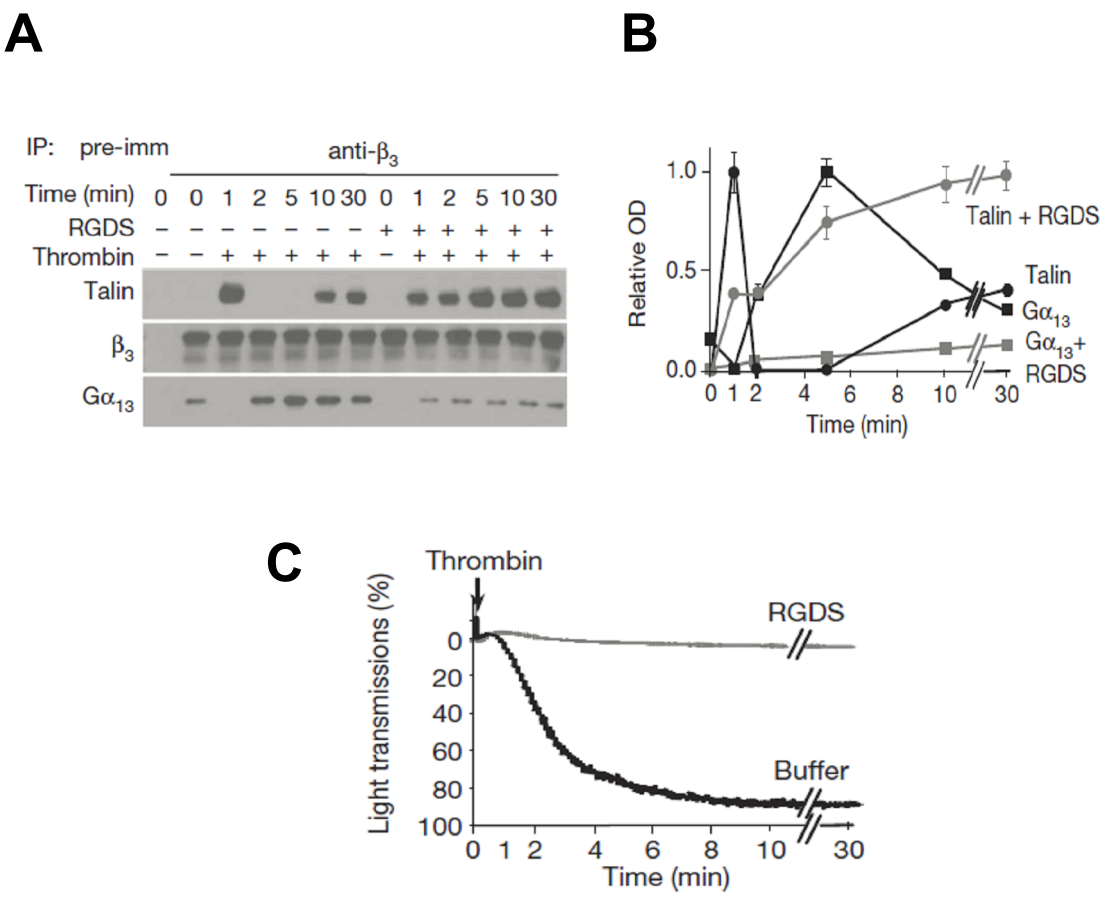

Fig. 9. Dynamics of talin and Gα13 binding to β3 (A-C) Human platelets were stimulated with 0.025 U/ml α-thrombin (in an aggregometer) with or without 2mM integrin inhibitor RGDS, solubilized at various time points, immunoprecipitated with anti-β3 or pre-immune rabbit serum, and immunoblotted for Gα13, talin and β3. (A) Typical immunoblots. (B) Quantification of immunoblots (mean +/- s.d., three experiments). OD, optical density. (C) Turbidity changes indicating integrin-dependent platelet aggregation.

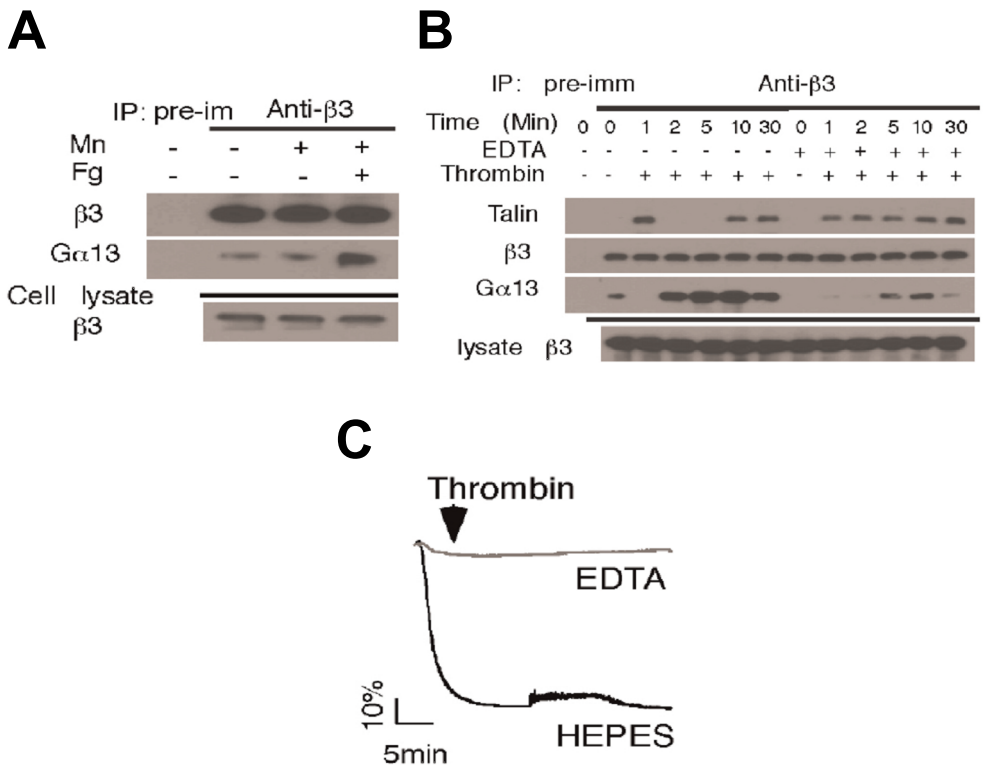

Fig. 10. Ligand occupancy induces switch of integrin αIIbβ3 from the talin-bound to the Gα13-bound state. (A) To determine the effect of integrin activation and ligand occupancy on Gα13-β3 association, human platelets were incubated with or without 1mM $MnCl_2$ and 30 μg/ml fibrinogen for 5 min at 22 degrees. Platelet lysates were then immunoprecipitated with anti-β3 or pre-immune rabbit serum. Lysates (10%) and immunoprecipitates were immunoblotted with anti-β3 or anti-Gα13. (B, C) Washed human platelets were stimulated with 0.025U/ml α-thrombin with or without adding 2mM EDTA (an inhibitor of the ligand binding function of integrins), stirred (1,000 r.p.m.) at 37 degrees, solubilized at various time points, and immunoprecipitated with anti-β3 or equal amounts of pre-immune rabbit serum. Lysates (10%) and immunoprecipitates were immunoblotted with anti-Gα13, anti-talin or anti-β3 antibodies. (B) Western blot results. (C) Turbidity changes in platelet suspension indicating integrin-dependent platelet aggregation. Note the inhibitory effect of EDTA on talin dissociation and Gα13 binding to β3.

4. The selective role of Gα13 EXE binding in integrin outside-in signaling

The opposing waves of talin and Gα13 binding to β3 suggest that the interaction of these two proteins with β3 selectively mediates inside-out and outside-in signaling, respectively. This hypothesis was tested using talin knockout{Petrich, 2007} and shRNA-induced talin knockdown platelets, which are defective in adenosine diphosphate (ADP)/fibrinogen-induced, integrin-dependent aggregation(Fig. 11A, 11B and Fig. 12A, 12B). Their defective aggregation was fully corrected with manganese or an integrin-activating antibody (LIBS6) (Fig. 11B and Fig. 12B), which activate integrins independently of inside-out signaling. These data confirm a role for talin in inside-out signaling(Tadokoro *et al.*, 2003; Petrich *et al.*, 2007; Wegener *et al.*, 2007). It is established that inside-out signaling is not the only pathway of αIIbβ3 activation. Integrin–fibrinogen interaction may occur independently of inside-out signaling when fibrinogen changes conformation, either by immobilization or conversion to fibrin(Coller, 1980; Ugarova *et al.*, 1993). This is because the initial contact of the exposed ligand recognition sequence, RGD, with resting integrins triggers ligand-induced integrin activation(Ugarova *et al.*, 1993). Interestingly, adhesion of resting talin-knockout or -knockdown platelets to immobilized fibrinogen was defective (Fig. 11C and Fig. 12C), indicating the importance of talin in platelet adhesion to immobilized fibrinogen in the absence of inside-out signaling. However, addition of manganese or integrin-activating antibody fully corrected talin-knockout and -knockdown platelet adhesion and spreading (and also the spreading of talin-binding-defective mutant β3-expressing Chinese hamster ovary (CHO) cells(Arias-Salgado *et al.*, 2005b)) on immobilized fibrinogen (Fig. 11D and Fig. 12D). Thus, the role of talin in resting platelet adhesion to fibrinogen is

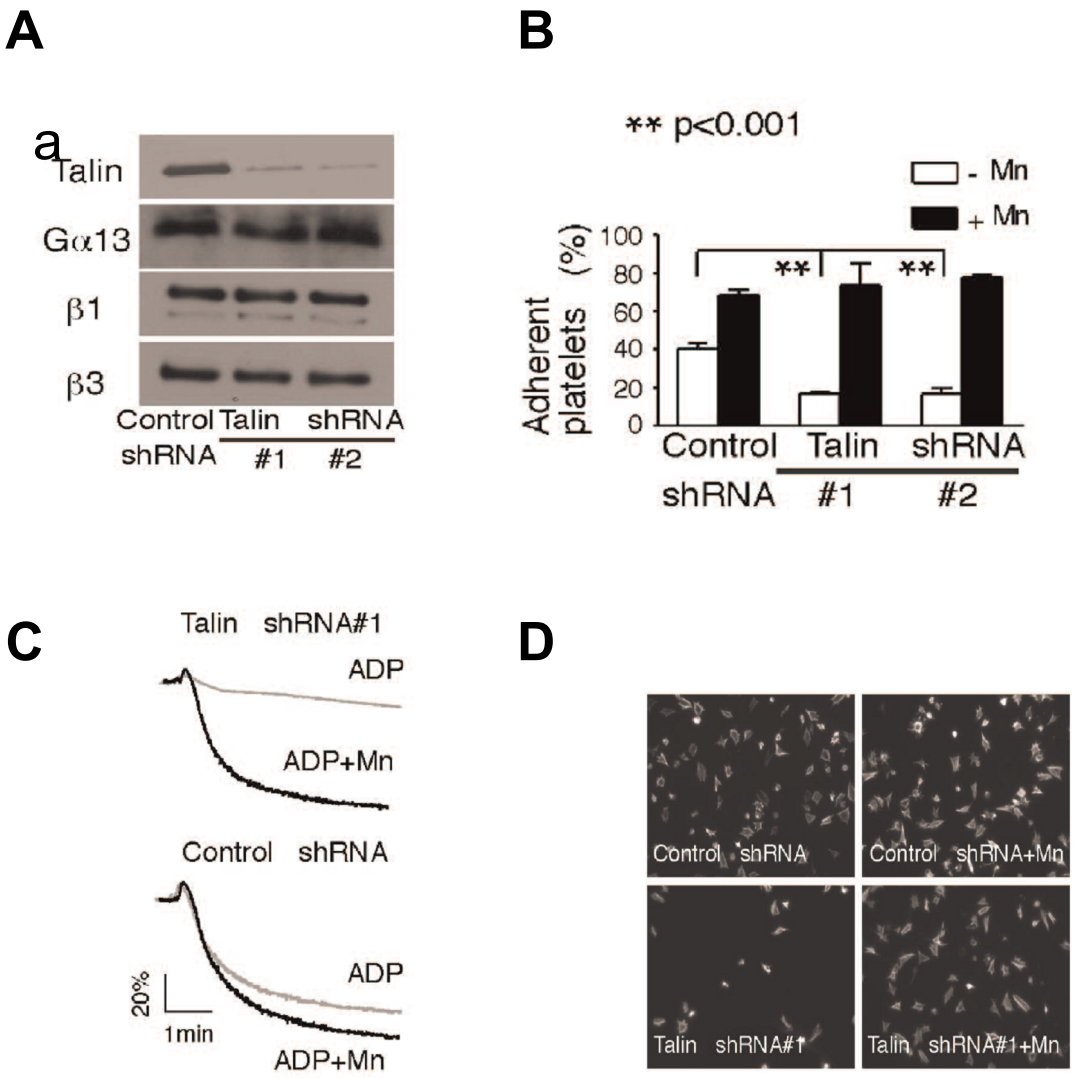

Fig. 11. Effects of shRNA-induced talin knockdown on integrin signaling. (A) Western blot comparison of talin1 expression levels in mouse platelets derived from control shRNA- or talin shRNA- transfected bone marrow stem cells. Western blots of Gα13, and integrin β1 and β3 are also shown. (B) Adhesion of unstimulated mouse platelets to immobilized fibrinogen for 1 h. Adherent platelets were quantified as percentage of total platelets loaded (mean +/- s.d., n=4). (C) Turbidity changes in mouse platelet suspension stimulated with 5 μM ADP in the presence of 20 μg/ml fibrinogen, with or without 1mM $MnCl_2$, as detected using an aggregometer. (D) Fluorescence microscopy images of phalloidin-stained mouse platelet spreading on fibrinogen for 1 h, with or without 1 mM $MnCl_2$.

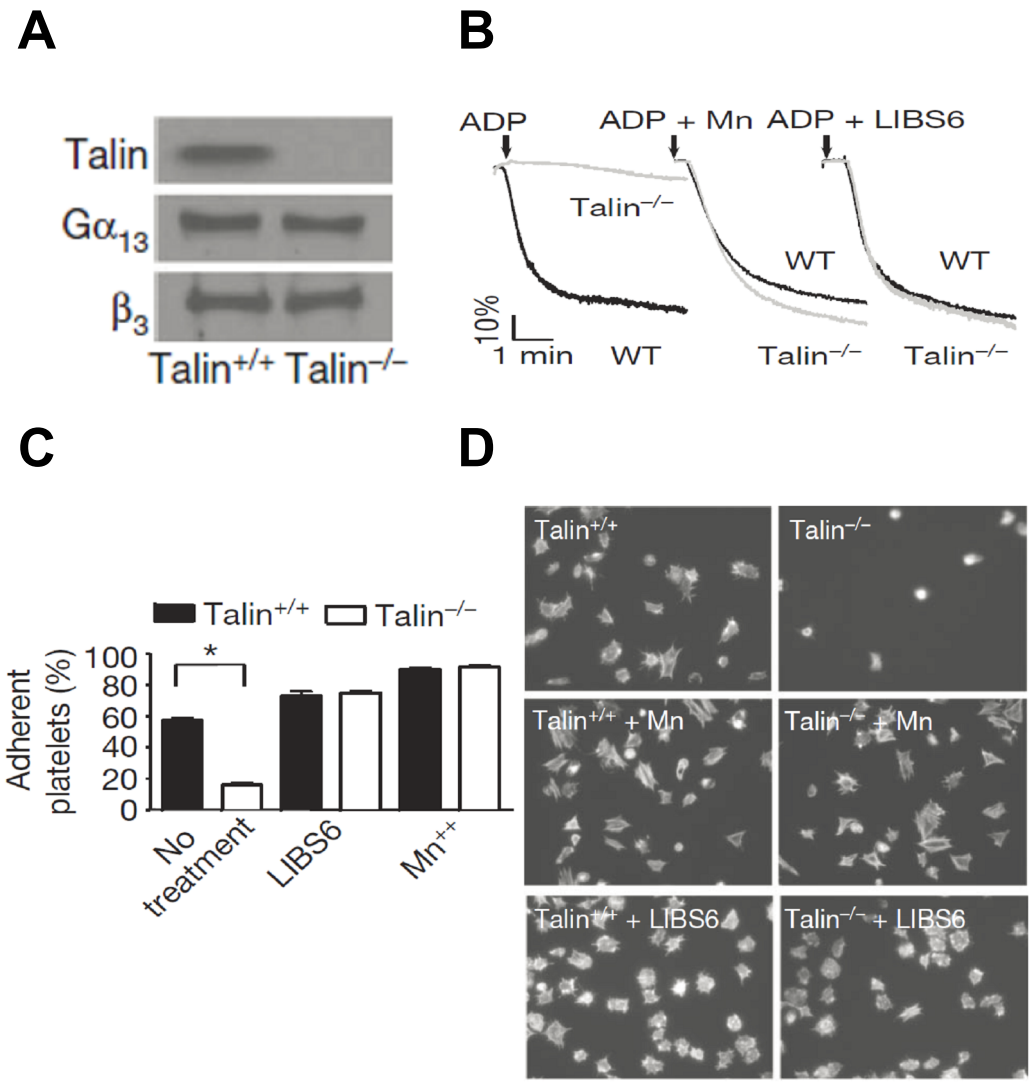

Fig. 12. Effects of shRNA-induced talin knockout on integrin signaling. (A) Immunoblotting of talin1 in wild-type and Tln1$^{-/-}$ (talin1$^{-/-}$) mouse platelets. (B) Aggregation of wild-type and talin1$^{-/-}$ platelets stimulated with 5 μM ADP in the presence of 20 μg/ml fibrinogen, with or without 1mM $MnCl_2$ or 0.3 μg/ml LIBS6. (C) Adhesion of unstimulatedmouse platelets to immobilized fibrinogen for 1 h, with or without 1mM $MnCl_2$ or 0.18 μg/ml LIBS6 (quantified as percentage of loaded platelets, mean +/- s.d., n=4, *P<0.001). (D) Images of phalloidin-stained mouse platelets spreading on fibrinogen for 1 h, with or without 1mM $MnCl_2$ or 0.18 μg/ml LIBS6.

solely due to its importance in ligand-induced integrin activation. Because cell spreading requires the early phase of outside-in signaling, these data further demonstrate that talin is not required for the early phase of outside-in signaling leading to cell spreading once its role in integrin activation is bypassed.

5. A new anti-thrombotic that does not cause bleeding

To assess whether Gα13 binding to the EXE motif selectively mediates outside-in signaling without perturbing talin-dependent integrin function, wild-type and AAA mutant β3-transfected ITGB3 $(\beta3)^{-/-}$ bone marrow stem cells (from $\beta3^{-/-}$ mice) were transplanted into irradiated β3-/- mice. The platelets from the recipient mice expressed similar levels of wild-type or AAA mutant β3 (Fig. 13A). The AAA mutation inhibited β3 interaction with Gα13, but not talin (Fig. 13B), during integrin signaling. The AAA mutation also had no effect on agonist induced soluble fibrinogen binding (Fig. 13C). Thus, the EXE motif is not required for talin-dependent inside-out signaling. By contrast, the AAA mutant β3-expressing platelets were defective in spreading on immobilized fibrinogen (Fig. 13D). Thus, Gα13-binding deficiency in β3 causes a selective defect in integrin outside-in signaling and platelet spreading.

Similarly, AAA and more conserved DED or QSE β3 mutants expressed in CHO cells, all were defective in Gα13 binding (Fig. 7C), and were also defective in spreading on fibrinogen (Fig. 14).

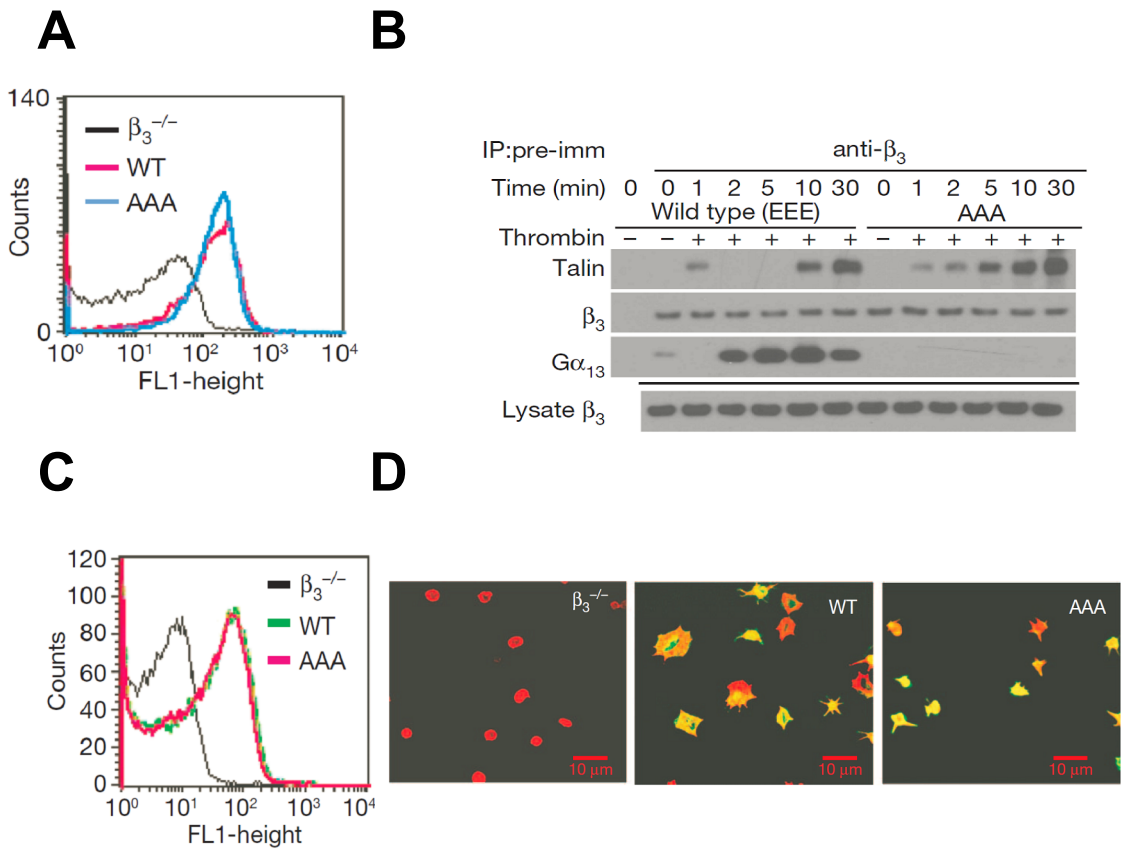

Fig. 13. The selective role of Gα13 EXE binding in platelet αIIbβ3 outside-in signaling. (A) Flow cytometric analysis of β3 expression in platelets from $\beta3^{-/-}$ mice transplanted with wild-type or AAA mutant β3-transfected bone marrow stem cells. $\beta3^{-/-}$ platelets served as negative control. Picture shows relative fluorescence (FL1) on the x axis and number of events (count) on the y axis. (B) Mouse platelets expressing wild-type (EEE) or AAA mutant β3 were stimulated with 0.025 U/ml thrombin, solubilized at various time points, immunoprecipitated with anti-β3 or pre-immune rabbit serum and immunoblotted for Gα13, talin and β3. (C) PAR4 agonist peptide (PAR4-AP)- induced binding of Oregon Green-labelled fibrinogen to wild-type or AAA mutant αIIbβ3- expressing platelets with $\beta3^{-/-}$ platelets as a negative control. (D) Confocal images of $\beta3^{-/-}$ platelets and $\beta3^{-/-}$ platelets expressing wild-type or AAA-mutant β3 spreading on fibrinogen and surface area quantification (mean +/- s.e.m.). Merged anti-β3 (green) and Alexa Fluor 546- conjugated phalloidin (red) fluorescence.

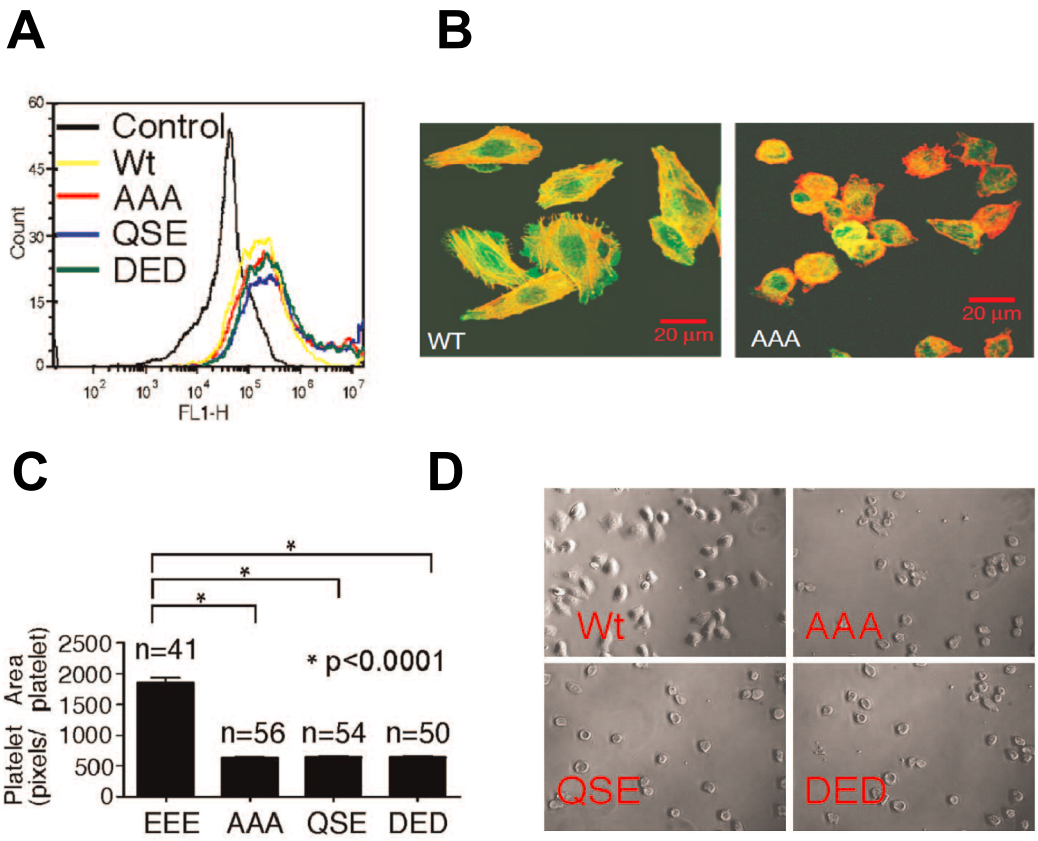

Fig. 14. Effects of mutational disruption of the EXE motif on integrin outside-in signaling. (A) Expression levels of wild-type or the EXE motif (QSE, DED or AAA) mutants of β3 in complex with wild-type αIIb in CHO cells, as determined by flow cytometry. Mouse IgG was used as a negative control. (B) Confocal images of phalloidin (red)/ anti-β3 (green)-double stained wild-type and AAA-mutant αIIbβ3- expressing CHO cell spreading on fibrinogen (45 min). (C, D) Spreading of CHO cells expressing wild-type αIIbβ3, and QSE, DED or AAA mutant αIIbβ3 on fibrinogen for 1 h. (C) Quantification of surface areas of individual cells (mean +/- s.e.m.). (D) Typical microscopy images.

However, AAA mutant β3 expressed in CHO cells had no negative effect on THD binding, in contrast to the Y747A mutant (Fig. 15). In addition, AAA-expressing cells showed defects in integrin-dependent activation of SRC (as shown by phosphorylation at Tyr 416) and transient inhibition of RHOA during cell spreading (Fig. 16), both of which are important elements of outside-in signaling. Together with previous studies that identified β3 sequences mediating talin binding (Fig. 5A)(Tadokoro *et al.*, 2003; Petrich *et al.*, 2007; Wegener *et al.*, 2007; Goksoy *et al.*, 2008), our data suggest that talin and Gα13 dynamically interact with distinct recognition sequences in the same region of β3 to serve as a molecular switch controlling the direction of integrin signaling.

The specific role of the EXE motif in outside-in signaling prompted us to design selective inhibitors of outside-in signaling. We synthesized several myristoylated (Myr) EXE-motif-containing β3 peptides: mP5 (Myr-EEERA), mP6(Myr-FEEERA) and mP13(Myr-KFEEERARAKWDT). These peptides inhibited co-immunoprecipitation between Gα13 and β3 (Fig. 17A), indicating that the minimal sequence of EEERA is sufficient to inhibit Gα13 binding. By contrast, only mP13, but not mP6 (or mP5), inhibited talin association with β3 (Fig. 17A), indicating that mP6 does not interact with talin. mP6 inhibited platelet spreading on fibrinogen (Fig. 17B), but had no effect on either agonist-induced fibrinogen/PAC1 (an ligand-mimetic antibody recognizing activated αIIbβ3) binding to platelets (Fig. 18) or platelet adhesion to immobilized fibrinogen (Fig. 19A). Interestingly, mP6 did not inhibit, but rather accelerated, platelet-dependent clot retraction, possibly due to the enhanced talin binding (Fig. 17A, 19B). These data indicate that the EXE-based inhibitor mP6 selectively inhibits the early phase of outside-in signaling without affecting talin-dependent inside-out signaling,

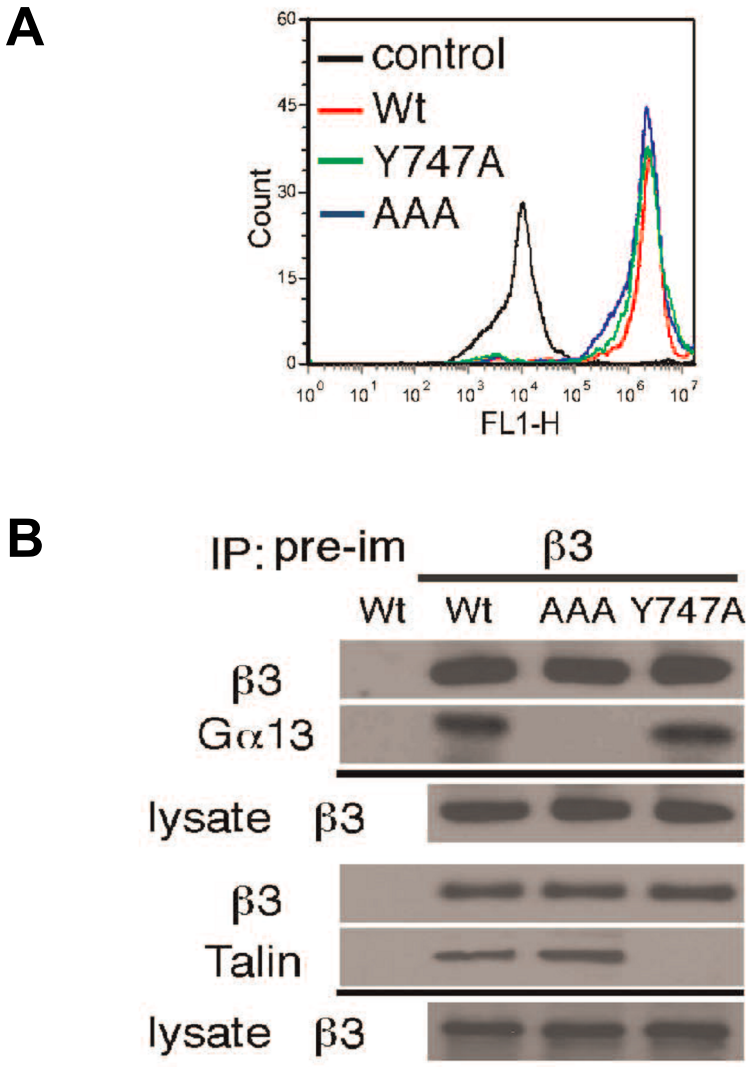

Fig. 15. Effects of mutational disruption of the EXE motif on talin binding to integrin β3. (A) Flow cytometric analysis of wild-type αIIbβ3, AAA or Y747A mutant αIIbβ3 expression in CHO cells. Mouse IgG was used as a control. (B) CHO cells expressing wild-type, AAA or Y747A β3 without (top panels) or with (bottom panels) co-expression of recombinant THD were solubilized and immunoprecipitated with anti-β3 or pre-immune serum. 10% lysates and immunoprecipitates were immunoblotted with anti-talin, anti-Gα13 or anti-β3 antibodies.

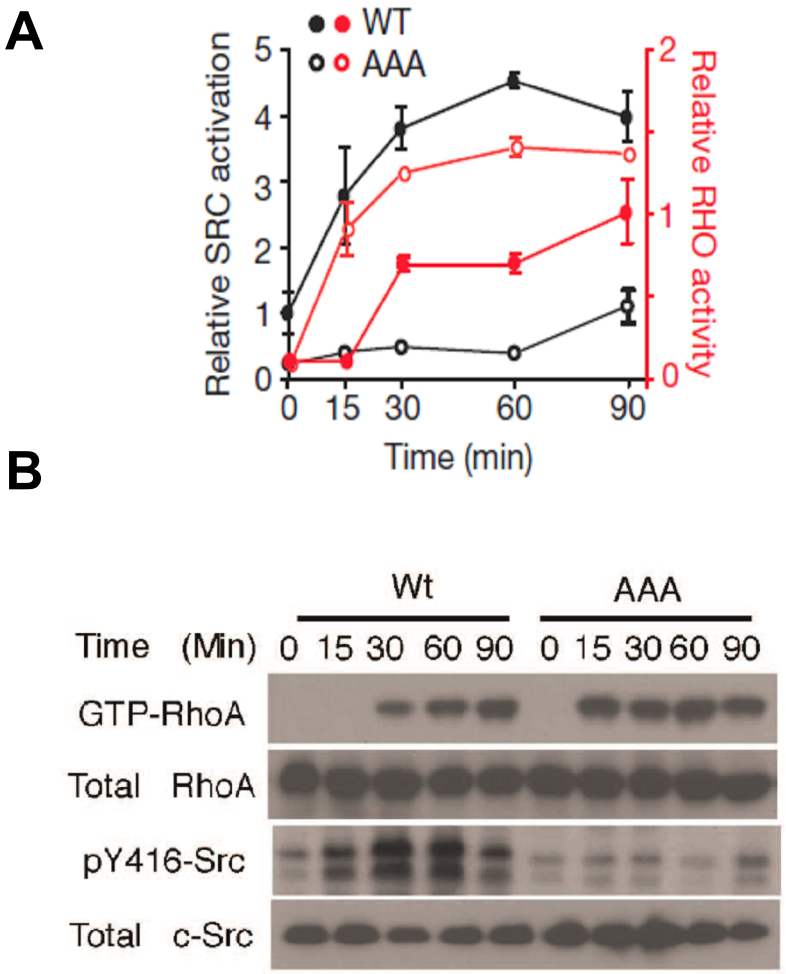

Fig. 16. Gα13 binding mediates SRC activation and transient RhoA inhibition. (A) Wild-type or AAA mutant αIIbβ3-expressing CHO-1b9 cells were allowed to adhere to immobilized fibrinogen, solubilized at various time points, and analysed for RHOA activation and SRC Tyr 416 phosphorylation (mean +/- s.d, n=3). (B) Typical western blots for (A).

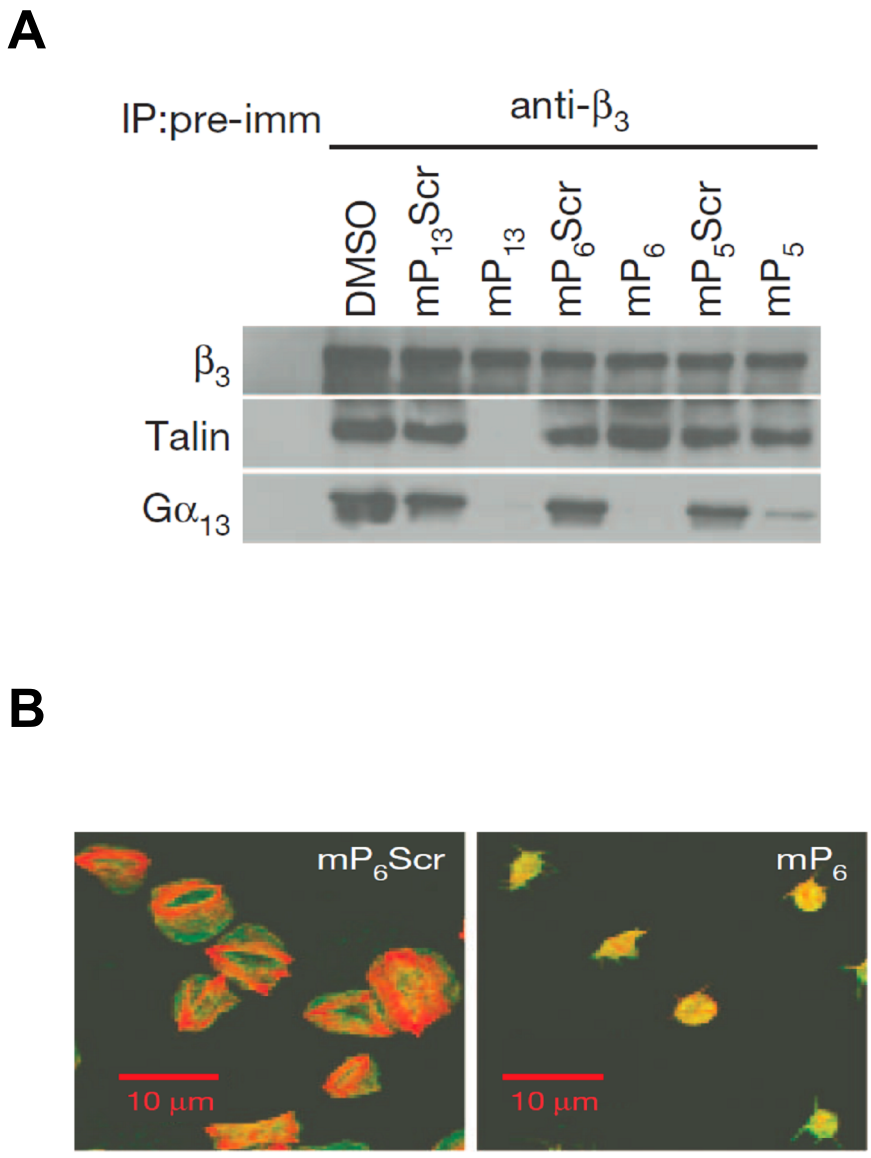

Fig. 17. EXE motif-based inhibitors inhibit Gα13 interaction with integrin β3. (A) The effects of 500μM mP13, mP6 or mP5 on co-immunoprecipitation of β3 with Gα13 or talin in thrombin-stimulated platelets in comparison with scrambled (Scr) controls. (b) Confocal images of phalloidin (red)/anti-β3 (green)-double-stained human platelets treated with 100μM mP6 or mP6Scr spreading on immobilized fibrinogen (1 h).

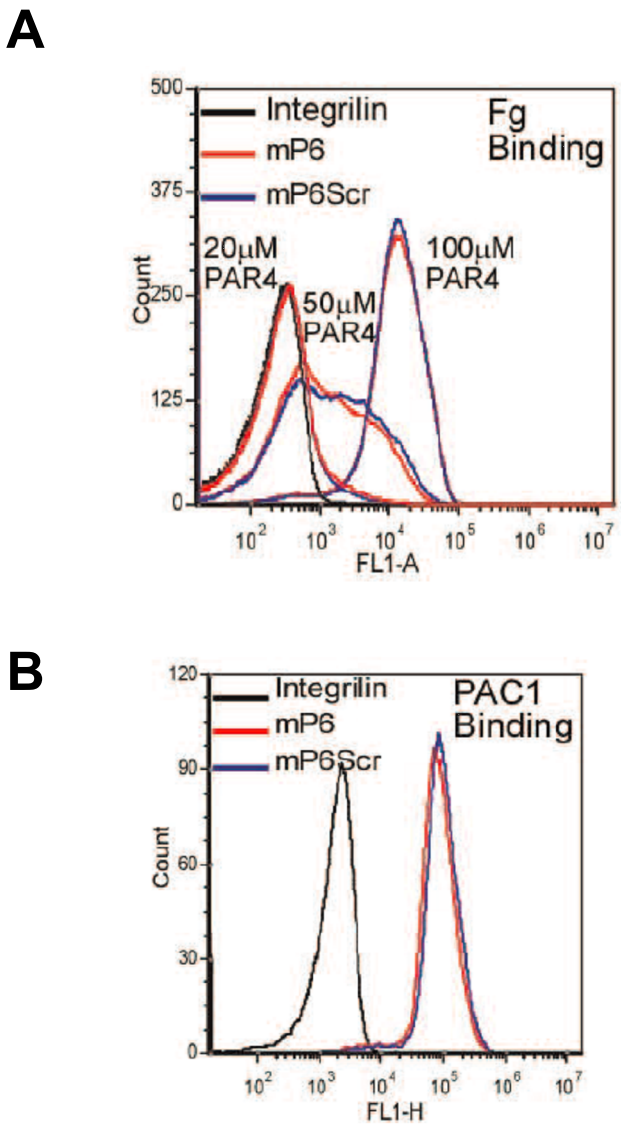

Fig. 18. EXE motif-based inhibitor mP6 does not affect ligand binding function of integrin β3. (A) Flow cytometric analysis of PAR4-AP-induced Oregon Green-labelled soluble fibrinogen binding to human platelets pre-treated with 100μM mP6Scr or 100μM mP6 stimulated with increasing concentrations of PAR4-AP. Integrilin-treated platelets were used as a negative control. (B) Flow cytometric analysis of 100μM PAR4-AP-induced PAC1 binding to human platelets pretreated with 100μM mP6Scr or mP6. Integrilin-treated platelets were used as negative control.

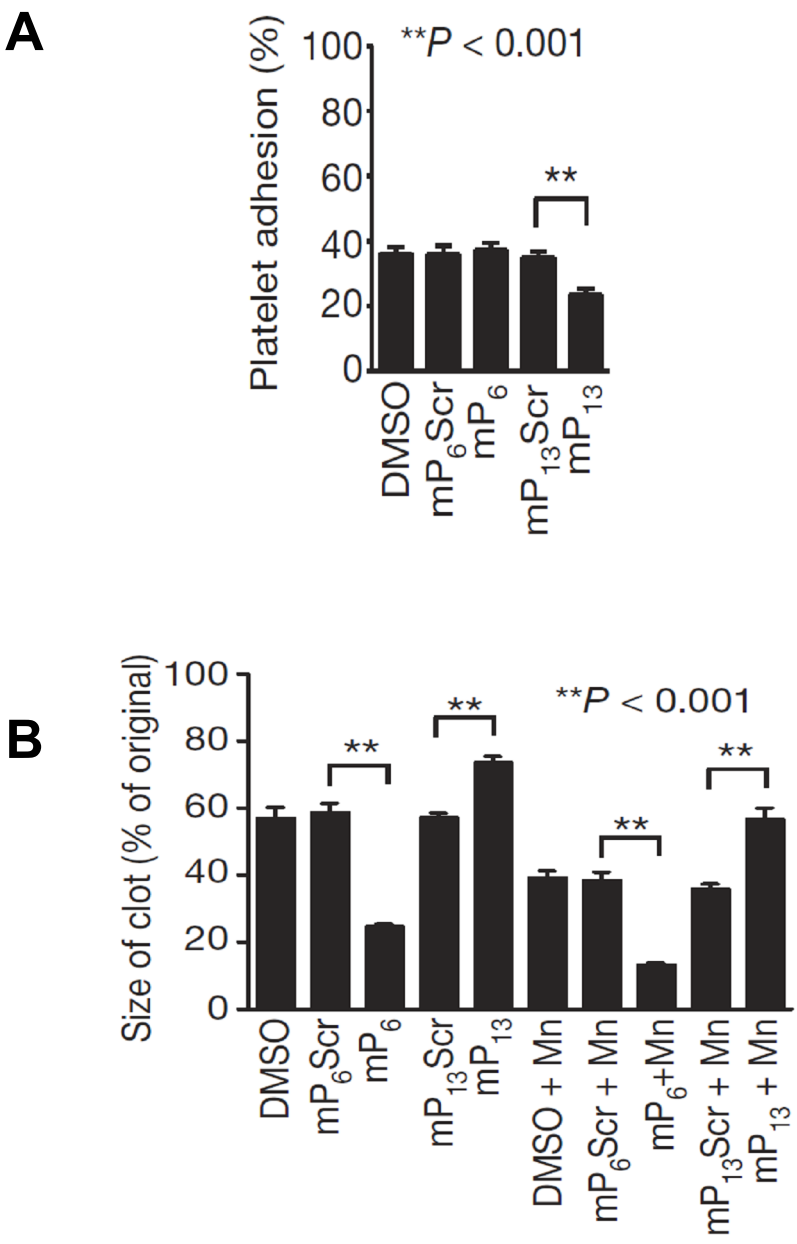

Fig. 19. EXE motif-based inhibitor mP6 inhibits platelet adhesion but accelerates clot retraction. (A) Effect of mP6 and mP13 (250 μM) on resting platelet adhesion to immobilized fibrinogen as compared with scrambled peptides (mean +/- s.d., n=4). (B) Effect of mP6 or mP13 (250 μM) on clot retraction of human platelet-rich plasma, with or without 1mM manganese (Mn) (mean +/- s.d., n=3).

ligand-induced integrin activation, or the late phase of outside-in signaling associated with the second wave of talin binding. By contrast, mP13 inhibited inside-out and outside-in signaling, as it inhibited fibrinogen binding (Fig. 20A), platelet adhesion (Fig. 19A) and clot retraction (Fig. 19B) (not reversed by manganese, as previously shown using talin-/- platelets(Haling *et al.*, 2011)). Thus, mP6 selectively interferes with the early phase of outside-in signaling, but mP13 affects all phases of integrin signaling.

Importantly, mP6 inhibited the second wave of thrombin-induced platelet aggregation *in vitro* (Fig. 21A), and when injected into mice as micelles, was as potent as the currently used integrin antagonist Integrilin in inhibiting laser-induced arteriolar thrombosis (Fig. 21B) and FeCl3-induced occlusive carotid artery thrombosis (Fig. 22). Notably, at the concentration at which both Integrilin and mP6 similarly inhibited occlusive thrombosis, Integrilin considerably prolonged tail bleeding and increased blood loss, whereas mP6 had no such adverse effect (Fig. 23). Thus, we have discovered a novel anti-thrombotic that prevents thrombosis without causing bleeding.

6. Discussion

Together, our study provides a conceptual advance by revealing a molecular switch controlling the directions and consequences of integrin signaling. We show that the switch between inside-out and outside-in signaling is mediated by coordinated but opposing waves of talin and Gα13 binding to distinct yet adjacent sequences within the β3 cytoplasmic domain. The discovery of this signaling switch forms a conceptual basis for selectively inhibiting outside-in signaling without perturbing the ligand-binding

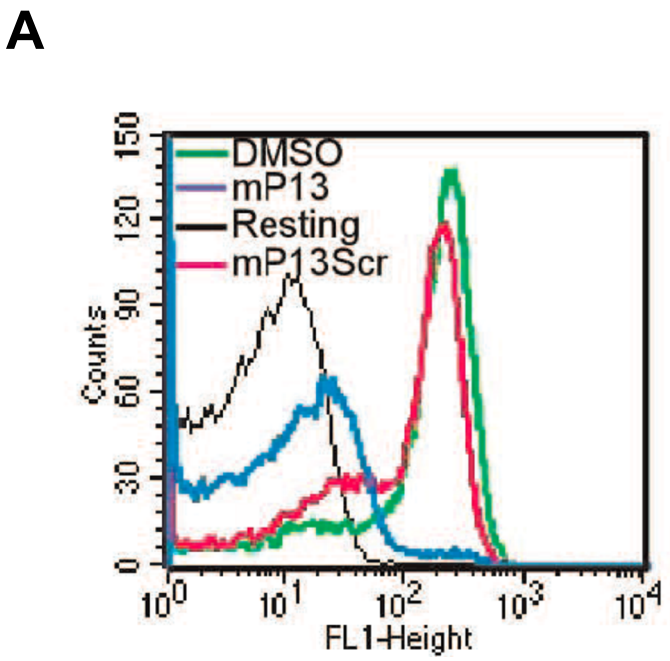

Fig. 20. EXE motif-based inhibitor mP13 inhibits ligand binding function of integrin β3. (A) Flow cytometric analysis of PAR4-AP-induced Oregon Green-labelled soluble fibrinogen binding to human platelets pre-treated with solvent DMSO, mP13Scr or mP13. Resting platelets were used as a negative control.

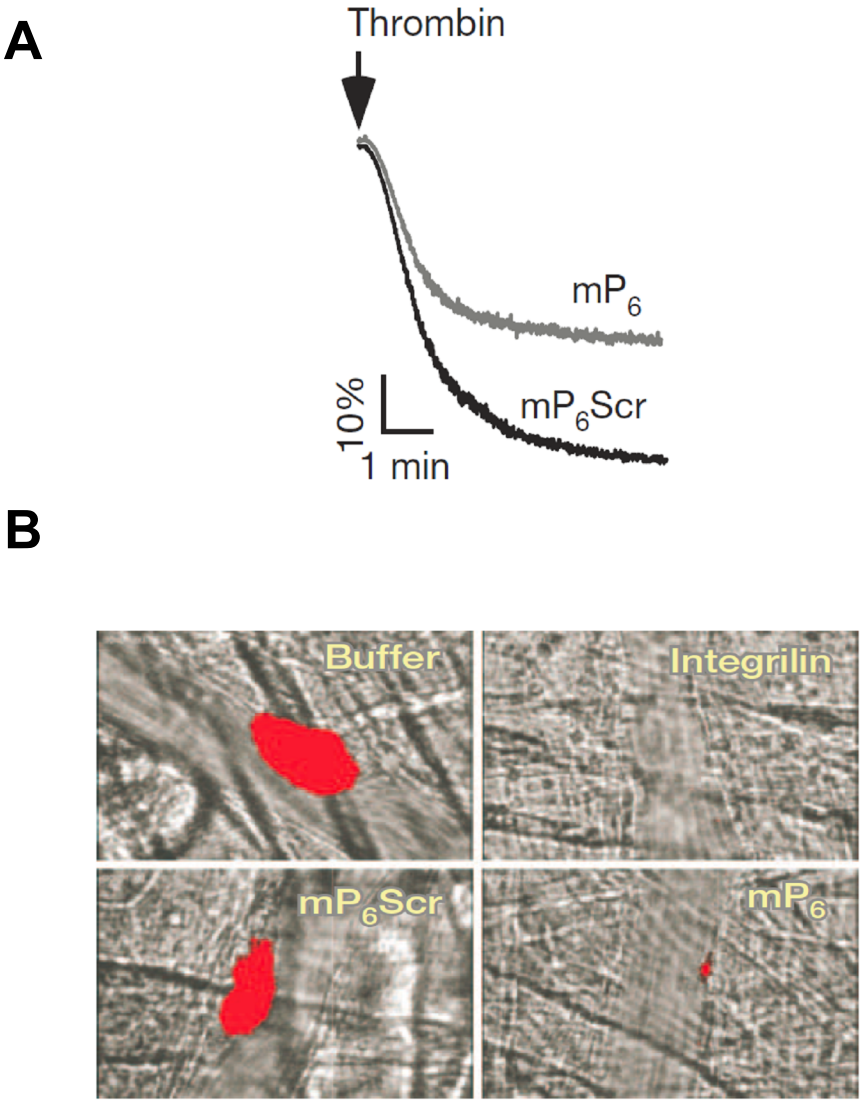

Fig. 21. mP6 micelle inhibits platelet aggregation and laser-induced arteriolar thrombosis (A) Effects of 10μM mP6 or mP6Scr micelles on platelet aggregation induced by 0.03 U/ml thrombin. (B) Comparison of mP6 micelle (5 μmol/kg) with Integrilin (12 μmol/kg) and their respective controls in inhibiting laser-induced arteriolar thrombosis in mice. Representative images at 60 s after injury are shown. Platelet thrombi were indicated by DyLight 649-labelled nonblocking rat anti-mouse GPIbβ (red).

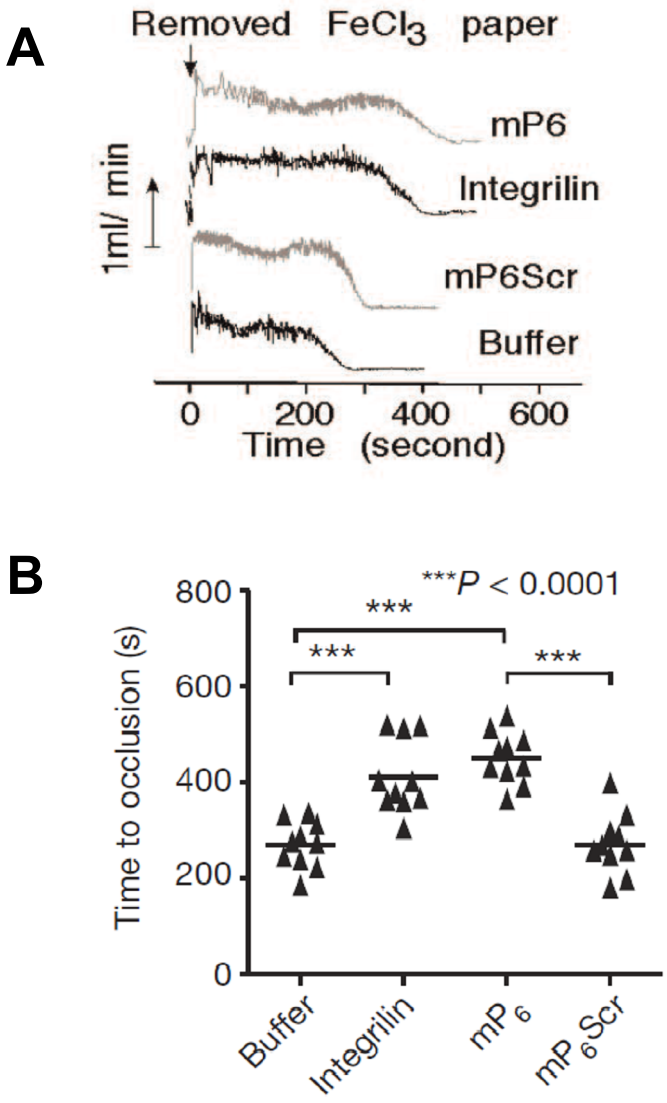

Fig. 22. mP6 micelle inhibits $FeCl_3$-induced occlusive thrombosis. (A, B) Comparison of mP6 (5 μmol/kg) with the same dose of Integrilin and their respective controls in occlusion time of $FeCl_3$-induced carotid artery thrombosis in mice. Typical arterial blood flow charts of $FeCl_3$-induced occlusive thrombosis are shown in (A), and quantification is shown in (B)

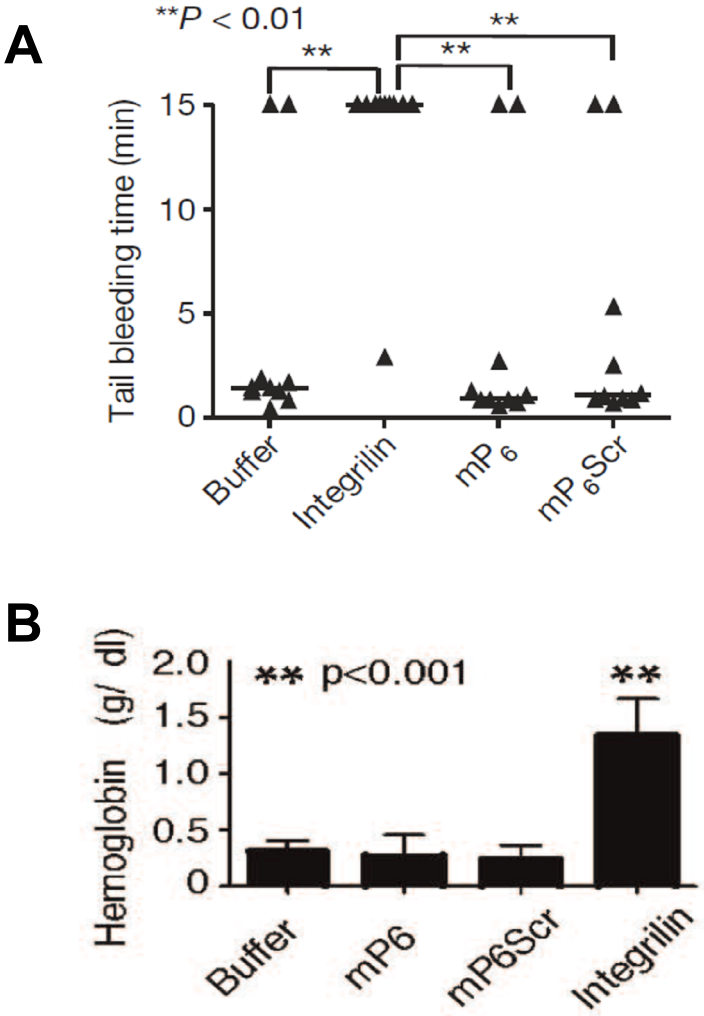

Fig. 23. mP6 micelle does not have bleeding side effect. (A, B) Comparison of mP6 (5 μmol/kg) with Integrilin (5 μmol/kg) and controls in mouse tail bleeding analysis. (A) Times to cease bleeding were used as a parameter to assess blood loss (mean +/- s.d., n=10). (B) Released haemoglobin levels were used as a parameter to assess blood loss (mean +/- s.d., n=10).

function of integrins. Importantly, we translated this new concept into a potent novel anti-thrombotic, which, unlike currently available integrin antagonists or other anti-thrombotics, potently inhibits arterial thrombosis without the adverse effect of causing bleeding, a potentially life-threatening problem that limits the clinical use of current anti-integrin and anti-thrombotic therapies (Fig. 24).

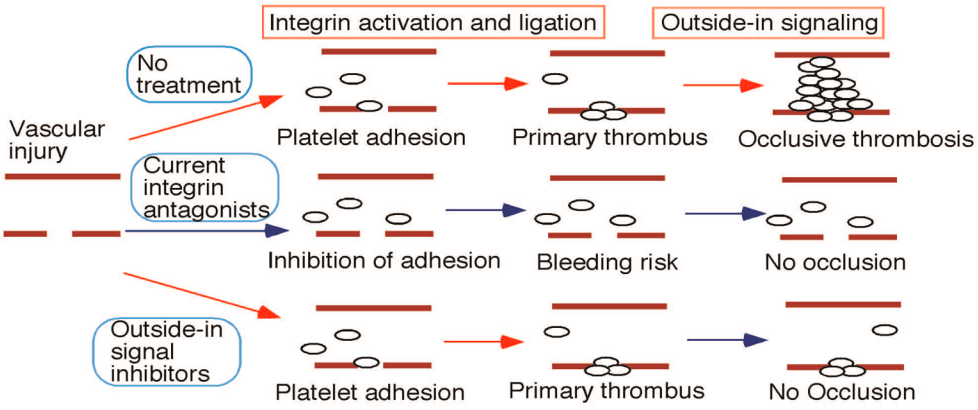

Fig. 24. A schematic showing how selective inhibitors of integrin outside-in signalling work as anti-thrombotics. Blue arrows indicate steps that are inhibited.

IV. THE INTERACTION OF Gα13 WITH INTEGRIN β1 MEDIATES CELL MIGRATION BY DYNAMIC REGULATION OF RHOA

(Previously published as Bo Shen, Brian Estevez, Zheng Xu, Barry Kreutz, Andrei Karginov, Yanyan Bai, Feng Qian, Urao Norifumi, Deane Mosher, Xiaoping Du (2015) The interaction of Gα13 with integrin β1 mediates cell migration by dynamic regulation of RhoA. Molecular Biology of the Cell. 26,20, 3658-3670)

1. The importance of Gα13 in cell migration

To assess the role of Gα13 in cell migration, we have developed two Gα13-specific shRNA and their corresponding non-specific scrambled control shRNA using a lentiviral vector. Transfection of the Gα13-specific shRNA, but not the scrambled control resulted in ~90% knockdown of Gα13 in CHO cells (Fig. 25A), which express endogenous β1 and β5 integrins but not β3 integrins. We employed a scratched wound healing assay to assess the role of Gα13 in cell migration. As shown in Fig. 25B, 20 hours after the scratch, CHO cells and CHO cells transfected with control shRNA had migrated to almost seal the gap. In contrast, Gα13 knockdown cells were defective in migration (Fig. 25B, 25C).

The process of scratched wound healing consists of both cell proliferation and cell migration. To exclude the possibility that the phenotypes we observed were attributed to suppression of cell proliferation, we demonstrated that CHO cells with Gα13 knockdown were not defective in cell proliferation, but rather cell proliferation was slightly increased

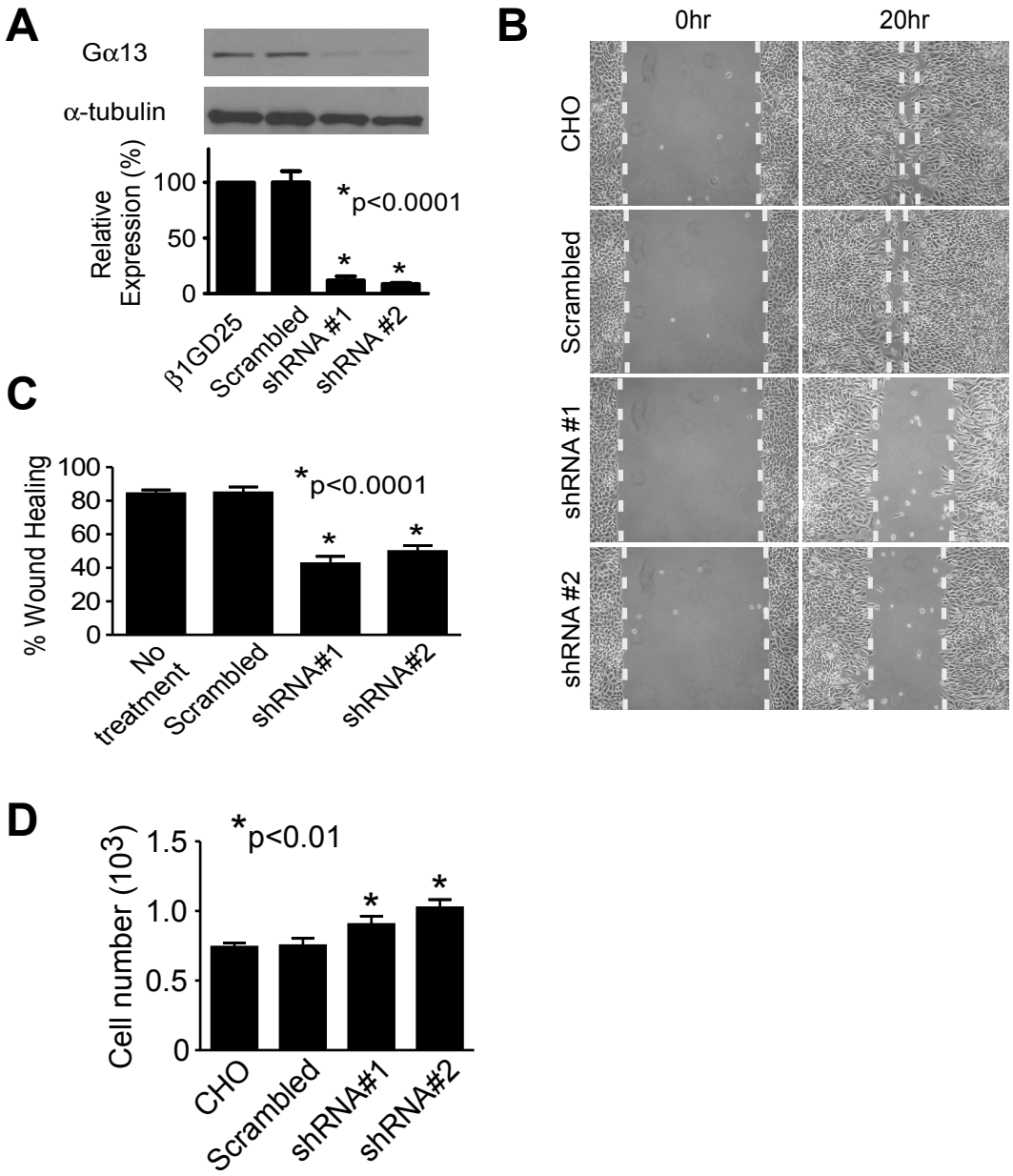

Figure. 25. The importance of Gα13 in cell migration. (A) Western blot comparison of Gα13 expression levels in CHO cells, and CHO cells transfected with Gα13-specific shRNA#1, shRNA#2, or scrambled control shRNA lentivirus. α-tubulin was used as loading control. Quantitative data are shown as mean +/- SD. (B) Phase contrast images of CHO cells before and after 20 hours migration following wound scratches. (C) Quantification of data as shown in (B), n=6. (D) Total cell counts at 24-hour time point (5 experiments). Cell counts at 0 hours are 0.5 x 103.

as compared to control (Fig. 25D). Thus, these data indicate that Gα13 plays an important role in migration of CHO cells.

2. The role of Gα13 in integrin β1-dependent cell migration

To specifically determine the role of Gα13 in β1 integrin-dependent cell migration, we analyzed cell migration in GD25 cells, a fibroblast cell line derived from β1 knockout mice(Wennerberg *et al.*, 1996), and GD25 cells transfected with wild type (Wt) or mutant integrin β1 subunits (Fig. 26A).

$\beta1^{-/-}$ GD25 cells displayed severely impaired migration in a scratched wound healing assay after 20 hours (Fig. 26B). In contrast, expression of β1 integrin in GD25 cells corrected the defective migration (Fig. 26B). These data are consistent with a previous study showing that β1 expression in β1-knockout cell lines enhanced cell migration(Sakai *et al.*, 1998a; Sakai *et al.*, 1998b; Gimond *et al.*, 1999; Sakai *et al.*, 1999), indicating that GD25 cell migration in the scratched wound healing analysis is dependent upon integrin β1. Moreover, knockdown of Gα13 in the β1-expressing GD25 cells using either of the two Gα13 shRNA caused significant defects in cell migration (Fig. 26B-D), indicating that Gα13 plays a critical role in β1-integrin-mediated cell migration. Similar to the above described experiments using CHO cells, Gα13 knockdown did not inhibit, but rather mildly increased cell proliferation as compared to control (Fig. 26E), indicating that the defective wound healing in Gα13 knockdown cells was not due to inhibited cell growth but rather suppressed cell migration.

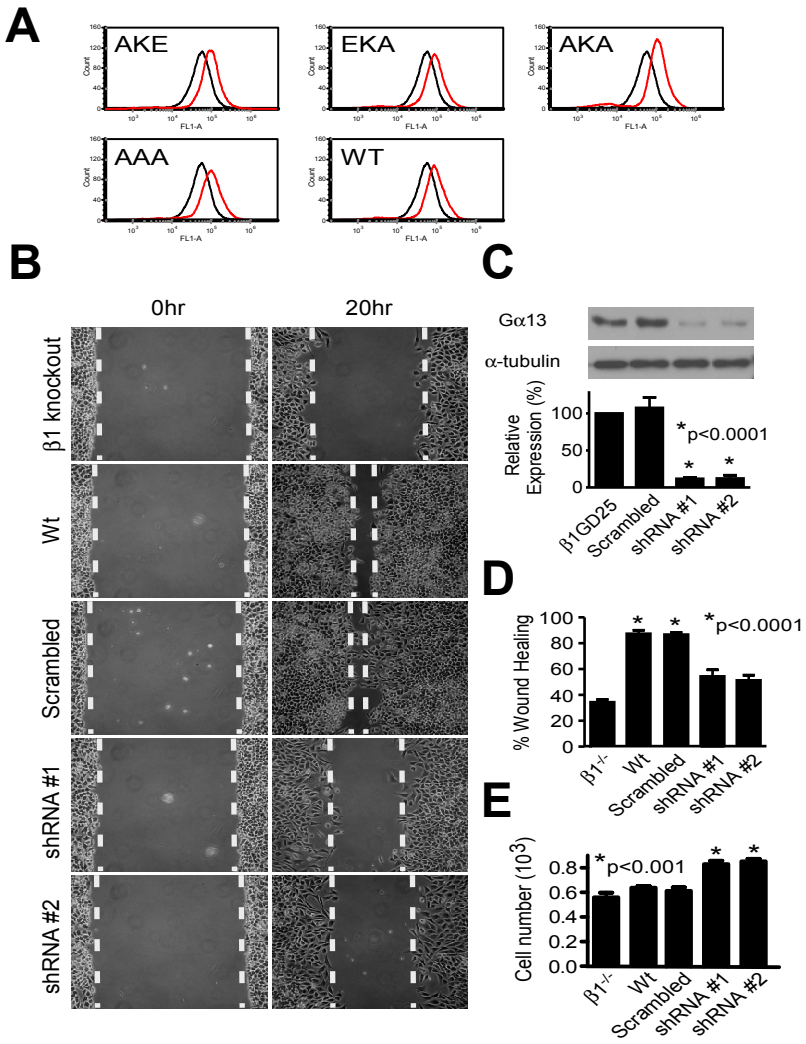

Figure 26. The role of Gα13 in integrin β1-dependent cell migration. (A) Flow cytometric analysis of β1 expression on GD25 cell surface after lentiviral transfection of various integrin β1 mutants. Similar expression levels of these mutants were achieved by cell sorting. (B) Phase contrast images of β1(Wt)GD25 cells, with or without Gα13-specific or scrambled control shRNA lentiviral transfection, before and after 20 hours migration following wound scratches. (C) Western blot comparison of Gα13 expression levels in β1(Wt)GD25 cells, and β1(Wt)GD25 cells transfected with Gα13-specific or scrambled control shRNA. α-tubulin was used as loading control. Quantitative data are shown in the bar graph (mean +/- SD, n=3). (D) Quantification of data as shown in (B) (mean +/- SD, n=6). (E) Total cell counts at 24-hour time point (5 experiments). Cell counts at 0 hours are 0.5 x 10^3.

3. The interaction of Gα13 with β1 integrins via the ExE motif

As discussed in Chapter 3, we showed that Gα13 directly interacts with integrin β subunits via a conserved ExE motif. To determine whether Gα13 interacts with β1 via the 767EKE sequence, we incubated purified Gα13 with sepharose beads conjugated with purified GST-Wt β1 integrin cytoplasmic domain fusion protein (GST-β1CD) or with GST-β1CD carrying mutations that changes the 767EKE sequence to AKA or to AAA. Gα13 binds directly to the GST-β1CD sepharose beads, whereas Gα13 failed to bind to the AKA or AAA mutants (Fig. 27A). Thus, the ExE motif is crucial in the direct binding of Gα13 to integrin β1CD. To study whether the interaction between intact β1 subunit and Gα13 occurs in cells, we also introduced a set of mutations in the 767EKE sequences, changing EKE to AKE, EKA, AKA or AAA, and expressed these mutants in GD25 cells at similar levels (Fig. 26A). We then performed a co-immunoprecipitation experiments using these cells. There was little integrin β1 associated with Gα13 in suspended cells (Fig. 27B). However, following cell adhesion on fibronectin for one hour, the interaction becomes prominent (Fig. 27B). This result suggests that integrin ligation is required for the binding of Gα13 to β1 in these cells. Furthermore, AKE or EKA mutants showed significantly reduced Gα13-β1 association, and mutation of both glutamic residues (AKA or AAA) almost totally abolished the interaction (Fig. 27B). Thus, the β1 integrin ExE motif is critical for Gα13-β1 interaction in cells.

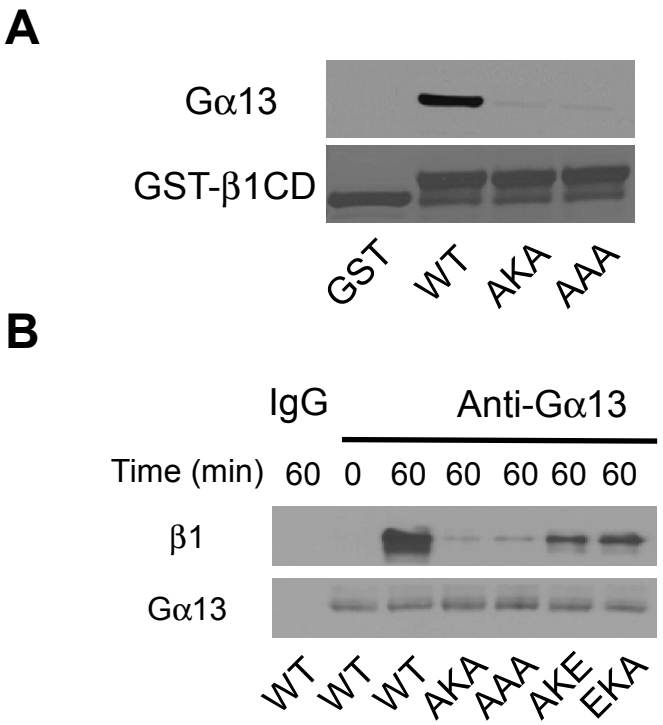

Figure 27. The critical role of the β1 ExE motif in Gα13-β1 interaction. (A) Binding of purified recombinant Gα13 to glutathione bead-bound glutathione S-transferase (GST), GST-Wt β1 cytoplasmic domain fusion protein (GST-β1Wt), GST-(AKA)β1 and GST-(AAA)β1 mutants. (B) β1GD25 cells expressing similar levels of wild-type (Wt) β1 and various ExE mutant β1 (AKE, EKA, AKA, or AAA) were allowed to adhere to fibronectin for 1 hour. After that, cells were lysed and cell lysates were immunoprecipitated with anti-Gα13 antibody or equal amount of control rabbit IgG. Immunoprecipitates were immunoblotted with anti-Gα13 and anti-β1 antibodies.

4. The interaction between Gα13 and β1 integrins mediates β1-dependent cell migration

To determine the importance of Gα13-β1 interaction in β1-dependent cell migration, we used the scratched wound healing assay to examine the migration of GD25 cells expressing wild type β1 or various β1 ExE motif mutants. The β1/Gα13-dependent cell migration was partially abolished in cells with AKE mutation in integrin β1 and was totally abolished in cells expressing AKA, AAA or EKA mutants (Fig. 28A, 28B). Meanwhile, mutations in the β1 ExE motif did not affect proliferation of these β1-expressing cells (Fig. 28C). Thus, it appears that Gα13-integrin interaction is responsible for the role of Gα13 in cell migration and Gα13-β1 interaction is critically important for the β1-dependent cell migration.

To determine the role of Gα13-integrin interaction in cell migration under different conditions, we also used a transwell migration assay. The integrin β1 ligand fibronectin was coated on the bottom side of a transwell insert with 8 μm pores, which would allow cells on the top side to migrate through the pores to the bottom side of the insert. As shown in Fig. 29, transwell migration of GD25 cells requires β1 integrin expression. Gα13 knockdown abolished transwell migration almost completely. Similarly to the result in a scratched wound healing assay, the AKE mutant of β1 only partially supported transwell migration, and the EKA, AKA or AAA mutants had very little activity (Fig. 29A, 29B). Based on these results, we conclude that Gα13-β1 interaction is required for the β1-dependent cell migration.

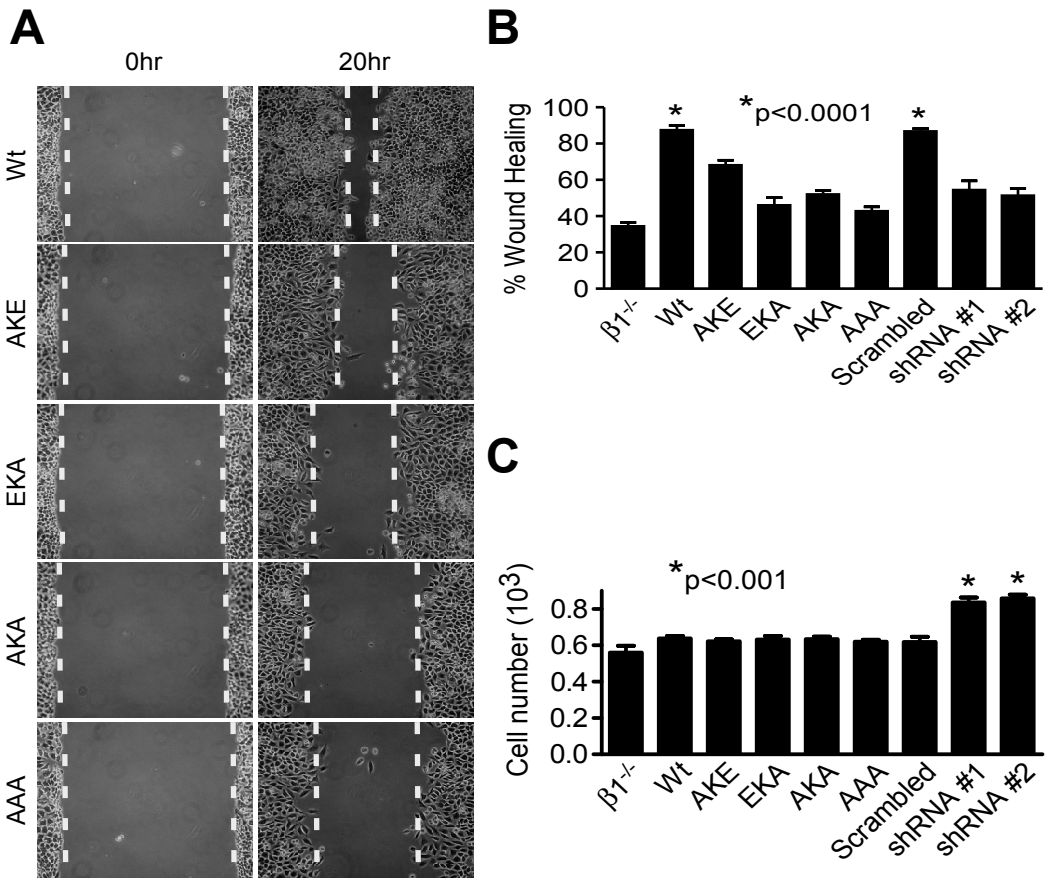

Figure 28. The interaction between Gα13 and β1 integrins mediates β1-dependent migration. (A) Phase contrast images of β1GD25 cells expressing similar levels of wild-type (Wt) β1 or various ExE β1 mutants (AKE, EKA, AKA, or AAA), before and after 20 hours migration following wound scratches. (B) Quantification of migration data in (A) (mean +/- SD, n=6). (C) Total cell counts at 24-hour time point (5 experiments). Cell counts at 0 hours are 0.5 x 10^3.

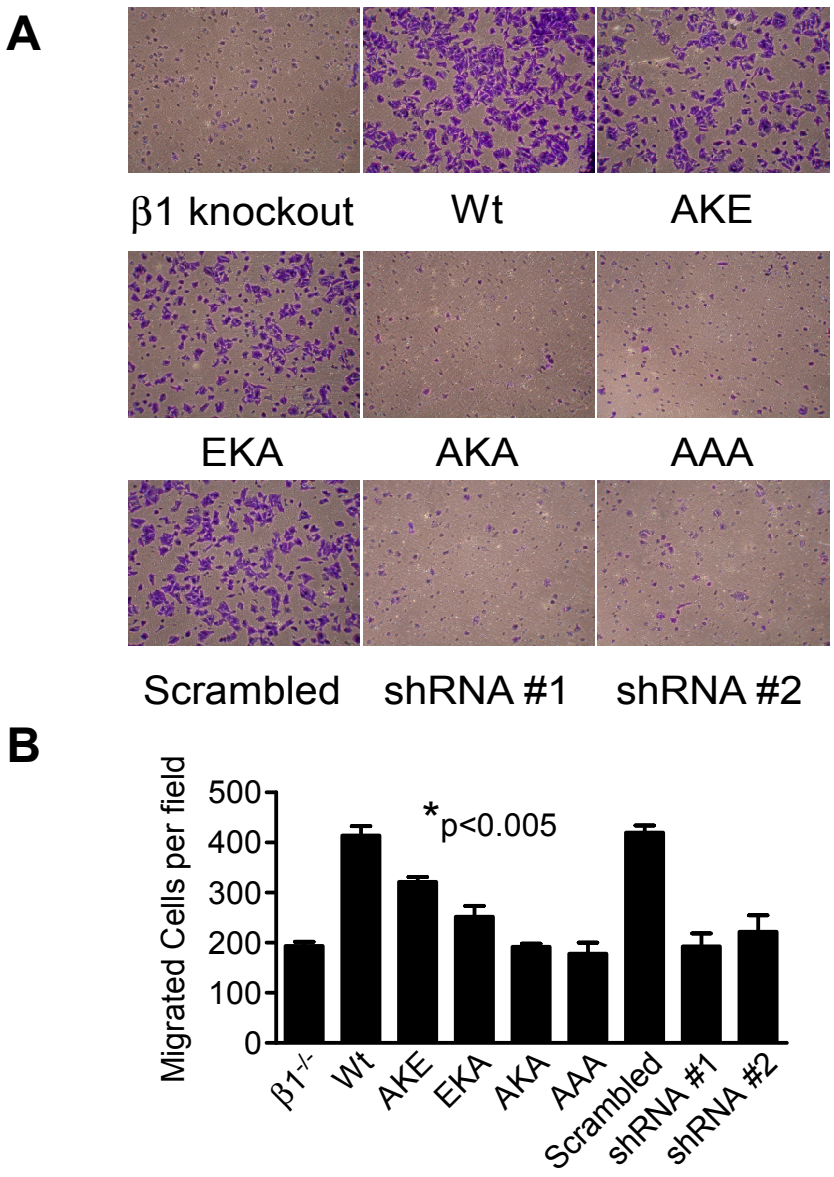

Figure 29. The interaction between Gα13 and β1 integrins mediates β1-dependent transwell migration. (A) GD25 cells without or with expression of similar levels of wild-type (Wt) β1 and various ExE β1 mutants (AKE, EKA, AKA, or AAA), or β1(Wt)GD25 cells transfected with scrambled or Gα13-specific shRNA were compared in a transwell migration assay. Cells were fixed and stained with crystal violet after 6 hours of migration. (B) Quantification of migrated cells in (A) (mean +/- SD, 4 experiments)

5. The role of Gα13-β1 interaction in mediating β1 integrin outside-in signaling leading to cell spreading

Next, we wanted to determine the mechanism responsible for the importance of Gα13-integrin interaction in β1-dependent cell migration. We have shown in Chapter 3 that Gα13 binding to integrin β3 is important in αIIbβ3 outside-in signaling and consequent cell spreading. Hence, we hypothesized that Gα13-mediated β1 integrin outside-in signaling and the consequent cell membrane movement (spreading and retraction) are responsible for their functions during cell migration. Thus, we investigated how Gα13 knockdown affects integrin-dependent cell spreading. CHO cells with Gα13 knockdown were defective in spreading on immobilized integrin ligand fibronectin (Fig. 30A, 30B), suggesting the importance of Gα13 in integrin-dependent cell spreading. Previous studies have shown that early phase cell spreading on fibronectin was defective in β1-/- GD25 cells(Wennerberg *et al.*, 1996; Pankov *et al.*, 2003; Green *et al.*, 2009). Our data also showed that early phase cell spreading occurred in Wt β1-expressing GD25 cells. GD25 cells expressing the β1 ExE motif mutants exhibited a significant reduction in early phase cell spreading to a similar level as was observed in β1-deficient GD25 cells (Fig. 30C). These results suggest that the Gα13-integrin interaction is important in β1-mediated outside-in signaling leading to β1-dependent cell spreading.

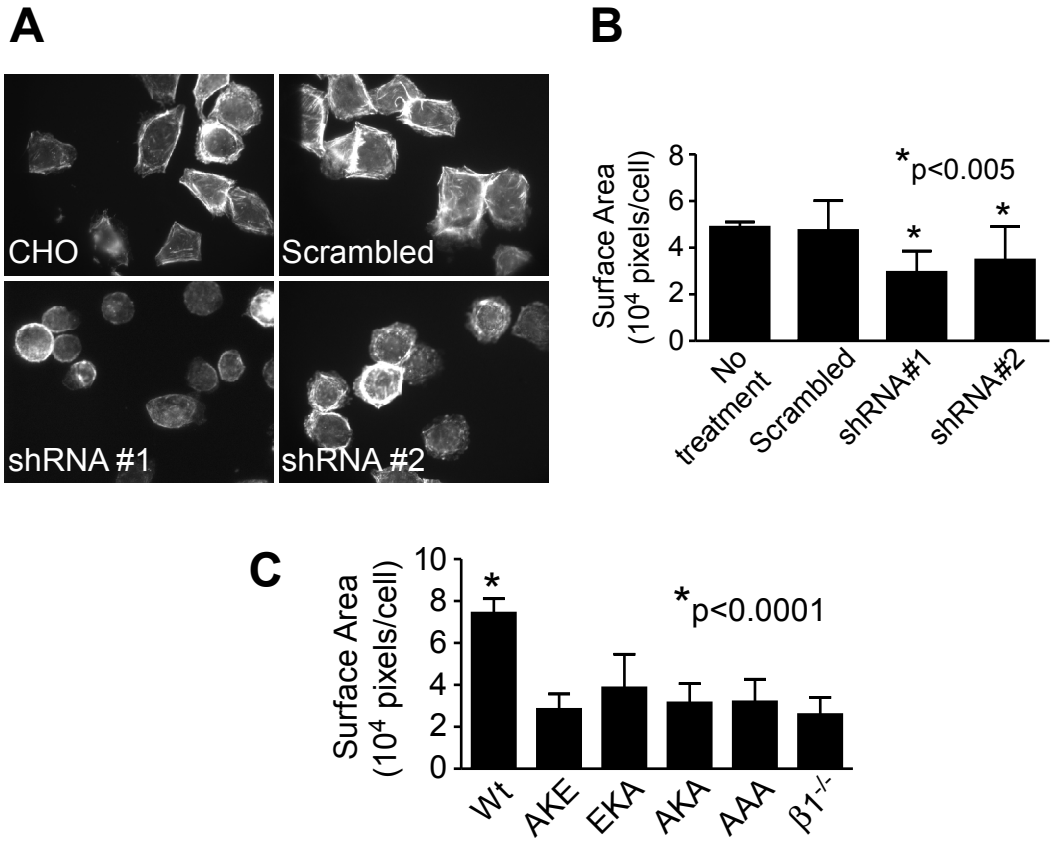

Figure 30. The role of Gα13-β1 interaction in mediating β1 integrin outside-in signaling leading to cell spreading. (A) Fluorescence microscopy images of phalloidin-stained CHO cells spreading on fibronectin for 1 hour. (B) Quantification of surface areas of individual cells (mean +/- SE, n=31, 24, 28, 29 for CHO, and CHO transfected with scrambled or Gα13-specific shRNAs). (C) Quantification of surface areas of individual GD25 cells spreading on fibronectin for 30 minutes (mean +/- SE). n= 23, 25, 26, 24, 28, 31 for Wt, AKE, EKA, AKA, AAA, and β1 knockout GD25 cells respectively.

6. Gα13-β1 binding mediates outside-in signaling through activation of c-Src and transient inhibition of RhoA

To determine whether and how Gα13 binding to β1 mediates integrin outside-in signaling, we seeded Wt and mutant β1-expressing GD25 cells onto immobilized fibronectin, and measured Src activation (indicated by phosphorylation at Y416) and RhoA activity (indicated by GST-rhotekin Rho-binding domain protein(RBD) pulldown(Ren *et al.*, 1999)), which specifically binds to active RhoA) at different time points following adhesion. Adhesion of the β1(Wt)GD25 cells on fibronectin resulted in a robust but transient RhoA inactivation (Fig. 31A, 31B). In contrast, this transient RhoA inactivation was not observed in β1(AAA)GD25 cells, which showed constant activation of RhoA (Fig. 31A, 31B). Furthermore, Src was activated in Wt β1-expressing GD25 cells following adhesion to fibronectin, and Src activation was inhibited in the AAA mutant β1-expressing GD25 cells (Fig. 31C, 31D). Since it is known that transient RhoA inhibition following β1 outside-in signaling is Src-dependent(Arthur *et al.*, 2000), our data indicate that Gα13 binding to the ExE motif of β1 integrins mediates Src activation and Src-mediated inhibition of RhoA signaling. To explore further the role of the Gα13 binding β1767ExE motif in regulating RhoA activity following cell adhesion, GD25 cells adherent to immobilized fibronectin were stained with the GST-rhotekin RBD(Ren *et al.*, 1999) (Fig. 32). Indeed, Wt but not AAA mutant β1-expressing cells showed a transient reduction in GST-RBD staining (Fig. 32A). The staining of the GST-RDB was specific as it was abolished by Rho inhibitor C3-transferase, and GST protein alone failed to stain

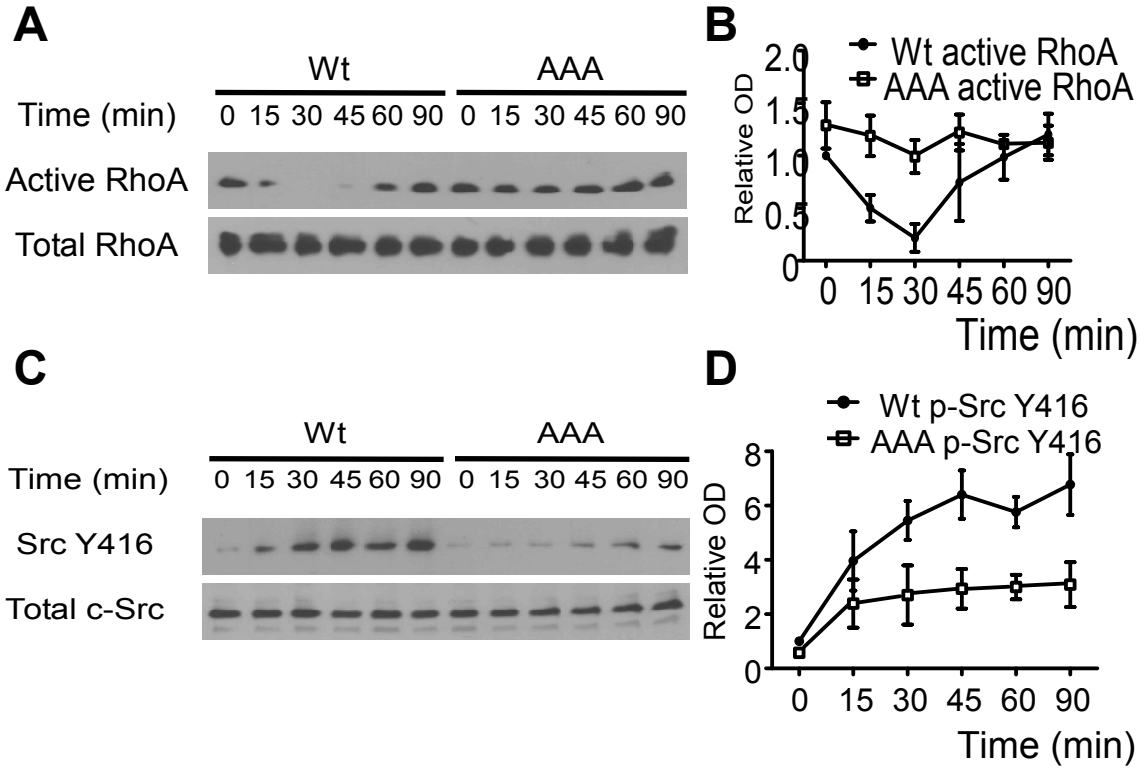

Figure. 31. Gα13-β1 binding mediates activation of c-Src and transient inhibition of RhoA. (A - D) GD25 cells expressing similar levels of Wt or AAA mutant β1 were allowed to adhere to immobilized fibronectin, solubilized at various time points, and analyzed for RhoA activation (A) and c-Src Tyr416 phosphorylation (C). Quantification of three independent experiments was shown in (B) and (D).

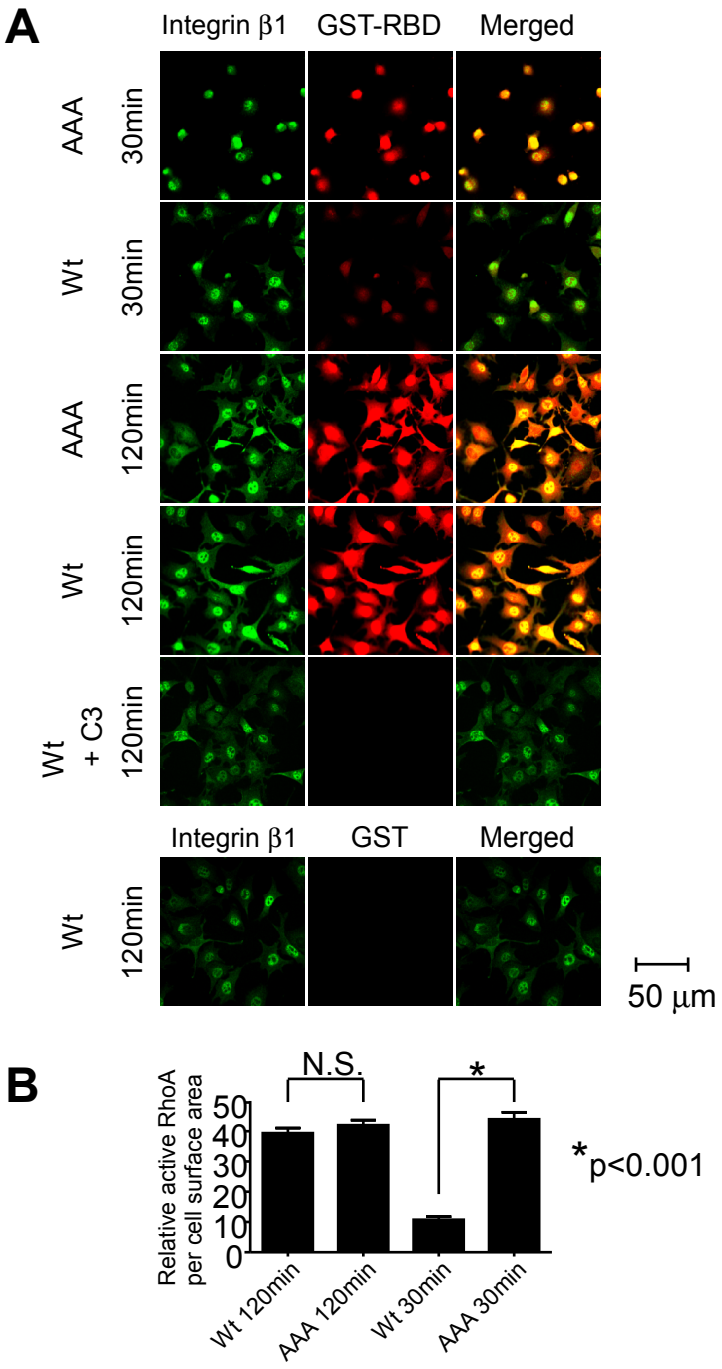

Figure. 32. In situ RhoA activation analysis of the role Gα13 binding to β1. (A - B) GD25 cells were allowed to adhere to immobilized fibronectin, fixed, and stained with anti-β1 antibody (green) and GST-RBD proteins (red). β1(Wt)GD25 cells were also pre-incubated with 20 μg/ml C3-transferase, and stained with anti-β1 (green) and GST-RBD (red). Cells stained with anti-β1 antibody (green) and GST proteins (red) served as additional negative controls. (B) Quantification of the active RhoA in (A) (mean +/- SD, n=3).

the cells (Fig. 32A). Thus, these data indicate that transient RhoA inhibition shortly following cell adhesion (30 min) was dependent upon Gα13-β1 interaction (Fig. 32B).

7. The effect of an inhibitor peptide based on the Gα13-binding sequence in β1

The above results indicate that integrin β1-Gα13 binding plays an important role in β1 integrin mediated activation of Src and transient inhibition of RhoA, and in β1-dependent cell migration. Thus, we hypothesized that an inhibitor of β1-Gα13 interaction should block β1-dependent integrin outside-in signaling and cell migration. To test this hypothesis, we synthesized a myristoylated, cell-permeable peptide containing the Gα13-binding ExE motif, mβ1P6 (Myr-FEKEKM). Pre-incubation of this peptide with β1(Wt)GD25 cells abolished the interaction of Gα13 to integrin β1 in the co-immunoprecipitation assay (Fig. 33A). In contrast, the scrambled control peptide mβ1P6Scr (Myr-EKMFEK) had no effect. These data indicate that mβ1P6 is effective in inhibiting Gα13-β1 interaction (Fig. 33A). Pre-incubation of mβ1P6 but not the scrambled peptide also significantly inhibited transwell migration in β1(Wt)GD25 cells (Fig. 33B, 33C). Furthermore, c-Src activity and transient RhoA inactivation were both inhibited by this inhibitor (Fig. 34A-D), mirroring the results of ExE mutation (Fig. 31). Thus, these results suggest that this inhibitory peptide effectively blocks integrin β1-mediated outside-in signaling and cell migration.

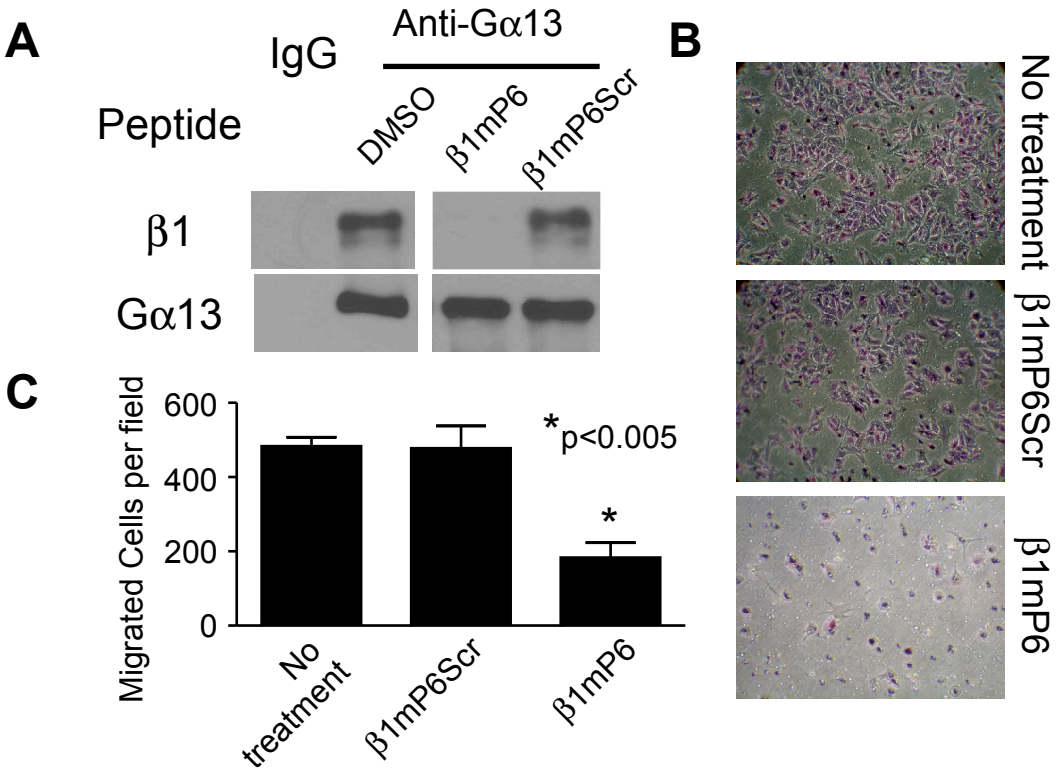

Figure. 33. The inhibitory effects of myristoylated peptide mβ1P6 on Gα13-β1 interaction and cell migration. (A) β1(Wt)GD25 cells treated with 100 μM β1mP6 or scrambled control peptide β1mP6Src were allowed to adhere to fibronectin for 1 hour. Cells were lysed and cell lysates were immunoprecipitated with anti-Gα13 antibody or equal amount of control rabbit IgG. Immunoprecipitates were immunoblotted with anti-Gα13 and anti-β1 antibodies. (B) Representative images of transwell migrated β1(Wt)GD25 cells 6 hours after migration. Cells were treated with 100 μM β1mP6 or scrambled control peptide β1mP6Src prior to migration and were stained with crystal violet after fixation. (C) Quantification of migrated cells in (B) (mean +/- SD, n=3).

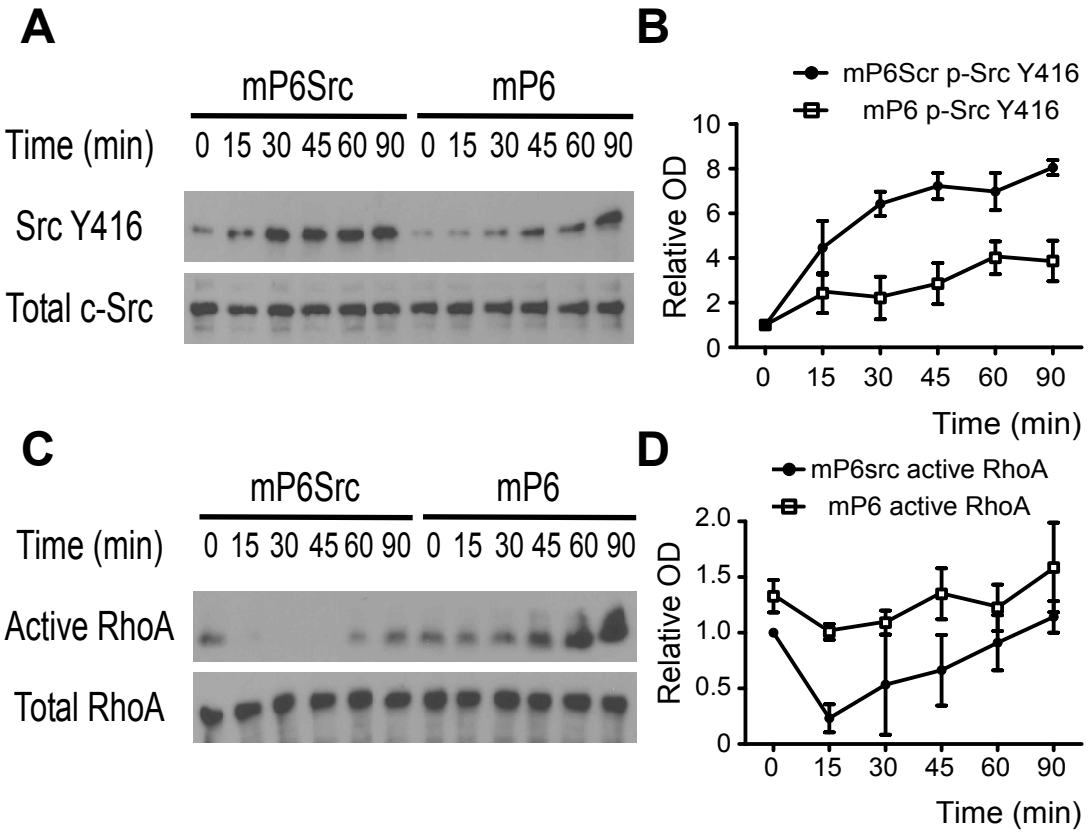

Figure. 34. The inhibitory effects of myristoylated peptide mβ1P6 on c-Src activation and transient RhoA inactivation. (A - D) β1(Wt)GD25 cells treated with 100 μM β1mP6 or scrambled control peptide β1mP6Src were allowed to adhere to immobilized fibronectin, solubilized at various time points, and analyzed for RhoA activation (A, B) and c-Src Tyr416 phosphorylation (C, D). (A, C) typical gels. (B, D) Quantification of the western blots (mean +/- SD, n=3).

8. Discussion

In this chapter, we have demonstrated that Gα13 directly binds to the ExE motif of integrin β1 cytoplasmic domain, and this binding is required for β1 outside-in signaling and cell migration on β1 integrin ligand fibronectin. Importantly, we show that Gα13-integrin interaction mediates transient RhoA inhibition, which is in contrast to the well-known role of Gα13 in mediating RhoA activation by binding to RhoGEFs(Goulimari *et al.*, 2005; Chen *et al.*, 2012). Thus, our data suggests that Gα13-mediated dynamic regulation of RhoA activity is a novel mechanism responsible for the role Gα13 during β1 integrin-dependent cell migration.

The importance of integrin β1 in cell migration has been reported in various cell types(White *et al.*, 2004; Liu *et al.*, 2010). Separately, the evidence of a role for Gα13 in directed cell migration has also been shown(Radhika *et al.*, 2004; Shan *et al.*, 2006; Tan *et al.*, 2006). However, it was not previously appreciated why and how β1 integrins and Gα13 play important roles in mediating cell migration and whether there is a connection. Here we show that integrin β1-dependent cell migration requires direct binding of Gα13 to the cytoplasmic domain of β1, establishing a direct connection between these two important molecules in cell migration. In the previous Chapter, we have shown that Gα13 interacts with the platelet integrin αIIbβ3 and plays an important role in αIIbβ3 outside-in signaling. Migration is not ordinarily considered or studied as an activity of platelets, and it remains unclear whether Gα13-β3 interaction is a common mechanism of integrin signaling that is shared with β1 integrin signaling and important in cell migration. Furthermore, it is known that there are major differences in outside-in

signaling mechanisms between platelet β3 integrins and β1 integrins in nucleated cells. In particular, β3 outside-in signaling requires Src binding to the C-terminal site of β3, which can be cleaved by calpain(Arias-Salgado *et al.*, 2003; Flevaris *et al.*, 2007). Calpain cleavage of β3 cytoplasmic domain abolishes c-Src binding and switches β3 integrin signaling from mediating cell spreading to retraction(Flevaris *et al.*, 2007). However, Src does not appear to bind to β1 C-terminus(Arias-Salgado *et al.*, 2003), and thus its interaction with β1 is likely to be differently regulated. Furthermore, β1 signaling was reported to involve complex formation of focal adhesion kinase (FAK) with Src and integrin, and FAK-dependent Src activation(Xing *et al.*, 1994; Thomas *et al.*, 1998), which has not been shown in platelet αIIbβ3. As discussed in the previous Chapter, platelet αIIbβ3-dependent activation of Src requires Gα13 binding to β3. Here we further show that Gα13 directly binds to the ExE motif in β1. Disruption of Gα13-β1 binding through mutations in the ExE motif or using an ExE motif peptide abolished β1-dependent Src activation, and inhibited β1-dependent outside-in signaling and cell spreading. These data indicate that, despite of the difference between β1 and β3 signaling, the ExE motif-containing integrins β1 and β3 share similar Gα13-dependent mechanisms of Src activation and outside-in signaling in platelets and in migrating nucleated cells. Importantly, our data for the first time indicate that the interaction between Gα13 and β1 ExE motif plays a critical role in integrin-dependent cell migration.

Our data not only indicate the important role for the Gα13-β1 interaction in cell migration, but also suggest a novel mechanism of Gα13-dependent dual regulation of RhoA activity in migrating cells (Fig. 35). Cell migration on β1 integrin ligands involves

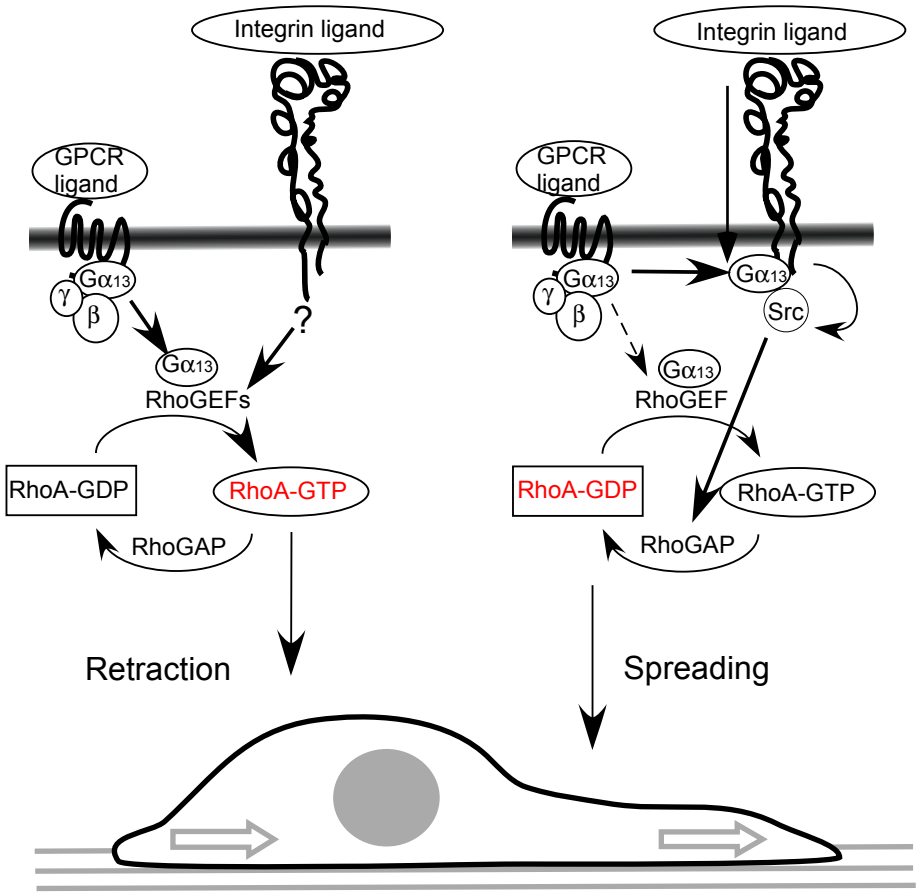

Figure 35. A new model for the Gα13-dependent dynamic regulation of RhoA and cell migration. GPCR-dependent activation of Gα13 stimulates the activation of RhoGEFs, leading to Rho activation, which has been suggested to be important for cell retraction in response to GPCR stimuli. Integrin ligation induces the interaction between the cytoplasmic domains of β subunits and the activated Gα13, which mediates Src-dependent transient inhibition of RhoA, and activates the Rac1 and PI3K pathways. These events lead to spreading of cells (lamellipodia and filopodia) towards the direction of migration. Late phase integrin signaling results in reactivation of RhoA and cell retraction driving the cell movement towards the direction of migration. Thus, Gα13-dependent dynamic regulation of RhoA results in coordinated cell spreading and retraction.

coordinated integrin-dependent cell spreading and retraction. The alternate protrusion and retraction occur during cell spreading at the leading edge(Machacek *et al.*, 2009; Tkachenko *et al.*, 2011). Retraction in the rear of a cell pulls the cell forward(Lauffenburger and Horwitz, 1996; Ridley *et al.*, 2003). The Rho family GTPase, RhoA, is a major regulator of cell retraction. RhoA activates Rho kinase. Rho kinase inhibits myosin light chain (MLC) phosphatase, increases MLC phosphorylation, resulting in acto-moysin-mediated cell retraction(Kimura *et al.*, 1996), which drives inward movement of cell membranes. Thus, RhoA activity inhibits cell spreading, and stimulates cell retraction(Vega *et al.*, 2011). Consequently, RhoA-dependent retractile signaling needs to be dynamically activated and inhibited in order for cells to migrate. Gα13, upon activation by GPCRs, directly stimulates RhoGEFs, and activation of RhoA(Kozasa *et al.*, 1998), which is thought to be the reason why Gα13 is important in cell migration(Bian *et al.*, 2006; Patel *et al.*, 2014). Here we for the first time demonstrate the other aspect of this dynamic regulation. We show that Gα13 binding to β1 ExE motif mediates the β1 integrin-dependent activation of Src and transient inhibition of RhoA in migrating cells. This finding is not only consistent with the previous data suggesting that integrin β1 outside-in signaling transiently inhibit RhoA activity via Src during cell spreading(Arthur and Burridge, 2001), but also provide a plausible mechanism that is responsible for initiating the Src-dependent transient RhoA inhibition. Thus, our data, together with the previously reported role of Gα13 in activating RhoA, suggest a novel mechanism through which dynamic regulation of RhoA activation by Gα13 is achieved: Gα13 binding to ligand-bound integrins induces Src-dependent transient inhibition of RhoA, which is required for cell spreading. On the other hand,

Gα13 binding to RhoGEFs stimulates RhoA and thus drives cell retraction together with RhoA activation induced by late phase integrin signaling(Dubash *et al.*, 2007). Dynamic regulation of RhoA activation by Gα13 thus provides a novel mechanism explaining the importance of Gα13 in driving coordinated cell spreading and retraction, leading to cell migration.

V. CONCLUSIONS

Integrins are adhesion receptors which mediate many important physiological and pathological processes, such as development, immunity, inflammation, thrombosis and cancer. They are known to transmit signaling in two directions: intracellular signaling induces the binding of key molecules, such as talin, to the cytoplasmic domain of integrins and stimulate conformational changes in the extracellular domain, leading to increased ligand affinity, which is called inside-out signaling. Conversely, ligand binding in the extracellular domain of integrins transmit signals into the cell, leading to a cascade of signaling events that stimulates cell spreading, retraction, migration, and proliferation, which is called outside-in signaling. The mechanism of integrin outside-in signaling has been unclear. We found that the heterotrimeric guanine nucleotide–binding protein (G protein) Gα13 directly bound to the integrin β3 cytoplasmic domain and that Gα13-integrin interaction was promoted by ligand binding to the integrin αIIbβ3 and by guanosine triphosphate (GTP) loading of Gα13. We therefore hypothesized that integrins could be noncanonical Gα13-coupled receptors, and Gα13 binding to integrins might be important in integrin outside-in signaling and integrin functions.

Antagonists of the platelet integrin αIIbβ3, which mediate thrombosis and hemostasis, are potent anti-thrombotic drugs, but also have the life-threatening adverse effect of causing bleeding. It is therefore desirable to develop new antagonists that do not cause bleeding. Integrins transmit signals bidirectionally. Inside-out signaling activates integrins through a talin-dependent mechanism.

Integrin ligation mediates thrombus formation and outside-in signaling, which requires Gα13 and greatly expands thrombi. Our studies demonstrate that Gα13 and talin bind to mutually exclusive but distinct sites within the integrin β3 cytoplasmic domain in opposing waves. The first talin-binding wave mediates inside-out signaling and also ligand-induced integrin activation, but is not required for outside-in signaling. Integrin ligation induces transient talin dissociation and Gα13 binding to an EXE motif (in which X denotes any residue), which selectively mediates outside-in signaling and platelet spreading. The second talin-binding wave is associated with clot retraction. An EXE-motif-based inhibitor of Gα13–integrin interaction selectively abolishes outside-in signaling without affecting integrin ligation, and suppresses occlusive arterial thrombosis without affecting bleeding time. Thus, we have discovered a new mechanism for the directional switch of integrin signaling and, on the basis of this mechanism, designed a potent new antithrombotic drug that does not cause bleeding.

We showed that Gα13 binds to most β integrins which contain a conserved EXE motif (except β8). Furthermore, we showed that the Gα13-integrin binding is also important in integrin-dependent cell migration. Gα13 is known to transmit G protein–coupled receptor (GPCR) signals leading to activation of RhoA and plays a role in cell migration. The mechanism underlying the role of Gα13 in cell migration, however, remains unclear. We successfully show that a direct interaction between Gα13 and the cytoplasmic domain of the integrin β1 subunit plays a critical role in β1-dependent cell migration. Point mutation of either glutamic acid in the Gα13-binding 767EKE motif in β1 or treatment with a peptide

derived from the Gα13-binding sequence of β1 abolished Gα13–β1 interaction and inhibited β1 integrin–dependent cell spreading and migration. We further show that the Gα13-β1 interaction mediates β1 integrin–dependent Src activation and transient RhoA inhibition during initial cell adhesion, which is in contrast to the role of Gα13 in mediating GPCR-dependent RhoA activation. These data indicate that Gα13 plays dynamic roles in both stimulating RhoA via a GPCR pathway and inhibiting RhoA via an integrin signaling pathway. This dynamic regulation of RhoA activity is critical for cell migration on β1 integrin ligands.

Conclusively, we demonstrate, for the first time, that integrins are a class of non-canonical G-protein-coupled receptors (GPCRs), which requires Gα13 binding to transmit integrin outside-in signaling. The binding of Gα13 to integrins play a common role in integrin-dependent cell functions, including platelet spreading and aggregation (platelet integrin αIIbβ3), cell migration (integrin β1), and potentially all other cell functions mediated by EXE motif-containing integrins. This finding helps to develop new therapeutic methods in the prevention and treatment of integrin-related diseases such as stroke, heart attack, and cancer.

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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

March 28, 2014

Xiaoping Du
Pharmacology
M/C 868

Dear Dr. Du:

The protocol indicated below was reviewed at a convened ACC meeting in accordance with the Animal Care Policies of the University of Illinois at Chicago on **2/18/2014**. *The protocol was not initiated until final clarifications were reviewed and approved on* **3/28/2014**. *The protocol is approved for a period of* ***3*** years with annual continuation.

Title of Application: Signaling Mechanisms of Platelet Activation

ACC Number: 14-017

Initial Approval Period: 3/28/2014 to 2/18/2015

Current Funding: ***Portions of this protocol are supported by the funding sources indicated in the table below.***
Number of funding sources: 3

Funding Agency	**Funding Title**			**Portion of Proposal Matched**
NIH	*Signaling Mechanism of Platelet Glycoprotein Ib-Ix*			*All matched*
Funding Number	**Current Status**	**UIC PAF NO.**	**Performance Site**	**Funding PI**
RO1 HL062350 (A1 version years 10-14)	*Funded*	*201000091*	*UIC*	*Xiaoping Du*
Funding Agency	**Funding Title**			**Portion of Proposal 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**
RO1 HL080264 (A1 version Years 5-8)	*Funded*	*201102188*	*UIC*	*Xiaoping Du*
Funding Agency	**Funding Title**			**Portion of Proposal 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**
HHSN268201400007C	*Funded*	*201300887*	*UIC*	*Xiaoping Du*

This institution has Animal Welfare Assurance Number A3460.01 on file with the Office of Laboratory Animal Welfare (OLAW), 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 funding proposal are matched to this ACC protocol.**

In addition, all investigators are responsible for ensuring compliance with all federal and institutional policies and regulations related to use of animals under this protocol and the funding sources listed on this protocol. Please use OLAW's "*What Investigators Need to Know about the Use of Animals*" (http://grants.nih.gov/grants/olaw/InvestigatorsNeed2Know.pdf) as a reference guide. Thank you for complying with the Animal Care Policies and Procedures of UIC.

Sincerely yours,

Bradley Merrill, PhD
Chair, Animal Care Committee

BM/*mbb*
cc: BRL, ACC File, Aleksandra Stojanovic, PAF *201000091, 20110218, 201300887*

UIC UNIVERSITY OF ILLINOIS AT CHICAGO

May 12, 2014

Xiaoping Du
Pharmacology
M/C 868

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

Dear Dr. Du:

The protocol indicated below has been reviewed in accordance with the Institutional Biosafety Committee Policies of the University of Illinois at Chicago on 3/13/2014**.** *The protocol was not initiated until final clarifications were reviewed and approved on* ***5/7/2014. Protocol expires 3 years from the date of review (3/13/2017). This protocol replaces protocol 11-014 which has been terminated.***

Title of Application: Signaling Mechanisms of Platelet Activation

IBC Number: 14-008

Highest Biosafety Level: 2

Condition of Approval: The enclosed report indicates the training status for bloodborne pathogen (BBP) training. Only those personnel who have been trained and whose training has not expired are approved for work that may involve exposure to bloodborne pathogens. Please note that federal regulations require yearly training for BBP.

You may forward this letter of acceptable IBC verification of your research protocol to the funding agency considering this proposal. **Please be advised that investigators must report significant changes in their research protocol to the IBC office via a letter addressed to the IBC chair prior to initiation of the change. If a protocol changes in such a manner as to require IBC approval, the change may not be initiated without IBC approval being granted.**

Thank you for complying with the UIC's Policies and Procedures.

Sincerely,

Randal C. Jaffe, Ph.D.
Chair, Institutional Biosafety Committee

RCJ/mbb

Enclosures

Cc: IBC file, Aleksandra Stojanovic-Terpo

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
Amendment to Research Protocol and/or Consent Document – Expedited Review
UIC Amendment # 22

November 19, 2014***; Revised***

Xiaoping Du, MD, PhD
Pharmacology
835 S. Wolcott Ave., 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:

Revision: The new research protocol was inadvertently omitted.

Please note that the research training for ***Bo Shen*** **will** expire on **11/26/2014** and he must complete a minimum of two hours of continuing education prior to the expiration date in order to continue to participate in the conduct of the research. You may refer him to the OPRS website, where continuing education offerings are available:
http://tigger.uic.edu/depts/ovcr/research/protocolreview/irb/education/2-2-2/ce_requirements.shtml

Members of Institutional Review Board (IRB) #3 have reviewed this amendment to your research and/or consent form under expedited procedures for minor changes to previously approved research allowed by Federal regulations [45 CFR 46.110(b)(2)]. The amendment to your research was determined to be acceptable and may now be implemented.

Please note the following information about your approved amendment:

Amendment Approval Date: November 11, 2014
Amendment:

Summary: UIC Amendment #18 dated 11/5/14 and received 11/7/14 is an investigator initiated amendment to add Xiang Shen as key personnel, remove Michael Keegan Delaney as key personnel, and to remove the National Institute of Health - National Heart Lung and Blood Institute as a funding source (Grant # HL 062350-08). The revised appendix P and Z have been submitted.

Research Protocol:

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

Please note the Review History of this submission:

Receipt Date	Submission Type	Review Process	Review Date	Review Action
11/07/2014	Amendment	Expedited	11/11/2014	Approved

Please be sure to:

→ **Use only the IRB-approved and stamped consent document(s) and/or HIPAA Authorization form(s) enclosed with this letter when enrolling subjects.**

→ 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 enclosure,
"**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 #3 has the right to ask further questions, seek additional information, 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 the OPRS at (312) 996-1711 or me at (312) 355-2939. Please send any correspondence about this protocol to OPRS at 203 AOB, M/C 672.

Sincerely,

Jewell Hamilton, MSW
IRB Coordinator, IRB # 3
Office for the Protection of Research Subjects

Enclosure(s): None

cc: Asrar Malik, Pharmacology, M/C 868

VITA

EDUCATION

2015, Ph.D, Pharmacology, University of Illinois College of Medicine, Chicago
2008, Bachelor of Science, Life Science, National University of Singapore, Singapore

HONORS AND AWARDS

Undergraduate Scholarship, National University of Singapore & Ministry of Education, Singapore (2004 – 2008)
Student Presenter Award, UIC (2011, 2013)
UIC Graduate College Medical Research Fellowship (2012 - 2014)
Albert and Doris Woeltjen Award, Department of Pharmacology, UIC (2013)
Dean's Scholar Award (2013 - 2014)
ATVB Scientific Sessions Travel Awards for Young Investigators (2014)
ASH Abstract Achievement Award (2014)
Chinese Government Award for Outstanding Students Abroad, with Special Award for Excellence (2014)
Klaus Unna Award, Department of Pharmacology, UIC (2014)

PUBLICATION

Brian Esteves, Kyungho Kim, M. Keegan Delaney Aleksandra Stojanovic-Terpo, **Bo Shen**, Changgeng Ruan, Jaehyung Cho, Zaverio M. Ruggeri, Xiaoping Du. Signaling-Mediated Cooperativity between Glycoprotein Ib-IX and Protease-Activated Receptors in Thrombin-Induced Platelet Activation. ***Blood***, under press

Bo Shen, Brian Esteves, Barry Kreutz, Andrei Karginov, Yanyan Bai, Feng Qian, Urao Norifumi, Deane F. Mosher, Xiaoping Du. The interaction of Gα13 with integrin β1 mediates cell migration by dynamic regulation of RhoA. ***Mol Biol Cell*** 2015 Oct 15;26(20):3658-70.

Brian Estevez, **Bo Shen**, Xiaoping Du**.** Targeting Integrin and Integrin Signaling in Treating Thrombosis. ***Arteriosclerosis, Thrombosis, and Vascular Biology*** 2015 Jan; 35(1):24-9

M. Keegan Delaney, Junling Liu, Kyungho Kim, **Bo Shen**, Aleksandra Stojanovic-Terpo, Yi Zheng, Jaehyung Cho, Xiaoping Du. Agonist-induced platelet procoagulant activity requires shear and a Rac1-dependent signaling mechanism. ***Blood*** 2014 Sep 18;124(12):1957-67

Bo Shen, Xiaojuan Zhao, Kelly A. O'Brien, Aleksandra Stojanovic-Terpo, M. Keegan Delaney, Kyungho Kim, Jaehyung Cho, Stephen C-T Lam, and Xiaoping Du. A mechanism for the directional switch of integrin signaling and selective inhibition of outside-in signaling as a new anti-thrombotic strategy. ***Nature*** 2013 Nov 7;503(7474):131-5.

Bo Shen, M. Keegan Delaney, Xiaoping Du. Inside-out, outside-in, and inside-outside-in: G protein signaling in integrin-mediated cell adhesion, spreading, and retraction. ***Curr Opin Cell Biol*** 2012 Oct;24(5):600-6.

Qiang Guo, Jun Jina, , Jason X.-J. Yuan, Amy Zeifman, Jiwan chen, **Bo Shen**, Jianan Huang. VEGF, Bcl-2 and Bad regulated by angiopoietin-1 in oleic acid induced acute lung injury. ***Biochem Biophys Res Commun*** 2011 Oct 7;413(4):630-6..

Haixia Gong; **Bo Shen**; Panagiotis Flevaris; Christina Chow; Stephen C-T Lam; Tatyana A Voyno-Yasenetskaya; Tohru Kozasa; Xiaoping Du. G protein subunit Galpha13 binds to integrin alphaIIbbeta3 and mediates integrin "outside-in" signaling. ***Science*** 2010;327(5963):340-3.

CONFERENCE PRESENTATIONS

Bo Shen, Brian Esteves, Barry Kreutz, Andrei Karginov, Yanyan Bai, Feng Qian, Urao Norifumi, Deane F. Mosher, Xiaoping Du. The interaction of Gα13 with integrin β1 mediates cell migration by dynamic regulation of RhoA. **ASCB 2015 Meeting** in San Diego, Dec 12-16, 2015

Bo Shen, Brian Estevez, Barry Kreutz, Andrei Karginov, Deanne Mosher, Yanyan Bai, Feng Qian, Urao Norifumi, Deane F. Mosher, Xiaoping Du. "The Interaction of Integrin beta1 to Galpha13 Mediates RhoA Inhibition and Cell Migration." **AHA ATVB/PVD Scientific Sessions** in San Francisco, May 7–9, 2015

Bo Shen, Xiaojuan Zhao, Kelly A O'Brien, Aleksandra Stojanovic-Terpo, M Keegan Delaney, Kyungho Kim, Jaehyung Cho, Stephen C-T Lam, Xiaoping Du. "A directional switch of integrin signalling and a new anti-thrombotic strategy." **15th Biennial Midwest Platelet Conference** in Chicago, October 8-10, 2014.

Bo Shen, Xiaojuan Zhao, Kelly A O'Brien, Aleksandra Stojanovic-Terpo, Michael Keegan Delaney, Kyungho Kim, Jaehyung Cho, Stephen C.-T Lam, and Xiaoping Du. 'Selective Inhibition of Outside-In Signaling for Platelet Integrin alphaIIbbeta3 as a Novel and Safer Anti-Thrombotic Strategy.' **2014 Thrombosis and Hemostasis Summit of North America (THSNA)** in Chicago, IL, Apr, 2013.

Bo Shen, Xiaojuan Zhao, Kelly A O'Brien, Aleksandra Stojanovic-Terpo, Michael Keegan Delaney, Kyungho Kim, Jaehyung Cho, Stephen C.-T Lam, and Xiaoping Du. 'Selective Inhibition of Outside-In Signaling for Platelet Integrin alphaIIbbeta3 as a Novel Anti-Thrombotic Strategy Without Adverse Effect of Bleeding.' ***Circulation***. 2013; 128: A16050. **AHA Scientific Sessions** in Dalls, TX, Nov, 2013.

Bo Shen, Xiaojuan Zhao, Kelly A O'Brien, Aleksandra Stojanovic-Terpo, Michael Keegan Delaney, Kyungho Kim, Jaehyung Cho, Stephen C.-T Lam, and Xiaoping Du. 'A Mechanism For Switch Of Integrin Signaling Direction and a New Anti-Thrombotic Strategy Through Selective Outside-In Signaling Inhibition.' ***Blood*** November 15, 2013 vol. 122 no. 21 2295. **55th ASH Annual Meeting and Exposition** in Dalls, TX, Nov, 2013.

Bo Shen, Xiaojuan Zhao, Aleksandra Stojanovic-Terpo, Kelly A. O'Brien, M. Keegan Delaney, Stephen C.-T. Lam, Xiaoping Du. 'A mechanism for directional switch of integrin signaling and the concept of selective inhibition of outside-in signaling as a new anti-thrombotic strategy.' **Gordon Research Conference (Fibronectin, Integrins & Related Molecules)** in Ventura, CA, Feb 9th to 16th, 2013.

Bo Shen, Xiaojuan Zhao, Kelly A. O'Brien, Stephen C.-T. Lam, and Xiaoping Du. 'Coordinated and opposing waves of talin and Galpha13 binding to integrin cytoplasmic domain switch directions of integrin signaling.' 14th **Biennial Midwest Platelet Conference** in Cleveland, OH. Oct 18th-19th 2012.

Bo Shen, Xiaoping Du. 'The Interaction between Galpha13 and Integrin beta1 Cytoplasmic Domain Mediates Cell Migration.' ***Circulation*** 2011; 124: A16570. **AHA Scientific Sessions** in Orlando, FL, Nov 12th-16th, 2011