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ADP-RIBOSYLATION FACTOR 6 (ARF6) REGULATES INTEGRIN αIIbβ3 TRAFFICKING, PLATELET SPREADING, AND CLOT RETRACTION

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ADP-RIBOSYLATION FACTOR 6 (ARF6) REGULATES INTEGRIN αIIbβ3 TRAFFICKING, PLATELET SPREADING, AND CLOT RETRACTION

DISSERTATION

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the College of Medicine at the University of Kentucky

By

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ABSTRACT OF DISSERTATION

ADP-RIBOSYLATION FACTOR 6 (ARF6) REGULATES INTEGRIN αIIbβ3 TRAFFICKING, PLATELET SPREADING, AND CLOT RETRACTION

Endocytic trafficking of platelet surface receptors plays a role in the accumulation of granule cargo (*i.e.* fibrinogen and VEGF) and thus could contribute to hemostasis, angiogenesis, or inflammation. However, the mechanisms of platelet endocytosis are poorly understood. The small GTP-binding protein, ADP-ribosylation factor 6 (Arf6), regulates integrin trafficking in nucleated cells; therefore, we posited that Arf6 functions similarly in platelets. To address this, we generated platelet-specific, Arf6 knockout mice. $Arf6^{-/-}$ platelets had a storage defect for fibrinogen but not other cargo, implying Arf6's role in integrin αIIbβ3 trafficking. Additionally, platelets from $Arf6^{-/-}$ mice injected with biotinylated-fibrinogen, showed lower accumulation of the modified protein than did WT mice. Resting and activated αIIbβ3 levels, measured by FACS, were unchanged in $Arf6^{-/-}$ platelets. $Arf6^{-/-}$ platelets had normal agonist-induced aggregation and ATP release; however, they showed faster clot retraction and enhanced spreading, which appears due to altered αIIbβ3 trafficking since myosin light chain phosphorylation and Rac1 activation, in response to thrombin, were unaffected. $Arf6^{-/-}$ mice showed no hemostasis defect in tail-bleeding or $FeCl_3$–induced carotid injury assays. These data suggest a role for Arf6 in integrin αIIbβ3 trafficking in platelets.

Additionally, the regulation of Arf6 in platelets was also investigated, focusing on integrin αIIbβ3 outside-in signaling which was suggested to be responsible for the second wave of Arf6-GTP loss. G protein-coupled receptor kinase-interacting protein 1 (GIT1), a GTPase-activating protein (GAP) toward Arf6, is suggested to be involved in αIIbβ3 downstream signaling. I found that GIT1, complex with β-PIX, was translocated to the detergent-insoluble pellet upon human platelet activation, a process that is blocked by RGDS and myrArf6 peptide treatment. Moreover, tyrosine-phosphorylation of GIT1 was impaired by treatment with both peptides or with actin polymerization inhibitors. GIT1's role in

platelets was further studied using platelet-specific, GIT1 knockout mice. GIT1$^{-/-}$ platelets failed to show any defect, including clot retraction or fibrinogen storage. Unlike human platelets, GIT1 expression levels were much lower in mouse platelets, suggesting that GIT2 may be the functionally relevant Arf6-GAP in mouse platelets. The data in this dissertation identify that Arf6 mediates fibrinogen storage, implying its role in integrin αIIbβ3 trafficking in platelets.

KEYWORDS: Platelets, Arf6, GIT1, Integrin αIIbβ3, Trafficking

Yunjie Huang

March 16th, 2015

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To Fanmuyi, Francis, Irene

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Platelet Biology Overview

Platelets are anuclear cytoplasmic fragments released in bone marrow from their precursor cells, megakaryocytes [1, 2]. They are the smallest blood cells in the blood stream and are important players for thrombosis and hemostasis [3, 4]. Platelets have several featured characteristics to fulfil their functions. First, platelets express a unique set of receptors on their surface, including P2Y purinergic receptors, PAR (protease activated receptor) receptors, GPVI (glycoprotein VI), and integrins, which are adhesion molecules linking the intracellular actin cytoskeleton network to the extracellular matrix. These receptors, upon stimulation by their ligands, induce different degrees of platelet activation in a spatiotemporally coordinated manner [5]. This is essential for the regulation of platelet activity. Additionally, endocytic trafficking of these receptors is an important mechanism for platelets to communicate with the surrounding environment by taking up or releasing small molecules and proteins, or by changing the surface level of these receptors at the plasma membrane [6-11]. Secondly, platelets contain three main types of granules, α-granule, dense granule and lysosome [12, 13]. Upon platelet activation, granule content is released by fusion of granule membranes to the plasma membrane. This fusion is mediated by SNAREs (soluble N-ethylmaleimide-sensitive fusion protein attachment receptor) proteins. The released material (releasate) from the three granules can either affect platelet activity in an autocrine or paracrine manner or affect other cells in the surrounding microenvironment. Lastly, human and mouse platelets have a unique membrane structure, the open canalicular system (OCS) which is thought to be a membrane reservoir that is important for platelet spreading on a wounded site [14, 15]. Therefore, platelets are unique cells, whose activity is highly regulated and whose function actually has been explored extensively beyond thrombosis and hemostasis.

Platelet Structures

Platelets are produced from megakaryocytes in bone marrow. Their production is a tightly regulated process, involving a complex of transcription factors, signaling

molecules and microenvironment [16, 17]. Each day, about 1 X 10^{11} platelets are produced in a healthy adult to maintain a normal platelet count in the body. Once released from megakaryocytes, platelets have a lifespan of approximately 10 days [18]. Resting platelets are of discoid shape, which is maintained by both microtubule and filamentous-actin cytoskeletons [19-21]. They contain three major granules, dense granule, α-granule, and lysosome [12]. In addition to these three major granules, platelets also contain mitochondria, peroxisomes, a dense-tubular system [22], and multivesicular bodies (MVBs) which are suggested to be an intermediate stage in α-granule and dense-granule biogenesis [23]. There are approximately three to eight dense granules per platelet [24]. These granules contain small molecules such as calcium, serotonin, ADP, and ATP, *etc*. ADP is important for amplification of platelet activation during thrombosis. Each platelet has about fifty to eighty α-granules [25]. These granules house various biologically active proteins, which are involved in a vast array of physiological processes, including adhesion, angiogenesis and inflammation. α-Granule cargo are from two major sources. One is derived endogenously by protein synthesis in the megakaryocyte, *e.g.* β-thromboglobulin [26], platelet factor 4 (PF4) [27], and von Willebrand Factor (vWF) [28]. The other is taken up from plasma via receptor-mediated endocytosis, fluid phase pinocytosis or perhaps even phagocytosis. As an example, fibrinogen uptake is dependent on the major membrane glycoprotein IIbIIIa (integrin αIIbβ3) [29]. Alpha granules also contain a number of membrane proteins essential for platelet functions, including integrin αIIbβ3, P-selectin (CD62P) and CD36 [25]. Upon granule exocytosis, following platelet activation, α-granules not only release their contents but also become part of the plasma membrane thus facilitating the exposure of the granule membrane proteins on the plasma membrane. Lysosomes are another type of organelle/granule in platelets, which contain degradative enzymes, including β-hexosaminidase, cathepsin, and acid phosphatases [30]. These enzymes are also released upon platelet activation, but only in response to strong agonists [31]. Activation-dependent granule secretion is mediated by two sets of SNARE proteins, vesicle SNAREs (v-SNAREs) and target membrane SNAREs (t-SNAREs) [32, 33]. Using genetic animal model and platelets from patient with secretion defects, it has been demonstrated that VAMP8 (vesicle-associated

membrane protein 8, v-SNARE) [34], Syntaxin11 (t-SNARE) [13], and SNAP23 (t-SNARE) [35] are the major SNARE proteins critical for platelet exocytosis.

Another unique but obvious structure in platelets is the open canalicular system (OCS) [15]. The OCS is characterized by the invagination of the plasma membrane. It was thought to have three major functions [14, 36]. The first one is as a membrane reservoir which is important for the formation of filopodia and for spreading following platelet adhesion to an activating surface. Second is to be a route through which external elements could enter into platelets or granule cargo could release to platelet exterior. It is thought that the OCS is one of the places where fibrinogen could be bound before being internalized in an integrin αIIbβ3-dependent manner [37]. Lastly, the OCS could be used as a cryptic store of plasma membrane glycoproteins [38]; although exposure of many of these proteins is now thought to occur subsequent to granule fusion. Recent unpublished data from the Storrie group has suggested that the OCS may be more complex. Tomography analysis of platelets has suggested that the OCS may, in fact, represent endosomal compartments that are not connected to the plasma membrane but that fuse to the plasma membrane at the earliest stages of platelet activation. These new findings greatly affect previous visions of the OCS and suggest that platelets may contain a much more extensive endosomal membrane system than previously thought. This interpretation is supported by data presented in this thesis.

Platelet Receptors and Their Signaling during Platelet Activation

Platelets express many receptors on their plasma membrane. The number of the receptors is still increasing due to recent proteomic analyses [39]. Here, only the receptors related to this thesis work are discussed. Interactions between the ligands and their corresponding receptors induce different downstream signaling pathways which are essential for platelet activation, *e.g.* adhesion, aggregation, spreading, and clot retraction [5]. In vivo, these receptors are working cooperatively rather than independently in order for platelets to do what they are needed to do.

<u>G protein-coupled receptors</u>

Platelets express multiple G protein-coupled receptors (GPCRs), a family of seven-transmembrane domain receptors that bind to and transmit signals through

heterotrimeric G proteins [40]. The heterotrimeric G proteins consist of three subunits, α, β, and γ. Upon ligand binding to the receptor, the α subunit (Gα) is activated, converting from the inactive GDP-bound form to the active GTP-bound form, and is dissociated from the receptor and the Gβγ complex. By interacting with their own specific downstream targets, Gα and Gβγ transmit the receptor's signals [41]. Based on functional specificity, Gα subunit can be classified into G_q, G_{12}/G_{13}, G_i and G_s subfamilies. The GPCRs expressed on platelet surface include protease-activated receptors (PARs), purinergic receptors (P2Y receptors), and thromboxane A_2 (TXA_2) receptor (TP).

There are four PAR receptors, PAR1 – PAR4 [42]. Human platelets express PAR1 and PAR4, while mouse platelets express PAR3 and PAR4. These receptors are activated by thrombin, one of the most potent platelet agonists. PAR1 and PAR4 in human platelets are activated by low and high concentrations of thrombin, respectively [43]. In contrast, PAR3 in mouse platelets functions as a co-receptor for PAR4 [44]. These PARs are coupled to G_q- and G_{12}/G_{13}-mediated signaling transductions. Since thrombin is generated on a cellular surface when the subendothelial Tissue Factor is exposed to plasma coagulation factors [45], PARs-mediated platelet activation occurs after platelet adhesion and some level of platelet activation. During the thrombus formation, thrombin is further generated and released [46]. Thus, thrombin-mediated platelet activation acts in a positive-feedback manner to greatly amplify platelet activation and to recruit more platelets to the growing thrombus. This positive-feedback mechanism is also shared by other GPCRs-mediated platelets activation as discussed below.

Platelets contain two major purinergic receptors, $P2Y_1$ and $P2Y_{12}$ [47-51]. Activation of these receptors is mediated by adenosine diphosphate (ADP) which is stored at high concentrations in dense granules in platelets and is released upon platelet activation. Released ADP activates platelets in either an autocrine manner or a paracrine manner. $P2Y_1$ and $P2Y_{12}$ are coupled to G_q and G_i, respectively. Like thrombin, ADP is important to amplify platelet activation and to recruit more platelets to the growing thrombus. Moreover, P2Y receptors have a unique feature – endocytic trafficking [52]. Endocytic trafficking of P2Y receptors allows platelets to be desensitized and resensitized to ADP.

It has been shown that $P2Y_1$ and $P2Y_{12}$, upon ADP stimulation, rapidly desensitize in a kinase-dependent manner [53]. Endocytosis of $P2Y_1$ is dependent on protein kinase C (PKC) activity, whereas $P2Y_{12}$ requires G protein-coupled receptor kinase (GSK). After removal of agonist, P2Y receptors recycle back to the cell surface, a process that is called resensitization. ADP-ribosylation factor 6 (Arf6) has been suggested to be important for $P2Y_{12}$ internalization by regulating Nm23-H1, an NDP (nucleoside diphosphate) kinase which facilitates dynamin-dependent fission of clathrin-coated vesicles [54]. However, it is still unclear what physiological significance this desensitization and resensitization cycling of P2Y receptors has.

Thromboxane A_2 is another soluble agonist that mediates the amplification of platelet activation and recruitment [5]. It is generated from arachidonic acid by cyclooxygenase-1 [41], and is released by activated platelets, though the mechanism of this release is unknown. TxA_2 receptor (TP) is coupled to G_q and G_{12}/G_{13} [55, 56]. Mice lacking TP have prolonged bleeding times and are unable to form stable thrombi [57]. Both ADP and TxA_2 are thought to be weaker agonists that which serve to enhance thrombosis but are generally not sufficient to initiate thrombosis unless present in high local concentrations.

GPIb-IX-V complex (GPIb complex)

GPIb complex is a transmembrane receptor, almost exclusively expressed on platelets [58]. The interactions of GPIb complex with von Willebrand factor (vWF) are crucial for the initial capture (transient adhesion) of circulating platelets to the exposed subendothelial matrix [59, 60]. VWF deposited on the subendothelial matrix is either synthesized by endothelial cells or recruited by collagen fiber from plasma [28]. Binding of GPIb complex to vWF activates downstream signaling cascade, leading to integrin activation (see below) and integrin-dependent stable platelet adhesion and aggregation [61-66]. GPIb complex also functions to be a binding site for thrombin, allowing platelet activation by low concentrations of thrombin [67].

Glycoprotein VI (GPVI)

GPVI is a member of the immunoglobulin (Ig)-like receptors and is one of the collagen receptors expressed on platelets [68]. GPVI is exclusively expressed in platelets where it non-covalently associates with a transmembrane adapter protein, FcRγ [69].

Collagens are the most abundant proteins in the subendothelial matrix. Dimerized GPVI has high affinity for collagen [70]. Interaction of GPVI with collagen initiates downstream signaling that is important for platelet activation [9]. In addition to collagen, convulxin, a C-type lectin venom, is a potent, specific ligand to GPVI and induces platelet activation by similar downstream signaling as triggered by collagen [71].

Integrins

Integrins are heterodimeric membrane proteins consisting of two subunits, α subunit and β subunit, and are cell adhesion receptors mediating the binding of cells to extracellular matrix, cell-surface ligands, and soluble ligands [72]. In vertebrate, there are 18 α subunits and 8 β subunits, generating 24 heterodimers. The subunits are non-covalently associated [73, 74]. Usually, integrins have big extracellular domain, a single transmembrane domain from each subunit, and small cytoplasmic tail, although the α and β subunits have distinct domain structures. Both subunits contribute to the ligand-binding site in their extracellular domain, with specific binding ligand for individual integrin. Integrin functions are regulated by both integrin signaling and integrin trafficking, and integrin dysfunction can cause severe diseases like cancer.

Platelets express multiple integrins for different ligands (Table 1, [75]). In resting platelets, integrins are kept in a bent or low-affinity state. Upon platelet activation, they are transformed into an extended or high-affinity state.

Integrin α2β1

Integrin α2β1 is another collagen receptor (in addition to GPVI) on platelets [76]. It plays a role in the adhesion of platelets to collagen and for subsequent optimal activation [77]. Deletion of α2 subunit or β1 subunit in mice does not cause bleeding defects, although these platelets show minor defects in platelet adhesion and aggregation to collagen [77, 78]. However, combined deficiency of α2β1 and GPVI in mice causes complete inhibition of thrombus formation [79]. Thus, it is widely accepted that α2β1 and GPVI function synergistically for optimal platelet adhesion and activation by collagen.

Integrin α5β1

Integrin α5β1 is the principal platelet receptor for fibronectin [80]. It supports adhesion of resting platelet to fibronectin in static conditions [81]. However, α5β1-fibronectin interaction does not cause platelet tyrosine phosphorylation of focal adhesion kinase (FAK), calcium oscillation and lamellipodia formation [81]. Since this interaction is sensitive to shear stress, it is thought that α5β1 may play a role in injuries in the larger blood vessels where shear forces are low [82].

Integrin α6β1

Integrin α6β1 is the principal laminin receptor in platelets [80]. Binding of platelets to laminin via α6β1 does not lead to platelet aggregation. But, α6β1-mediated platelet adhesion to laminin induces signaling pathways, leading to filopodia formation [81].

Integrin αvβ3

Integrin αvβ3 prefers vitronectin as its ligand, although it can also bind osteopontin and fibronectin [83]. It has been shown that integrin αvβ3 mediates platelet adhesion to vitronectin and osteopontin *in vitro* [84]. Integrin αvβ3 support adhesion on fibronectin and promote formation of filopodia but not lamellipodia or FAK phosphorylation [81]. However, the in vivo role of integrin αvβ3 is unknown.

Integrin αIIbβ3

Integrin αIIbβ3 binds several Arg-Gly-Asp (RGD)-containing ligands, including fibrinogen (the major ligand), vWF, vitronectin, and fibronectin. It mediates platelet aggregation by crosslinking integrin αIIbβ3 on different platelets through fibrinogen, and serves as the principle receptor for platelet adhesion in vivo. In human, mutation on αIIb and β3 leads to the so-called Glanzmann thrombasthenia (GT) syndrome, a bleeding disorder due to quantitative or qualitative defects in αIIbβ3. Deletion of β3 subunit in mice represents a good model for human GT, having prolonged bleeding times, abnormal platelet aggregation and clot retraction [85].

Platelets and Hemostasis

Platelets play an essential role in hemostatic plug formation at the vascular injury site, in a spatiotemporally coordinated manner [86, 87]. This process can be roughly divided into three phases: 1) initiation phase: in which platelets adhere to the exposed subendothelial extracellular matrix and are activated, 2) extension phase: where additional platelets are recruited and further activated by locally released platelet

Table 1 Integrins in Platelets

Integrin	Number per Platelet	Ligand(s)	Other Name	Reference
α2β1	2000 - 4000	Collagen	CD49b/CD29, VLA-2	[81, 83, 84, 88]
α5β1	2-4000	Fibronectin	CD49c/CD29, VLA-5	
α6β1	2-4000	Laminin	CD49f/CD29, VLA-6	
αvβ3	a few hundred	Vitronectin, Fibronectin, Osteopontin	CD51/CD61	
αIIbβ3	50,000 – 80,000	Fibrinogen, vWF, Fibronectin, vitronectin, Thrombospondin, CD40 ligand	CD41/CD61	

agonists, 3) stabilization phase: where a consolidated hemostatic plug is formed [5, 89].

In the initiation phase, the subendothelial extracellular matrix is exposed. This matrix contains several adhesive macromolecules, including collagen, vWF, laminin, fibronectin and thrombospondin. They interact with different platelet receptors, mediating platelets adhesion to the injury site. Under high shear conditions (>1000 s^{-1}), as in small arteries, platelets are arrested by interactions between platelet GPIb-IX-V and vWF [90]. VWF can be synthesized by both endothelial cells and megakaryocytes/platelets and stored in Weibel-Palade bodies and α-granules, respectively [91-93]. Endothelial cells constitutively secrete their vWF into the plasma. At the event of vascular damage, circulating vWF is rapidly recruited to the matrix by interactions with collagen. Recently, it has been reported that vWF from endothelial cells is sufficient to support hemostasis and vWF from megakaryocytes/platelets can also contribute to hemostasis in the absence of EC derived vWF [28]. In contrast, under low shear conditions (<1000 s^{-1}), as in veins, adhesion of platelets to subendothelial matrix is mediated by collagen, fibronectin, and laminin [94, 95]. Collagen receptors, including GPVI and integrin α2β1, are important for platelets to form stable adhesions [79].

In the extension phase, platelets are activated by different signal transduction pathways after stable binding to extracellular matrix. This causes increased cytosolic Ca^{2+}, cytoskeletal rearrangement, and integrin activation. The signaling pathways to activate platelets include both tyrosine kinase (e.g. GPVI, GPIb complex)-based signaling and G protein-coupled receptors (e.g. thromboxane A2, P2Y, PAR receptors)-based signaling. Upon platelet activation, a controlled release reaction (the fusion of platelet granules with plasma membrane) occurs. Platelets contain three main types of granules: dense granule, α-granule, and lysosome. The released contents, especially the ADP from dense granules, act in a para- or autocrine manner, to quickly amplify the platelet activation by activating nearby platelets. α-Granules contents are heterogeneous: chemokines, (anti-) angiogenic molecules, growth factors and both pro and anti-thrombotic molecules. Thus, the effects induced by α-granule releasate are diverse, including: primary hemostasis, coagulation, inflammation, angiogenesis,

and wound healing [25]. For hemostasis, released vWF and fibrinogen play roles in mediating the cross-linking of platelets via αIIBβ3 and GPIb complex. These interactions induce outside-in signaling which leads to further activation of αIIbβ3. It is known that there is an intracellular pool of integrin αIIbβ3 and GPVI on α-granule membranes [96-99]. Thus, upon α-granule exocytosis, these receptors are exposed on plasma membrane, which amplifies the platelet activation process. Dense granules contain serotonin, nucleotides, and Ca^{2+}, and play a direct role in platelet amplification. ADP secreted from dense granules interacts with two biochemically related purinergic G protein-coupled receptors, $P2Y_1$ and $P2Y_{12}$. Interaction between ADP and $P2Y_1$ causes platelet shape change and aggregation through G_q-mediated phospholipase C-β2 activation [100]. Meanwhile, ADP binding to $P2Y_{12}$ induces sustained platelet activation by G_i-mediated inhibition of adenylyl cyclase activity [101]. These two receptors are equally required for conversion of free arachidonic acid to the secondary activator, TxA2 [102]. The signaling discussed above leads to inside-out activation of integrin αIIbβ3 and platelet aggregation, which is facilitated by fibrinogen-mediated crosslinking of integrin αIIbβ3 on different platelets.

In the stabilization phase, irreversible platelet aggregates are formed. Fibrinogen, vWF and fibronectin all contribute to the formation of stable platelet aggregates by interacting with activated integrin αIIbβ3. Interestingly, mouse lacking both vWF and fibrinogen was still able to form fragile thrombi [103]. Wang *et al* reported that fibronectin was the first component deposited onto the subendothelial extracellular matrix of damaged vessel and plays an important role for stable thrombi formation in mouse [104, 105]. At the last stages, a fibrin network is formed to stabilize the platelet thrombus, which is mediated by thrombin-generating coagulation system.

At the other side of thrombus formation, the inhibitory signaling for platelet activation is required to limit the spontaneous platelet aggregation that leads to vessel occlusion and potential ischemia. These signaling pathways are also important to limit the thrombus size to the site of vascular injury. Nitric Oxide (NO) and prostacyclin (PGI_2) generated by endothelial cells are strong negative regulators pacifying platelet activation [106, 107].

Platelets and Cancer

In additional to the well-recognized role in hemostasis, increasing evidence supports that, platelets are also important players in multiple physiological or pathological conditions, including inflammation, angiogenesis, and cancer [108]. Although these conditions are distinct and unique in their development and progression, platelet contributions to these processes have similarity at certain levels.

The association between platelets and cancer progression has been known for over a century as Professor Armand Trousseau reported recurrent migratory thrombophlebitis as early as in 1865. However, interactions between tumor cells and platelets has only recently been studied more carefully [109]. It has been suggested that the ability of tumor cells, *e.g.* adenocarcinoma cells and SKNMC neuroblastoma, to undergo extensive interactions with platelets may be critical for the successful metastatic spread of tumor cells [109-111].

The ability of tumor cells to aggregate platelets, often called tumor cell-induced platelet aggregation (TCIPA), is tumor type-dependent, and varies due to the mechanisms used.

Cancer cells can stimulate the release of platelet contents, like ADP, which contributes to TCIPA. SKNMC [112] and neuroblastoma small-cell lung cancer [113], breast carcinoma [114] and fibroblastoma [115] can mediate TCIPA by tumor cell-dependent ADP release from platelets.

Cancer cells can also stimulate the production and release of thromboxane A2, which in turn leads to TCIPA probably by activating platelets through thromboxane receptors. It has been shown that TCIPA induced by osteosarcoma can be inhibited by BM-567 which is the original TXA_2 synthase inhibitor and TXA_2 receptor antagonist [116]. It has also been shown that ketoconazole, a thromboxane synthetase and 5-lipoxygenase inhibitor, is able to significantly inhibit thromboxane B2 formation and reduce hepatic metastasis from the human pancreatic carcinoma in the nude mouse model [117].

Cancer cells are able to stimulate TCIPA by generating serine proteinases (e.g. thrombin), cysteine proteinases (e.g. cathepsin B), and matrix metalloproteinases (MMPs). Human glioblastoma [118], neuroblastoma [119], and pancreatic cancer [120] cells can generate thrombin, and thus increase TCIPA. It was shown that cathepsin B can be released from cancer cells and mediate TCIPA [121-123]. It has been shown

that MMP-2 released from both platelets and cancer cells mediates fibrosarcoma and breast adenocarcinoma cells-induced TCIPA [114, 115, 124].

Lastly, cancer cells can stimulate TCIPA through adhesion receptors, including GPIb-IX-V, Integrin αIIbβ3 and P-selectin. It was shown that breast adenocarcinoma cells expressed GPIb-IX-V [114, 115], and that inhibition of GPIb-IX-V or vWF with blocking antibodies reduced platelet-cancer cell interaction [125, 126]. Integrin αIIbβ3 has been shown to play a crucial role in TCIPA induced by cancer cells of various origin [114, 121, 125-130]. Interaction between P-selectin and mucin was shown to mediate TCIPA in mucin-producing cancers [131-135].

Because of these effects of tumor cells on platelets, cancer patients are at higher risk for a thrombotic incident. Actually, the risk of venous thromboembolism (VTE), *i.e.* deep venous thrombosis or pulmonary embolism, is four- to sevenfold higher in cancer patients compared to healthy individuals [136].

Conversely, platelets act as a potential cancer modulator affecting tumor growth and progression [137]. Platelets play multiple roles in cancer progression, including promoting tumor cell proliferation, protecting tumor cells from immune system elimination, and helping tumor cells arrest at the vessel wall, supporting the establishment of secondary lesions (metastasis).

Platelets promote tumor growth by releasing many factors that affect angiogenesis, a crucial event for tumor growth [138]. These factors could be either anti-angiogenic (*e.g.* angiopoietin 1, sphingosine 1-phosphate, serotonin) or pro-angiogenic (*e.g.* vesicular endothelial growth factor, epidermal growth factor, basic fibroblast growth factor). However, how platelets balance the pro-angiogenic effects and the anti-angiogenic effects remains unclear. It has been suggested that these factors are differentially secreted by platelets upon different stimulation [139]. In addition to angiogenic factors, other enzymatic molecules secreted by platelets also affect cancer progression. Recently, platelet-derived autotaxin (taken up from plasma and stored in α-granules) was suggested to play an important role for tumor cell proliferation, adhesion, migration and thus metastasis [140].

Platelets can help the tumor cells survive in the blood stream by forming hetero-aggregates with tumor cells and leukocytes [138]. Due to the high shear rates and the

immune system, the microenvironment in the blood stream is hostile for tumor cells. TCIPA leads to formation of a 'platelet coat' on the surface of tumor cells and thus shields them from immune responses, creating a protective microenvironment [137]. After entering the blood stream, hetero-aggregates travel within the circulation and then tether and roll along the activated areas of the endothelium. P-selectin from both platelets and endothelial cells contribute to these transient interactions [133]. The firm arrest on the endothelium is mediated by integrins. Integrin αIIbβ3 plays an important role in this process [141, 142].

At last, platelets could assist tumor cells as they exit the bloodstream and invade target organs for metastasis. The release of platelet granule contents and microparticles enhances vascular permeability [143, 144], allowing tumor cells to cross the endothelial barrier and reach secondary lesion sites. Growth factors (*e.g.* platelet-derived growth factor, VEGF, EGF) [145], small lipids (*e.g.* S1P, LPA) [146], serotonin [143] and histamine [147] were shown to be involved in the regulation of vascular permeability for extravasation.

In summary, platelets are special cells circulating in the blood. They have unique structures with different cargo segregated in different granule compartments. They express various receptors controlling different signaling. Upon stimulation, they go through fundamental changes in the shape and in the intracellular structure to perform their functions. Their roles have been extended far beyond their primary role in which they arrest blood loss by forming thrombotic plugs; they are actually actively involved in various physiological and pathological conditions, including cancer.

Subsection Two: Integrin Signaling and Trafficking

Integrin Activation

As discussed above, integrins switch between the bent inactive conformation which has low affinity for its ligand, and extended active conformation, which has high affinity for its ligand. The shift of the integrin conformation from bent to upright is called integrin activation, which could be mediated by either direct binding to immobile substrate or by inside-out signaling. Upon integrin activation, outside-in signaling will transmit the signal into the cell by binding to its ligand.

Integrin inside-out signaling

There are many cellular proteins that have been reported to be important for integrin activation, including talin [148], kindlin [149], Rap1 [150], CalDAG-GEF [151], RIAM (Rab1-GTP interacting adaptor molecule) [152] and so on. Talin consists of a large N-terminal head domain and C-terminal rod domain [153, 154]. The head domain contains a PTB domain which has high-affinity for the integrin β tail and is sufficient to activate integrin [155]. Moreover, the head domain contains a second binding site for the membrane-proximal region of β-integrin cytoplasmic domain, which is required for integrin activation. Binding of talin to integrin destabilizes the interaction between the αIIb integrin transmembrane domain and the β3 integrin transmembrane domain, and thus mediates integrin conformational change and activation. Talin-deficient platelets have severe defects in integrin αIIbβ3 activation and platelet aggregation, and thus platelet-specific talin knockout mice have spontaneous hemorrhages and pathological bleeding [156]. Kindlin is another β-integrin-binding protein that acts as a co-activator of talin for integrin activation [157]. Kindlin binds to integrin at a site distal to the talin-binding region. There are three isoforms of kindlin, kindlin 1, kindlin 2 and kindlin 3. Kindlin 3 deficient platelets exhibit defective activation of αIIbβ3 integrin and impaired aggregation, and thus mice lacking kindlin3 in platelets have a thrombosis formation defect in $FeCl_3$-induced injury of mesenteric arteries [158]. Another player in integrin activation is Rap1, a small GTP-binding protein. Knockout of Rap1b or its exchange factor CalDAG-GEFI in mice results in the partial impairment of agonist-dependent fibrinogen binding to αIIbβ3 integrin and defective platelet aggregation [159, 160]. Also, Rap1 effector proteins, like RIAM, contribute to integrin activation in other cells [152], but not in mouse platelets [161]. A working model for integrin activation in platelets is proposed [162] in which thrombin-mediated activation of PAR receptors stimulates phospholipid hydrolysis, resulting in the generation of inositol triphosphate (IP_3) and diacylglycerol (DAG) which leads to an increased cytosolic free Ca^{2+}, activation of PKC and CalDAG-GEFI. Following that, Rap1 is activated, switching from the GDP-bound form to the GTP-bound form. Activated Rap1 recruits its effector, RIAM, and its binding partner, talin, to the plasma membrane, enabling the interaction between talin and the β3 integrin tail. Kindlin 3

seems to be recruited to the β3 integrin tail to strengthen the activation of integrin following talin binding.

Integrin outside-in signaling

Upon integrin activation and binding to its ligand, integrin outside-in signals are transmitted into cells. In cells attaching to the extracellular substrate, integrin outside-in signaling results in the recruitment of a variety of cytoplasmic proteins to integrin and the formation of a structure called focal adhesion which functions as a mechanical links between intracellular actin bundles and the extracellular substrate. These signals are important for the regulation of cell proliferation, differentiation, *etc* [163, 164]. Integrin cytoplasmic tails, of both α and β subunits, are generally short and lack enzymatic activities. Therefore, adapter proteins are required for transducing integrin signals to the cytoskeleton, cytoplasmic kinases, and transmembrane growth factor receptors. There are many proteins involved in integrin outside-in signaling [165-167], including caveolin-1, focal adhesion kinase (FAK), Src-family kinase, paxillin, and $G\alpha_{13}$. Caveolin-1, an oligomeric membrane protein, has been shown to associate with several integrins such as αvβ3 and α5β1 integrins [168-171]. Suppressing caveolin-1 expression inhibits the formation of focal adhesions, and integrin signaling [172]. It is hypothesized that caveolin-1 helps integrin to cluster on the plasma membrane, and facilitates concentrating integrin-associated structural and signaling proteins [173, 174].

Focal Adhesion Kinase (FAK) behaves as a phosphorylation-regulated signaling scaffold and is important for adhesion turnover [175]. Most integrin signals induce the FAK-mediated pathway [176, 177], although FAK activation is not well understood. Association between FAK and β-integrin may be mediated either by direct interaction or through the cytoskeletal proteins, talin and paxillin [176]. Upon activation, FAK autophosphorylates at Tyr^{397}, creating a binding site for the Src homology 2 (SH2) domain of Src and Fyn and thus resulting in phosphorylation of a number of focal adhesion components, like paxillin [178, 179]. The activation of these downstream effectors links FAK to signaling pathways that modify the cytoskeleton [180] and activate mitogen-activated protein kinase (MAPK) cascade [181]. Upon assembly of focal adhesions, FAK is phosphorylated on serine, which is different from the previous

tyrosine phosphorylation, and disassociates FAK from Src which may lead to a loosening of cell-substrate attachments [182].

Integrins also partner with growth factor receptors for optimal activation of these proteins [183]. It has been demonstrated that appropriate integrin-mediated cell attachment is necessary for optimal signaling downstream of the receptors, e.g. insulin receptor [184], platelet-derived growth factor (PDGF) receptor [185, 186], epidermal growth factor (EGF) receptor [187], and vascular endothelial growth factor (VEGF) receptor [188, 189]. Integrins appear to have preferences for the growth receptors they associate with. The αvβ3 integrin can be immune-precipitated in complexes with receptor for insulin [190] insulin, PDGF [191], and VEGF [192], whereas α5β1 integrin associates with the EGF receptor [193].

The fact that G proteins interact directly with integrin β subunit and regulates integrin-mediated signaling has been revealed recently [194, 195]. At least two β subunits, β1 subunit and β3 subunit, are able to interact with $G_{\alpha 13}$ [195]. Knockdown of $G_{\alpha 13}$ or inhibition of the $G_{\alpha 13}$ binding leads to inhibition of integrin-dependent activation of Src family kinases (SFK) and impairment of integrin-dependent cell spreading. Thus, it is likely that $G_{\alpha 13}$-mediated integrin signaling and cell spreading is through activation of SFK. The current model for $G_{\alpha 13}$-regulated platelet spreading and clot retraction is that following binding of the extracellular ligands to integrin αIIbβ3, $G_{\alpha 13}$-β3 interaction, and consequent activation of β3-bound c-Src induces c-Src-dependent inhibition of RhoA, which is required for cell spreading [196]. During this process, c-Src phosphorylates β3 at Y747 and Y759, preventing the cleavage of β3 cytoplasmic tail by calpain. Subsequent dephosphorylation of β3 integrin Y759 after cell spreading allows calpain to cleave β3 cytoplasmic tail, leading to removal of c-Src and freeing the inhibitory effect of c-Src on RhoA. RhoA is subsequently activated, initiating contractile signaling and cell retraction [195]. However, it is still unclear how the binding of Gα13 and other signaling molecules to integrin β subunits is coordinated during integrin signaling.

Integrin Trafficking and its Regulations

Integrin trafficking is another aspect of regulation of integrin function. It was discovered in the late 1980s and early 1990s. This work was pioneered by Mark

Bretscher who, using biochemical recycling assays, found that integrin heterodimers are continually recycling between the plasma membrane and endosomal compartments [197]. It has become increasingly clear that integrin endo-exocytic cycling is a rapid, selective, and highly efficient process, which has multiple cellular roles, such as mediating uptake of cargo to endosomal compartments and conducting cellular processes in a spatiotemporal manner. Consistently, integrin recycling has been shown to be an important element in controlling cell migration and tumor cell invasion. My dissertation work has attempted to address the role of this process in platelet function.

Integrin endocytosis occurs in both a clathrin-dependent and a clathrin-independent manner. For instance, integrin α5β1 and αvβ3 can enter the cell via both clathrin-dependent endocytosis [198-200] and caveolar-dependent endocytosis [168, 201]. Like other receptors following clathrin-dependent endocytosis, e.g. the low density lipoprotein receptor, many integrin β subunits contain a NXXY motif in their cytoplasmic domain. This mediates the recruitment of adaptor proteins for clathrin-coated pit formation. However, it is interesting that disruption or depletion of the NXXY motifs in the β3 cytoplasmic domain did not affect the internalization of β3 integrins, αIIbβ3 and αvβ3 [202], implying a potential role for the α subunit in the endocytosis process. Integrins could also be internalized via a caveolin-dependent process, which is dynamin-dependent and linked to the activation of protein kinase C (PKC)-α. Indeed, several integrins including αvβ3 and α5β1 are found to associate with caveolin-1 or with detergent-resistant membrane domain that contain caveolin [168, 169, 201, 203]. Moreover, both dynamin and PKC-α are found required for β1 integrin internalization [204, 205] and dominant negative dynamin expression has been shown to block disassembly of β1 integrin-containing focal adhesions [206], possibly by the disruption of a dynamin-dependent internalization of this integrin.

After internalization, integrins are recycled back to plasma membrane via two temporally and spatially distinct mechanisms, a short-loop and a long-loop [204]. After reaching early endosomes (EEs), integrins selected for short-loop recycling are sorted to particular subdomains of EEs and then rapidly returned to the plasma membrane in a Rab4-dependent manner. Alternatively, integrins may pass from EEs to the

perinuclear recycling compartment (PNRC), from where they return to the plasma membrane under control of the Rab11 GTPase, referred as long-loop.

The Rab11-positive PNRC accommodates a range of integrins (*e.g.* β1 integrins [207-209], αvβ3 [208, 210], and α6β4 [210], growth-factor/cytokine (CXCR2) [211], TGFβ-R [212], EGF receptors [213], transferrin receptor (TFN-R) [214] and other receptors [215]. Current studies demonstrate that PKB/Akt mediates the recycling of α5β1 and αvβ3 integrins, but not TFN-R, from PNRC to PM via the phosphorylation of GSK-3β [208], suggesting the selective recycling of integrins can be modulated by certain signaling pathways. It becomes clear that ADP-ribosylation factor 6 (Arf6) regulates the exit of β1 integrins from PNRC in conjunction with Rab11 [216]. It is interesting that effectors of Rab11 (FIP3 and FIP4) also bind to Arf [217].

In contrast to Rab11-dependent long-loop recycling of integrins back to PM, short-loop recycling requires Rab4. In fibroblasts treated with platelet-derived growth factor (PDGF), αvβ3 integrin is diverted into short-loop recycling pathway [218]. Moreover, the PKC-related kinase, PKD1 is required for short-loop recycling of αvβ3 [219], while mutant αvβ3, that is unable to bind to PKD1, does not recycle in response to PDGF. Supportively, proteomic studies indicate that PKD1 associates with an αvβ3 complex. Arf6 has been demonstrated to mediate the short-loop recycling of integrins in neuronal cells [220].

Integrin trafficking plays an important role in cell adhesion, migration, and tumor cell invasiveness. It has been shown that blocking all endosomal membrane recycling and Golgi transport by primaquine causes rapid disappearance of cycling adhesion receptors from the cell surface and consequent detachment of cells from the substratum. Selective inhibition of long-loop recycling by targeting Rab11, Arf6, or myosin Vb impairs migration and/or chemotaxis in a number of different cell types [211, 216]. Similarly, inhibiting short-loop recycling of αvβ3 by expressing dominant negative Rab4 reduces cell spreading on vitronectin, an αvβ3 ligand [221]. To address the specific role that integrin trafficking has in these processes, the cytoplasmic domain motifs of integrin were targeted since these motifs regulate integrin function during cell migration. It was shown that disruption of a YXXΘ motif in the membrane proximal region of the β2 integrin cytoplasmic domain has no effect on the receptor's

adhesive functions but impairs the recycling of αLβ2, leading to reduced cell migration on intercellular adhesion molecule-1 (a ligand for αLβ2) but not on other ligands, such as fibronectin [222]. The contribution of integrin endo-exocytosis trafficking to cell migration could be from 1) the simple return of binding competent integrins to the cell surface; of 2) the spatial redistribution of integrins toward the cell's leading edge. The role of integrin trafficking in cancer growth and invasiveness has been demonstrated through the connection of the RabGTPase and kinases responsible for controlling integrin recycling to cancer. The tyrosine kinase, Src, well-known for promoting cell transformation, is recruited to the plasma membrane by a Rab11-controlled recycling pathway [223]. Moreover, Rab11-FIP3 has been identified as tumor marker [224]. Consistently, a number of proteins, which regulate integrin trafficking, e.g. Arf6 [225], PKD1 [219], and dynamin [226], are abundant in the invadopodia of aggressive cancer cells. Integrins could also contribute to cancer cell invasiveness by directing MT1-MMP and uPAR/uPA, membrane-associated proteases, to invadopodia [227].

Integrin αIIbβ3 Trafficking in Platelets

Integrin αIIbβ3 cycling in platelets back and forth between plasma membrane and intracellular vesicles has been recognized for about two decades [228]. αIIbβ3 localizes in various cellular locations, including plasma membrane, α-granules [97], and dense-granules [229]. In fact, many platelet membrane proteins are present on both the plasma membrane and α-granules, *e.g.* integrins, immunoglobulin family receptors [99], leucine-rich repeat family receptors [230], tetraspanins [230], and other receptors (*e.g.* CD36) [231]. Due to the fact that the abundance of plasma membrane receptors reside in α-granule membranes, it has been suggested that endocytosis of plasma membrane contributes to the presence of adhesion molecules in α-granules [232]. It is well established that fibrinogen in α-granules is derived from plasma and the uptake process is mediated by integrin αIIbβ3 [29]. Therefore, fibrinogen content in α-granules is a good indication of trafficking of integrin αIIbβ3. However, the molecular machinery regulating the trafficking of integrin αIIbβ3 is largely unknown. Electron microscopy experiments suggested that integrin αIIbβ3 trafficking in platelets is a clathrin-dependent process [233]. Consistently, the clathrin-

associated adaptor proteins AP-1, AP-2 and AP-3 are found in platelets [234]. Interestingly, unlike other cells, the clathrin coat on the coated vesicles in platelets is retained throughout trafficking and for a period after fusion with α-granules [235]. Moreover, Src-family kinases may also play an important role in the endocytosis process in platelets based on the observation of co-localization of Src-family kinase with clathrin [235]. In fact, a knockout of lyn, a member of Src-family, in mouse platelets increased the surface level of αIIb integrin [236]. Recently, disabled-2 (DAB-2), an endocytic adaptor protein, was suggested to be required for cellular uptake of fibrinogen in megakaryocyte cell lines (Hung WS 2012). However, DAB-2 deletion in mouse platelets had only mild effects on fibrinogen uptake with only a 20% decrease in platelet fibrinogen levels [237]. This suggests that DAB-2 plays a limited role in integrin αIIbβ3 trafficking in platelets and may contribute to other aspects of integrin function.

Integrin αIIbβ3 trafficking in platelets is fast. Abciximab or c7E3 Fab, an antagonist to integrin αIIbβ3, is detectable within 3 hours post-infusion into a patient [233]. It requires much shorter time for abciximab to be detectable in vitro after incubation with washed platelets. It was suggested that the OCS is the entry site for these exogenous substances. Indeed, OCS channels were stained as early as 1 minute [238], suggesting a rapid diffusion of c7E3 into the internal platelet compartment, which is consistent with other reports. Studies using monoclonal antibody AP6 [239], which binds to integrin αIIbβ3 only when the receptor is occupied by intact fibrinogen, suggested the presence of a pool of ligand-bound αIIbβ3 in the α-granule membrane. Also, thrombin induced activation of the internal integrin αIIbβ3 prior to the surface pool, and then induced the exposure of αIIbβ3-ligand complexes to the surface. In fact, platelet activation by ADP or thrombin receptor activation peptide (TRAP) significantly increases the actin cytoskeleton-dependent internalization of αIIbβ3 [240]. Also, direct activation of αIIbβ3 with an anti-LIBS antibody caused an approximate 8-fold increase in the extent of receptor internalization. Consequently, upon stimulation while the stirring is delayed, internalization of fibrinogen-bound receptor complexes reduced platelet aggregation [241]. In summary, integrin αIIbβ3 trafficking is a continuous process in platelets that appears to play an important role in deposition of

α-granule cargo, like fibrinogen, and in modulation of platelet aggregation. However, it is unclear whether integrin trafficking plays roles in other integrin-mediated platelet functions, like spreading, clot retraction. Moreover, there is little known about the molecular mechanism(s) regulating the trafficking of integrin αIIbβ3.

Subsection Three: ADP-ribosylation Factor (Arf)

Arf Family

The Arf family of small GTP-binding proteins belongs to Ras superfamily [242, 243]. Like other Ras-related proteins, members of the Arf family cycle between a GDP-bound inactive form and a GTP-bound active form. The Arf family includes Arf proteins, Arf-like (Arl) proteins and Sar1. There are 6 mammalian Arf proteins that can be divided into three classes based on homology of primary protein sequence: Class I (Arfs 1-3), Class II (Arfs 4-5), and Class III (Arf6). They are all ubiquitously expressed and their amino-acid sequences seem to be well conserved in all eukaryotes, however, humans do not express Arf2. There are over 20 Arl proteins expressed in different systems, and Sar1 is present in all eukaryotes. For my thesis work, I focus on Arf proteins, particularly Arf6, the most divergent Arf protein.

Arf proteins were originally discovered as cofactors for cholera-toxin in catalyzing ADP-ribosylation of the α-subunit of heterotrimeric G proteins, Gs, in cell-free biochemical assays [244]. They have multiple cellular functions, including the recruitment of coat proteins that promote sorting of cargo into vesicles, the recruitment and activation of lipid-modification enzymes, actin cytoskeleton rearrangement, and membrane trafficking [245].

The cellular compartments in which Arfs reside include the plasma membrane, and the membranes of the secretory and the endosomal/lysosomal pathways, and the cytosol. The amino-terminus of Arfs features an amphipathic helix and myristoylation at the second glycine residue, which are both critical for membrane localization [246]. Upon GTP-binding, the myristoyl group and associated amphipathic helix are flipped out and inserted into the membrane, causing very close contact of Arfs with the membrane [247]. It seems that Arf1 and Arf3 are released from the membrane upon GTP hydrolysis in cells [248], while GDP-bound Arf6 still remains at the membrane

[249]. Arf1 and Arf6 are the most studied members of Arfs. Arf1 functions in transportation between the Golgi and Endoplasmic Reticulum (ER), while Arf6 functions between the plasma membrane and endosomal vesicles [242]. Despite the unique subcellular localizations of Arf1 and Arf6, they share similar functions as discussed below for Arf6.

Arf6 and Phospholipid Metabolism

The effect of Arf6 on membrane phospholipid modification is mediated by activation of two important phospholipid enzymes, phosphatidylinositol 4-phosphate 5 kinase (PIP5K) [250] and phospholipase D (PLD) [251-253]. PIP5K catalyzes the phosphorylation of phosphatidylinositol 4-phosphate [PI(4)P] at the D5 position of the inositol ring to produce the versatile lipid messenger phosphatidylinositol 4,5-bisphosphate [$PI(4,5)P_2$]. Honda *et al.* [250] shown that Arf6 directly activates PIP5K in phosphatidic acid-dependent manner, and, via PIP5K, Arf6 regulates various cellular functions by locally increasing the concentration of $PI(4,5)P_2$. First, $PI(4,5)P_2$ could serve as a precursor of lipid second messengers. $PI(4,5)P_2$ is hydrolyzed by phosphatidylinositol-specific phospholipase C to generate two lipid second messengers, diacylglycerol (DG) and inositol 1,4,5-triphosphate (IP3), which in turn activates protein kinase C and increases intracellular Ca^{2+}, respectively. Second, $PI(4,5)P_2$ could be further phosphorylated by phosphatidylinositol 3-kinase, generating another lipid second messenger phosphatidylinositol 3,4,5-triphosphate which is important for cell growth, survival and apoptosis [254, 255]. Lastly, PI(4,5)P2 itself could act as a signaling molecule by regulating the activities of target proteins and enzymes or by recruiting them to certain areas of specific subcellular compartments.

Indeed, Arf6/PIP5K signaling has been demonstrated to play various cellular functions via $PI(4,5)P_2$. Arf6/PIP5K is directly involved in $PI(4,5)P_2$-dependent exocytosis, including insulin secretion in MIN6 pancreatic cells [256], dense core vesicle exocytosis in PC-12 neuroendocrine cells [257], and neurotransmitter release from synaptosomes prepared from rat brain [258]. Arf6/PIP5K signaling also regulates clathrin-dependent endocytosis of synaptic vesicle by recruiting adaptor complex AP-2 to synaptic membranes [259, 260]. Moreover, the Arf6/PIP5K signaling pathway

plays a key role in endocytic trafficking. Overexpression of an constitutively active Arf6 mutant in Hela or COS cells results in accumulation of internalized vesicles and subsequent formation of elongated vacuolar structures [261], which mirrors the phenotype seen by overexpressing wild type PIP5K. Conversely, overexpression of a dominant negative Arf6 mutant blocks the transport of the recycling endosome to the plasma membrane [262, 263]. These observations suggest that the inactivation of Arf6 and thus the decreased level of $PI(4,5)P_2$ on the internalization vesicles are essential for formation of recycling endosomes, and that re-activation of Arf6 and subsequent PIP5K activation are critical for the endosomal recycling trafficking to and/or fusion with the plasma membrane. Also, the Arf6/PIP5K signaling complex plays an important role in the actin cytoskeleton reorganization responsible for neurite elongation by recruiting the actin-binding protein Mena [264], possibly through $PI(4,5)P_2$.

PLD is another lipid modification enzyme that is regulated by Arf6. PLD catalyzes the hydrolysis of phosphatidylcholine to generate phosphatidic acid (PA) and choline. PA plays various cellular roles, including exocytosis [265] and endocytosis [266] and reorganization of the actin cytoskeleton [267, 268]. There are two mammalian PLD genes, each capable of generating two splice variants [269, 270]. It has been demonstrated that PLD activation is mediated by the Rho family, the Arf family and the protein kinase C family (PKC) [271]. Single members from each of these activator families function in a cooperative way to increase PLD1 activation in vitro [271]. The direct interaction between Arf6 and PLD in vivo is suggested by surface plasmon resonance experiments [253] and it appears that PLD1b can concurrently interact with a single member from each regulator family in vivo [252]. In contrast to PLD1, it seems that PLD2 activity is less directly regulated by GTPases but is controlled via interaction with PIP5K [272].

Consistent with PA's role, Arf6/PLD signal complexes are involved in various cellular processes. Arf6/PLD1 has been shown to regulate the stimulation-regulated exocytosis in Chromaffin cells [273]. In Hela cells expressing an Arf6 mutant that are defective in activating PLD, Arf6N48R and Arf6N48I, block membrane recycling to the plasma membrane and induce accumulation of tubular endosomal membranes, suggesting that Arf6/PLD plays a role in endosomal membrane recycling [274]. In

addition to the roles in membrane trafficking, Arf6/PLD is involved in other cellular processes. Recently, it was found that Arf6/PLD promotes autophagosome formation by influencing endocytic uptake of plasma membrane into autophagosome precursors [275]. Also, Arf6 was demonstrated to play a role in cancer cell proliferation in a PLD-mTORC1- and p38MAPK (mitogen-activated protein kinase)-dependent manner [276].

Arf6 and Actin Cytoskeleton Reorganization

Arf6 regulates actin cytoskeleton rearrangement in different ways under different-conditions. Arf6 could trigger actin assembly at the membrane indirectly by recruiting the Arf guanine nucleotide exchange factor (GEF) ARNO that activates Arf1 to enable WRC [the WASP family veroprolin homolog (WAVE) regulatory complex]-dependent actin assembly [277]. This pathway is important for the invasion of the pathogen, Salmonella, into host cells. In Hela and CHO cells, Arf6 mediates the redistribution and activation of endosome-localized Rac1 to the plasma membrane [278, 279]. Upon stimulation by physiological stimuli, Arf6 in CHO cells facilitates the redistribution of Rac1 to the cell surface while inhibition of RhoA-mediated formation of stress fiber [280]. Arf6 can also modulate the actin cytoskeleton through its effect on lipid modification enzymes, PIP5K and PLD as discussed earlier. The direct or indirect lipid product, $PI(4,5)P_2$, has been shown to regulate actin capping and the activities of several actin-binding proteins [250, 261]. Using a chimera protein Arf1-6 (with the amino half of Arf1 and the carboxyl half of Arf6) which is unable to induce the formation of actin-containing protrusions, Al-AWAR *et al.* showed that substitution of two residues in the amino-terminal half of Arf6, Q37 and S38, into the chimera could result in formation of protrusions, suggesting that the functions of Arf6 in actin reorganization is controlled through specific sequence in Arf6 [281]. Arf6-mediated actin rearrangement is important for various cell functions, including cell spreading [282, 283], formation of pseudopods and membrane ruffles [278, 284], migration [285-287], and phagocytosis [288-291].

Arf6 and Vesicle Trafficking

Arf6 is well known for its role in endocytic trafficking, including the endocytosis pathway and the endocytic recycling pathway. Indeed, Arf6 are reported to participate

in many steps in the membrane trafficking, including recruitment of coat proteins, coated pit assembly, vesicle fission, and vesicle route.

Arf6 in endocytosis pathway

As discussed earlier, Arf6 activation could lead to activation of lipid modification enzymes, PLD and PIP5K, resulting in the local accumulation of $PI(4,5)P_2$ rapidly. It is well established that $PI(4,5)P_2$ is one of the most important lipids regulating clathrin mediated endocytosis [292]. It has been shown that Arf6-mediated increase of $PI(4,5)P_2$ by PIP5K leads to the recruitment of clathrin coats in synaptic vesicle preparations [259]. The role of Arf6 in coated-pits assembly was convincingly demonstrated by the evidence that Arf6-GTP and $PI(4,5)P_2$ function synergistically to recruit AP-2 onto liposomes [293]. At the lateral membrane of MDCK cells, an epithelial cell line, Arf6 binds to and recruits NM23-H1 [294], a nucleoside diphosphate kinase that functions as a GTP source for dynamin-dependent vesicle fission [295]. It has also been shown that Arf6-mediated activation of NM23-H1 plays an essential role in the internalization of P2Y purinoceptor receptors, a G protein-coupled receptor, in human platelets [54]. Blocking Arf6 activation inhibits P2Y receptor endocytosis, resulting in a failure to cycle between desensitization and resensitization in platelets. Arf6 is also involved in the internalization of other GPCRs, including β2-adrenergic receptor [296] and luteinizing hormone receptors [297]. Therefore, it is clear that Arf6 has an established role in in clathrin-mediated endocytosis.

Arf6's roles in other types of endocytosis process are also reported. It seems that Arf6 association with caveolae-enriched membrane fractions is important for signaling initiated by Hepatocyte growth factor (HGF) [285], vascular endothelial growth factor [298] and oncogenic H-Ras [299], because inhibition of Arf6 activity diminishes these signaling events. In addition to the endocytosis of GPCR and GFR (growth factor receptor), Arf6 also regulates the internalization of ligands through a unique clathrin- and caveolae-independent pathway [300]. These ligands include the major histocompatibility complex class I protein (MHC class I), M2-muscaranic acetylcholine receptors, the β1 integrins and the peripheral myelin-membrane protein (PMP22). After ligand internalization, Arf6 and other membrane components are recycled back to the cell surface, while cargo ligands, via the classic endocytic pathway, are further

sorted [301]. Arf6 inactivation seems to be required for this pathway [261]. Therefore, Arf6 participates in receptor internalization through the clathrin-dependent, the caveolae-dependent, and the clathrin- and caveolae-independent pathways.

Arf6 in endocytic recycling

Most of membrane components that are internalized by clathrin-dependent or -independent endocytic pathways are recycled back to the plasma membrane, either directly from the sorting endosome, a "fast" recycling route, or from a juxtanuclear, endocytic recycling compartment (ERC), a "slow" recycling route [302, 303]. In addition to Arf6, Rab GTPases also participate in the recycling processes. Among them, Rab4 and Rab11 localize to sorting endosomes and ERC, respectively, and function as important regulators for each recycling route [304-306]. Arf6's role in endosome recycling was first identified in Chinese Hamster Ovary (CHO) cells, where dominant negative Arf6 mutant affected the recycling of surface markers, such as MHC class I and ectopically expressed Tac antigen, but not the recycling of classic recycling protein, Transferrin receptor (TfR) [307, 308]. In contrast, in Hela cells, Arf6 plays a role in the fast, microtubule-dependent endocytic recycling of TfR by interaction with JNK-interacting proteins 3 and 4 (JIP3 and JIP4). Additionally, Arf6 in Hela cells also regulates the recycling of integral plasma-membrane proteins that lack cytoplasmic AP-2 and clathrin-sorting sequences, including the IL2 receptor α-subunit, MHC class I, and glycosylphosphatidylinositol (GPI)-anchored protein [262, 301]. These observations suggest that Arf6 can have different functions in different cell systems.

Arf6's role in the fast recycling pathway was also reported in the regulation of β2-adrenergic receptor (β2AR), a member of the GPCR, β1 integrins and others [220, 309, 310]. Arf6 activation by β-arrestin, upon the engagement of ligand with β2AR, leads to accumulation of β2AR in the degradation pathway and plays a negative role in Rab4-dependent fast recycling of β2AR for resensitization [309]. In neuronal cells, Arf6 regulates rapid axonal transport of α9β1 integrin and their trafficking at the cell surface. Arf6 activation enhances the integrin endocytosis but decreases axon growth, while Arf6 inactivation promotes the recycling of integrin and increases axon growth [220]. Arf6 also acts as an upstream negative regulator of Rab35-mediated fast

endocytic recycling pathway which is essential for cytokinesis. However, this Rab35-mediated pathway is not Rab4-positive [310].

In addition to the roles in the fast recycling, Arf6 is also involved in the slow recycling pathways and thus for diverse cell functions, including cell movement, cell adhesion and junction formation, cytokinesis, cell polarity, lipid homeostasis and cell fusion. [303, 311]. The slow recycling route is vesicle trafficking from a juxtanuclear, endocytic recycling compartment (ERC) to plasma membrane. The ERC is a tubular compartment having a resident marker protein, Rab11.

Arf6 and Rab11-mediated recycling of endosomal membrane is required for cytokinesis in several organisms, including vertebrates and invertebrates. In cultured mammalian cells, Arf6 is important for severing the final intercellular bridge during abscission, although it has less effect on furrowing [312, 313]. It is proposed that active Arf6 interacting with the exocyst mediates the tethering of Rab11-FIP3/4 containing recycling endosomes to the cleavage furrow/midbody. During abscission, the last step of cytokinesis, Arf6 was also suggested to regulate the endocytic trafficking direction by controlling the interactions of its downstream effectors, JIP3 and JIP4 (JNK-interacting proteins) with the motor proteins of opposing direction. In *Drosophila* spermatocytes, Arf6 is required during the early and late phase of cytokinesis [314].

E-cadherin trafficking is important for cell adhesion and morphogenesis. Both Arf6 and Rab11 are thought to be regulators of E-cadherin recycling from endosomes to the plasma membrane [285, 315]. Activation of Arf6 causes loss of adherens junction, leading to increased epithelial cell migration.

Arf6 and Integrin Trafficking

In cancer cells, active β1 integrin and inactive β1 integrin traffic through different endocytic recycling routes, although their endocytosis requires a same clathrin- and dynamin-dependent process [316]. Inactive β1 integrins are recycled to the plasma membrane through a Rab4a- and actin-dependent Arf6-positive compartment. It is unclear whether Arf6 is required for this process. In Hela cells, depletion of BRAG2, a GEF for Arf6, inhibits β1 integrin endocytosis whereas depletion of Arf6 inhibits β1 integrin recycling back to surface, suggesting that Arf6 may function on both endocytosis and recycling pathways of β1 integrins [317]. As a consequence,

knockdown of BRAG2 enhances cell adhesion and spreading, whereas knockdown of Arf6 inhibits cell spreading. Arf6 activation is also suggested to mediate the recycling of integrin-containing lipid rafts to the plasma membrane, and thus influences cell adhesion [318].

Arf6 is also involved in integrin trafficking in stimulated cells. In starved Hela cells, β1 integrins are recycled to the plasma membrane upon external stimulation upon serum addition, a process that is Arf6-, Rab11- and actin- dependent but Rab4-independent [216]. In these cells, β1 integrins are internalized to Arf6- and Rab11- positive compartment. External stimulation mediates the recycling of integrins to Arf6-enriched membrane ruffles. This Arf6-GTP-regulated integrin recycling is important for cell migration. In contrast, Arf6 activation in endothelial cells is reported to play a role in semaphorin 3E-stimulated integrin endocytosis, leading to disassembly of focal adhesion, retraction of filopodia, and inhibition of angiogenesis [319]. In summary, Arf6 plays a role in both endocytosis and recycling of integrins, regardless in resting cells or in stimulated cells, although Arf6 has different functions in different context. Given the vast effects of Arf6 discussed above, Arf6 global KO mice exhibit defective liver development, due to impairment in hepatic cord formation [320].

Arf6 Regulators, GEFs and GAPs

As other Ras GTPases, Arf6's role is regulated by its cycle between active GTP-bound state and inactive GDP-bound state. This cycle is regulated by two classes of proteins, guanine-nucleotide-exchange factors (GEF) mediating the loading of GTP on Arf6, and GTPase activating proteins (GAP), catalyzing the hydrolysis of GTP to GDP by Arf6. Given the diverse functions of Arf6, it is not surprising that its regulators are also diverse.

<u>Arf6 GEFs</u>

All Arf GEFs contain a conserved Sec7 catalytic domain in, which is named based on its homology to the yeast ArfGEF, sec7p [321, 322]. Therefore, Arf GEFs are also called a Sec7 family. In mammals, there are 15 Sec7 family members, which could be divided into five classes based on overall structure and domain organization [323]. They could also be grouped into Brefeldin A (BFA)-sensitive and BFA-insensitive members. BFA is a fungal toxin that binds tightly to the Arf-GDP-Sec7 domain complex and thus

sequesters the GEFs in an abortive reaction intermediate [324-326]. The Sec7 domain consists of approximately 200 amino acids. Crystal structures of this domain alone and the complex of Sec7 domain/Arf have revealed the details of the nucleotide exchange process, in which the glutamic finger from Sec7 domain is essential for the catalytic reaction [324, 327-329]. So far, all Arf6 GEFs identified are resistant to BFA due to the fact that Arf6 cannot accommodate BFA in its interface with GEFs [330]. The five families include Golgi BFA-resistance factor 1/BFA-inhibited GEF (GBF/BIG), Arf nucleotide binding site opener (ARNO)/cytohesin, exchange factor for Arf6 (EFA6), Brefeldin-resistant Arf GEF (BRAG) and F-box only protein 8 (FBX8). Among them, the cytohesin family [331-333], the EFA6 family [334, 335], and the BRAG family [317, 336-338] are reported to have Arf6 GEF activity.

Cytohesin family

There are four isoforms in the cytohesin family, cytohesin 1 - 4. They are relatively small (45-50 KD) and share a common domain organization consisting of an N-terminal coiled-coil domain, a central Sec7 domain followed by a PH domain and a short C-terminal extension rich in positively charged amino acids. They are ubiquitously expressed [339] and are primarily localized to the cell periphery [340]. In response to PI3K signaling, they can be acutely recruited to the plasma membrane [341, 342]. Cytohesin family members are ambiguous to the Arf specificity. In biochemical assays, Arf1 is the preferred substrate over Arf6 [340, 343-346]. However, in cells, ARNO can activate Arf6 and is recruited to the plasma membrane where Arf6 is localized [287, 340, 347]. Interestingly, ARNO, via its PH domain but not Sec7 domain, was recruited to by Arf6-GTP and, in turn, recruited and activated Arf1 at the PM [348]. Cytohesin-2 has been shown to promote both the migration of epithelial cells [287] and the outgrowth and branching of neurites [264, 349]. Cytohesin-2 has also been implicated in vesicular transport [296, 350, 351]. Recently, Torii and colleagues showed that expression of cytohesin-1 in Schwann cells in mice increases Arf6-GTP level in these cells, and enhanced myelin thickness [331], suggesting the GEF activity of cytohesin-1 on Arf6 in animals.

Proteomics study has shown that cytohesin-2 is present in human platelets [352]. Consistently, Arf6 was shown to be able to co-immunoprecipitate (IP) cytohesin-2 in

resting platelets and this interaction was decreased upon platelets activation [353]. Pretreatment of platelets with cytohesin inhibitor, SecinH3, enhanced dense core granule secretion. In another study, SecinH3 was shown to inhibit Arf6 activation upon P2Y receptors activation and thus to inhibit internalization of P2Y receptors [54]. Regardless of the discrepancy between these two studies, these results implicated a role for cytohesin in platelet functions.

EFA6 family

The EFA6 family in mammals consists of four isoforms, EFA6A, EFA6B, EFA6C, and EFA6D. They are characterized by a variable N-terminal region, a central Sec7 domain paired with a PH domain, and a coiled-coil motif containing C-terminal domain. EFA6A and EFA6C are predominantly expressed in the brain [336, 354]. EFA6B and EFA6D are more widely expressed [355]. Compared to the cytohesin family, the EFA6s are selective for Arf6 in cell-free assays and in cells [356]. The EFA6s also appear to interact selectively with $PI(4,5)P_2$, consistent with their PM localization [354]. Like Arf6, EFA6 family regulates membrane trafficking and the actin cytoskeleton [356, 357]. The EFA6 family, via activation of Arf6, plays various roles, including spine formation in dendritic cells [334], E-cadherin-mediated tight junction formation and stability [335, 358], and cytokinesis [359]. However, EFA6 was not detected by proteomics in human platelets in a recent study [352].

BRAG family

The last Arf6 GEFs discussed are members of the BRAG family. The BRAGs consist of an IQ-like domain, a central catalytic domain, and adjacent PH domain and C-terminal coiled-coil domain. In mammals, BRAG1 and BRAG3 are primarily expressed in the brain [360, 361] while BRAG2 is much more widely distributed [362]. BRAGs also behave as GEFs for Arf6 in cell-free assays and in cells [317, 362]. Although both BRAG1 and BRAG3 mainly function in the post-synaptic area, it seems that BRAG1 is at excitatory synapses and BRAG3 is at inhibitory synapses [338, 363]. BRAG2 acts as a downstream effector of AMPA receptor signaling, regulating endocytosis of AMPA and thus long term depression [364]. In endothelial cells, BRAG2 regulates the endocytosis of α5β1 integrin, mediating the disassembly of α5β1-containing focal adhesions and sprouting of endothelial cells [365]. BRAG2-mediated endocytosis of β1 integrins is

also important for cell motility [317]. BRAG2 also contributes to tumor cell metastasis by cooperating EGFR signaling and Arf6 activation [366, 367]. However, BRAG family was not detected by proteomics in human platelets in recent study [352].

Arf6 GAPs

Like Arf GEFs, all Arf GAPs contain a catalytic domain, called the ArfGAP domain which was first identified in rat ArfGAP1 [368] and is highly conserved. ArfGAP domains are about 130 amino acids in length and contain a characteristic C_4-type zinc finger motif and a conserved arginine that is required for activity, within a particular spacing ($CX_2CX_{16}CX_2CX_4R$). Arf GAPs display various degrees of specificity for the individual Arf family member both in vitro and in vivo. Based on sequence similarities of their ArfGAP domains, the 31 predicated human Arf GAPs have been divided into 10 subfamilies, including subfamilies of ArfGAP1, ArfGAP2, ADAP, SMAP, AGFG, GIT, ASAP, AGAP, ACAP and ARAP [369]. In the discussion that follows, subfamilies including ADAP, ASAP, ARAP, and ACAP will be described as these ArfGAPs have been studied more as Arf6 GAP proteins. GIT family proteins will be discussed in more details in an independent chapter.

ADAP subfamily

This subfamily consists of two members, ADAP1 and ADAP2. They are characterized as two PH domains immediately C-terminal of the ArfGAP domain. ADAP1 has high affinity to $PI(3,4,5)P_3$ and $Ins(1,3,4,5)P_4$ [370, 371], and was thought to be involved in PI3K-mediated cellular pathways [372]. It is a brain-enriched protein and localizes to the cytosol and nucleus [373]. It could be recruited to the PM in a PIP_3-dependent manner and functions as an Arf6 GAP protein regulating actin cytoskeleton organization [374]. ADAP1 has been reported to interact with various proteins, including Arf proteins [375], F-actin [375], KIF13B (a member of the kinesin superfamily, [376], Nardilysin (NRDc, N-arginine dibasic convertase, [377]) RanBPM (Ran binding protein in microtubule-organizing center, [378]), nucleolin [379], and casein kinase 1 [380, 381]. Consistent with that, ADAP1 plays multiple cellular functions, such as actin cytoskeleton reorganization [382], GPCR endocytosis [383], mitochondrial Ca^{2+} homeostasis [384]. ADAP1 has been shown to increase dendritic differentiation [385].

ASAP subfamily

Three human ASAPs share multiple domains, including a Bin/Amphiphysin/Rvs (BAR) domain, a PH domain, an Arf GAP domain, ANK repeats, and a proline-rich (Pro) domain, but differ in their C termini [369]. The BAR domain in ASAP1 negatively regulates its GAP activity [386] and binding of the BAR domain to FIP3, a Rab11 effector, stimulates its GAP activity [387]. ASAP1 and 2 prefer Arf1 and Arf5 to Arf6 in vitro [388, 389]. In vivo, overexpression of ASAP1 decreases Arf1-GTP level while increases Arf6-GTP level [390, 391]. However, there is other evidence showing that ASAP1 and ASAP2 also function with Arf6 through direct binding and slow catalysis [392, 393]. The crystal structure of Arf-ASAP suggests the importance of Ca2+ in the GAP activity [394], in an PI(4,5)P_2- and PA-dependent manner [388, 395]. ASAP1 has been shown to interact with Src, CrkL, FAK, CD2AP and CIN85 [396]. ASAP2 interacts with pyk2, a focal adhesion kinase, whereas ASAP3 associates with focal adhesions and regulates stress fibers [397]. ASAP1 has been suggested to be an important regulator of cellular structures, like FAs, CDRs, Invadopodia, and podosomes [398, 399], thus it is important for FA turnover, receptor recycling, cell migration, cell spreading and tumor invasion [396]. ASAP1 and 2 were recently found present in platelets using a proteomic approach [352].

ARAP subfamily

ARAPs consist of the most complex of domains compared to other ArfGAPs, including ArfGAP, RhoGAP, ankyrin repeats, and Ras association domains. The domain structure of ARAPs indicates that they are important coordinators of two or more GTPase signaling pathways. There are three members of this subfamily in humans, and they are distinct in functions and cellular locations with different Arf, Rho, and Ras binding specificity for individual ARAPs. ARAP1 uses Arf1 and Arf5 as substrates [400]. ARAP2 uses Arf6 preferentially as compared with Arf1 and Arf5 in vitro and in vivo [401, 402]. ARAP3 has been reported to function as an Arf6 or Arf5 GAP in vitro [403, 404] and to regulate Arf6-dependent event including membrane protrusions and ruffling in vivo [403, 405]. PI(3,4,5)P_3 could stimulate the Arf GAP activity of ARAPs [406, 407]. ARAPs affect EGFR-mediated signaling, FA dynamics, lamellipodia formation [396, 408] and

recently integrin α5β1 trafficking [402]. ARAP1 was also found in the platelet proteomics study [352].

ACAP subfamily

ACAP is an acronym for ArfGAP with coiled-coil (identified as a BAR domain later), ankyrin repeat, and PH domain [396]. ACAP1 and 2 have a preference for Arf6 over other Arfs in vitro and in vivo, and $PI(4,5)P_2$ and PA are important lipid components for their ArfGAP activity [409]. ACAPs are reported to regulate Arf6-dependent actin remodeling and endocytosis and receptor tyrosine kinase-dependent cell movement. ACAP1, as an effector of Arf6, functions as part of a clathrin coat complex, mediating the stimulation-dependent endocytic recycling of integrin and glucose transporter type 4 [410]. Thus, it is important for cell migration and glucose homeostasis. Also, overexpression of ACAP1 in Hela cells reduces FAs formation [411]. ACAP2 regulates neurite outgrowth by linking the two small GTPases, Rab35 and Arf6 [412]. ACAP1 and 2 were also found in the platelet proteomics study [352].

Arf6 Regulation and its Role In platelets

Although Arf6 was first identified in 1991 [413], the first study of roles of Arf6 in platelets occurred actually 15 years later by Choi *et al.* from our group [414]. Before our studies, it had been projected that Arf proteins may play some role in the αIIbβ3 integrin signaling [399, 415]. Oda and colleagues found that ASAP1, an ArfGAP protein, is present in platelets and can be recruited to peripheral focal adhesions via CrkL, a SH2 and SH3 adapter for WASP, syk and STAT5 in platelets [415, 416], suggesting a potential role for Arf in platelets. Our previous work, by Choi and Karim, showed that in resting platelets, Arf6 is present in the GTP-bound form, and upon platelet activation, Arf6-GTP is quickly converted to Arf6-GDP. This transition is regulated by two waves of platelet signaling pathways, primary signaling (e.g. PAR receptor, GPVI) and contact-dependent signaling (e.g. αIIbβ3 integrin) [417]. Blocking integrin αIIbβ3 outside-in signaling could partially reverse the loss of Arf6-GTP, suggesting a regulation of Arf6 by signaling downstream of integrin αIIbβ3. It was shown that Arf6 is downstream of many well-known platelet signaling steps, like PLC, PI3K, PKC, and Syk. Using an inhibitory, myristoylated Arf6 N-terminal peptide, loss of Arf6-GTP could be blocked, probably due to the inhibition of the Arf6 GAP activity by the peptide.

Pretreatment with this peptide inhibited platelet aggregation, adhesion and spreading on collagen-coated surface, and activation of Rho family members, suggesting the central role of Arf6 in platelet activation.

Recently, Arf6 was suggested to be involved in P2Y receptor endocytosis [54] and in dense granule secretion, but not α-granule or lysosome secretion [353] in platelets. It was shown that P2Y activation by ADP could stimulate Arf6 activation, and activated Arf6 stimulates Nm23-H1, a nucleoside diphosphate kinase, which in turn promotes dynamin-dependent internalization of P2Y receptors. It was also proposed that Arf6-GTP in resting platelets is maintained by cytohesin-2, an ArfGEF protein, and upon platelet activation, PKC-mediated phosphorylation of cytohesin-2 decreases the interaction between Arf6-GTP and cytohesin-2, leading to loss of Arf6-GTP and platelet secretion. However, it should be noted that these studies used Myr-Arf6 peptide and pharmacological inhibitors, and the dynamics of Arf6-GTP changes were not consistent with previous reports. Thus there is confusion over the role of Arf6 in platelets justifying the need for more experimental data and better systems to evaluate Arf6 function. Using conditional knockout mice as done in this dissertation may shed light on the role of Arf6 in platelet function and hemostasis.

Subsection Four: G Protein-coupled Receptor Kinase Interacting Protein (GIT)

GIT Family

GITs were originally identified as G protein-coupled receptor kinase interacting proteins. There are two members in this family, GIT1 and GIT2. GIT1 expression is prominent in endothelial cells, while GIT2 is almost ubiquitous [418]. They consist of N-terminal ArfGAP domain, three Ankyrin (ANK) repeats, a Spa-homology domain (SHD), a coiled-coil domain and a C-terminal paxillin-binding site (PBS). Through interactions with various proteins, GITs could form a big complex regulating membrane traffic and actin cytoskeleton-related structures, like FAs. GITs exhibit efficient and almost equivalent GAP activity toward Arf1 and Arf6 in vitro [419-421] and GITs prefers Arf6 as substrate which is supported by the colocalization of GITs with Arf6 in the cell periphery in vivo [422-424]. GITs are present in platelets shown by western blot [425] and by proteomic approach [352]. However, its role in platelet

is unknown, although it was suggested to be involved in integrin αIIbβ3 downstream signaling.

GIT Domains and Corresponding Interacting Partners

The ArfGAP domain

Like other ArfGAP proteins, GITs contain a zinc-finger-like motif essential for their GAP activity [368, 426]. GITs' ArfGAP domain could function on both Arf1 and Arf6 [419] and the ArfGAP activity could be stimulated by PI(3,4,5)P3 but not by PI5P, PI(4,5)P2 or diacylglycerol (DAG) [422]. A truncated GIT mutant lacking the ArfGAP domain, or ArfGAP activity deficient mutant (R39K) resulted in the formation of very large GIT1-containing recycling endosomes [427-429], suggesting that ArfGAP activity is necessary to maintain proper membrane trafficking.

The ANK repeats

ANK repeats usually mediate protein-protein interaction and have diverse binding partners. For GIT2, there is a potential paxillin binding site between the ANK repeat and the SHD domain, although this is not the dominant binding site for paxillin [430]. Truncated GITs mutant consisting of only the ArfGAP and the first ANK repeat localizes to endosome, and further depletion of the first ANK repeat results in diffused cytoplasmic distribution of the ArfGAP domain [427, 429], suggesting the first ANK repeat may regulate the binding of GITs to endosome.

The SHD domain

The SHD domain is an important binding site mediating formation of a large oligomeric complex. There are multiple binding partners for this domain, including p21-activated kinase interacting exchange factor (PIX), PLCγ and MEK1.

PIX is a Dbl-homology and pleckstrin-homology (DH/PH) domain-containing protein, and thus can serve as a GEF for Rac1 and Cdc42 [431-433]. PIX binds to GIT via its α-helical region of the C-terminus. GIT and PIX could form large aggregates in the cell [434, 435], consistent with the fact that both proteins can homodimerize. Disrupting their interaction causes diffuse cytoplasmic localization of both proteins [432, 436]. Through PIX, GITs can also interact with p21-activated kinase (PAK). PAK interacts directly with PIX and is thus recruited to GIT-PIX oligomers [427, 437, 438]. It was suggested that in response to extracellular stimuli, activated Rac1/Cdc42 induces a

conformational change in PAK which allows association of Rac1/Cdc42-PAK with GIT-PIX oligomers [438]. Local accumulation of PAK at GIT-PIX complex induces its autophosphorylation and subsequent activation of PAK [432]. PAK recruitment to GIT-PIX oligomers mediates translocation of this complex to focal complexes, and through unknown mechanism that requires ArfGAP activity, Rac1 is inactivated in a GIT-dependent manner [439, 440], providing a negative feedback regulation.

GIT1, via its SHD domain, binds to a PLCγ SH2 domain but does not bind to PLCδ and PLCβ [441, 442]. Knocking down GIT1 expression inhibits agonist-mediated PLCγ phosphorylation and Ca^{2+} release [441].

MEK1 is another interacting partner of GIT1 that binds the SHD domain [443] and is an upstream kinase for ERK1/2. Through MEK1, GIT1 may spatially regulate ERK1/2 activation. Overexpression of GIT1 prolongs ERK1/2 activation in response to epidermal growth factor (EGF) stimulation, and knocking down GIT1 inhibits EGF-mediated activation of ERK1/2 [443]. It has been shown that GIT1-mediated c-Src-dependent activation of Erk1/2 plays various roles in regulating focal adhesion and cell migration in vascular smooth muscle cells and Hela cells [443, 444].

The coiled-coiled domain

The coiled-coil domain resides between the SHD and the PBS, and mediates GIT1 and GIT2 homo- and hetero-dimerization [435, 445].

The PBS binding site

Paxillin is a focal adhesion adaptor protein containing five leucine-rich domains (LDs) and four C-terminal LIM domains. GIT1 and GIT2 bind to the LD4 region of paxillin through their PBS [427, 428, 438, 446]. In addition to that, LDs also mediates paxillin binding to vinvulin, FAK and integrin, tyrosine phosphorylated motifs that mediate binding to SH2 domains and proline-rich motifs that mediate binding to SH3 domains [447]. Through paxillin, GITs localize to focal complexes, and depletion of either the GIT PBS region or the paxillin LD4 motif prevents GITs from associating with focal complexes [428, 438, 440]. It has been proposed that association of PAK with GIT-PIX actually helps unmask the PBS region for paxillin binding and subsequent recruitment of GIT-PIX complex to focal complex. This model is supported by several lines of evidence [432, 438, 448].

GIT Regulation

GITs have multiple phosphorylation sites and it appears that phosphorylation of certain residues is important for GIT activity. The kinases known to mediate GIT phosphorylation include Src, FAK and protein kinase D3 (PKD3) [437, 443, 449, 450]. It has been shown that tyrosine phosphorylation of GIT1 at residue 392 is important for activation of PLCγ and podosome formation in vascular smooth muscle cells [451]. PKD3 was shown to directly phosphorylate GIT1 on serine 46, regulating its subcellular localization and cellular protrusive activity [450]. The only phosphatase regulating the dephosphorylation of GITs is protein tyrosine phosphatase ζ (PTPζ) [449].

GIT1 in Membrane Ttraffic

GIT1, via its GAP activity to Arf6, affects ligand-stimulated endocytosis of several GPCRs through either clathrin-dependent or clathrin-independent endocytic pathways [452]. Overexpression of GIT1 and GIT2 disrupts the internalization of some agonist-bound receptors from the plasma membrane, including the β1- and β2-adrenergic, adenosine 2B, μ-opioid, M1 muscarinic and EGF receptors [419, 420, 453]. GIT mutations affecting the ArfGAP domain diminish the effect of overexpressed GITs on receptor internalization [419].

GITs also regulate presynaptic and postsynaptic vesicle trafficking. Through the presynaptic proteins piccolo and scribble, GIT1 participates in organization and release of presynaptic vesicles [445, 454]. Moreover, interaction of GIT1 with liprin-α, a postsynaptic protein, anchors AMPA glutamate receptors to post-synaptic densities. GIT1 was also found to regulate synaptic vesicle recycling by direct association with the endocytic adaptor stonin2, linking the endocytic machinery to the cytomatrix at the active zone [455].

GITs may affect membrane trafficking at other points in the endocytic pathway. Point mutants that lack GAP activity and truncation mutants that lack the Arf GAP domain induce a membrane-delimited structure containing transferrin, paxillin and Rab11 [283, 428]. However, the physiologic function of this compartment is not yet determined.

GIT1 in Cytoskeleton Rearrangement

GITs' role in cytoskeleton rearrangement is mainly through their interacting partners during cell spreading and migration. It has been proposed that the GIT-containing complexes mediate the coordinated movement of actin regulators between sites of activity. It seems that a GIT-containing complex, consisting of paxillin, PAK and PIX, cycle among three intracellular sites: the cell edge, FAs and punctate cytoplasmic structures [456]. GIT, through its PBS, associates with the adhesive structures and the cell edge and interacts with paxillin; while, via its SHD domain, GIT associates with the cytoplasmic structures [423, 424, 456]. Given that the cytoplasmic structures are not associated with any membrane compartment markers, they are thought to be supramolecular complexes but not vesicles. This cytoplasmic structure could be the mediator regulating the movement of actin regulator proteins between FAs and the cell edge [456]. Overexpression of mutant GIT or interacting partners that prevent their association affect the function of GIT on actin remodeling and cell movement. For example, overexpression of the isolated PBS or the isolated N-terminus of GIT prevents association of PIX and PAK with FAs [456]. Expression of paxillin that does not bind GITs or a GIT2 mutant lacking PBS resulted in increased random motility, decreased directed motility, and formation of broad lamellipodia [440, 457], suggesting GITs play an important role in proper directionality of cell movement. This direction-determined function of GIT1 is also shown in endothelial cells during pathological angiogenesis by interacting with cortacin, a protein critical for directional migration [458]. It seems that the interaction between βPIX and MYO18 is important for focal adhesion turnover and cell migration [459]. GIT1 is also important for the formation of podosomes, actin-rich structures important for cell migration [460].

By modulating the F-actin cytoskeleton, the GIT1-βPIX-Rac1-PAK complex recently has been shown to play a crucial role in maintaining the stability of $GABA_A$ receptors, an essential mediator of inhibitory neurotransmission in central nervous system [461].

GIT1 in Other Functions

In endothelial cells, Robo4 (Roundabout homolog 4) interacts with paxillin, which recruits GIT1 to inactivate Arf6, causing vascular stability, blocking cellular protrusions and neovascular leak [462]. In the same cells, GIT1 is proposed to regulate NO production by interacting with endothelial nitric-oxide synthase [463]. In epithelial

cells, GIT1 is proposed to suppress EphA2 (Erythropoietin-producing hepatocellular carcinoma)-mediated Arf6 activity, which provides a positive feedback loop to enhance E-cadherin-based cell-cell contacts [464]. GIT1 is also identified as a novel regulator of vascular smooth muscle cell proliferation, migration, and apoptosis through PLCγ and Erk1/2 signaling pathways, and thus is important for vascular remodeling [465]. In breast cancer cells, GIT1 may enhance tumor cell invasion and metastasis by preventing paxillin and α5β1 integrin from degradation and thus enhancing α5β1 integrin-mediated cell adhesion to fibronectin and collagen [466].

GIT1's Physiological Roles

Given the effect of GIT1 in brain function, it is not surprising that GIT1 knockout mice are defective in fear learning [467] and dendritic spine formation [468]. GIT1 deficient mice also exhibited delayed fracture healing, resulting from reduced bone formation [468], and decreased secretion of VEGF, which is probably due to impaired BMP2 signaling [469]. GIT1 also regulates mitochondrial biogenesis in heart and global GIT1 knockout mice exhibit enhanced cardiomyocyte apoptosis and cardiac dysfunction, probably due to abnormal mitochondria function in heart [470]. Attention-deficit hyperactivity disorder (ADHD) is one of the most common psychiatric disorders in children. It has been shown that an intronic single nucleotide polymorphism in GIT1, which could cause reduced GIT1 expression, is associated with ADHD susceptibility in humans [471]. This cause-effect relation is supported by the observations in GIT1-deficient mice [471]. However, in a study of Brazilian children, GIT1 s (SNPs) are not associated with ADHD [472]. Further data will be needed to valid that. GIT1 was also demonstrated to participate in vascular pulmonary vascular development by regulating VEGF-mediated PLCγ and ERK1/2 activation [473]. Due to that, global GIT1 knockout mice has markedly reduced numbers of pulmonary blood vessels and increased alveolar spaces.

In summary, GIT1 plays important roles in the functions of multiple systems. It is not surprising that global GIT1 knockout mice exhibited ~60% perinatal mortality [473].

Summary

Platelets are important players in thrombosis, hemostasis and beyond. Our previous work has shown that Arf6 is tightly regulated in platelets and is important for platelet

activation (e.g. platelet adhesion, spreading, and aggregation) [414, 417]. However, it is still unclear what Arf6 does in platelets and how Arf6 achieves it?

An interesting phenomenon to Arf6 in platelets is that resting platelets maintain relatively high level of Arf6-GTP which goes down rapidly upon platelet stimulation. We have proposed that Arf6-GTP could help platelets keep in the resting state by cycling membrane protein (e.g. integrins) and preventing cell-cell or cell-matrix contact. Conversely, Arf6-GDP allows platelets to interact with each other or with matrix, for full platelet activation. Interestingly, most platelet receptors are implicated to actively cycle between endosomes and the plasma membrane. A well-studied one is integrin αIIbβ3 which is likely to cycle between the plasma membrane, α-granules, and dense-granules. Upon platelet stimulation, intracellular pools of integrin αIIbβ3 are exposed on the plasma membrane and, together with surface pools, mediate platelet aggregation. Recent findings suggested in platelets that Arf6 mediated P2Y receptor endocytosis and regulated dense-granule exocytosis. However, the molecular mechanism involved in these processes makes the role of Arf6 controversy. One shows that Arf6 is in GDP state in resting platelets; the other shows that Arf6-GTP level in resting platelets is sequestered by its regulator. Therefore, it becomes needed to have a better system and more experimental data to uncover the role of Arf6 in platelets. In the first part of this thesis, platelet-specific, Arf6 knockout mice were generated to explore the role of Arf6 in platelets.

Another interesting phenomenon in our previous findings is that the Arf6-GTP level is partially recovered by inhibiting integrin αIIbβ3 outside-in signaling and platelet aggregation. This raises an important question: how is Arf6 regulated in platelets? The observation can be simply explained by a scene in which an Arf6 GAP protein is activated following integrin αIIbβ3 outside-in signaling. Of course, other explanations are equally possible. Therefore, identifying the regulators, GEFs or GAPs, in platelets would help better understand the role of Arf6. Proteomics studies have shown the presence of multiple Arf6 regulators in platelets. During the preliminary search, GIT1, a GAP protein, stood out. GIT1 is a multi-domain protein, containing a C-terminal PBD which allows the association of GIT1 to integrin via paxillin. GIT1 tyrosine phosphorylation is regulated by integrin outside-in signaling. The second part of this

thesis is to study GIT1 by using human platelets and platelet-specific, GIT1 knockout mice.

Chapter Two: Reagents and Methods

Reagents and Antibodies

Custom Myr-Arf1/Arf6 peptide (residue 2-13 of its N-terminal), scrambled Myr-Arf6 peptide (scArf6, Myr-FINGLEVKKFSK) and RGDS peptide were from Anaspec (San Jose, CA). Apyrase, human fibrinogen, cytochalasin D, tetramethyl rhodamine isothiocyanate (TRITC)-phalloidin, poly-D-lysine, glutathione-agarose, hirudin and proteinase K were from Sigma (St. Louis, MO). latrunculin A was from Calbiochem (San Diego, CA). Thrombin, Collagen, ADP, and CHRONO-LUME reagent were from Chrono-log (Havertown, PA). U46619 and prostaglandin I_2 (PGI_2) was from Cayman (Ann Arbor, MI). A23187 was from Calbiochem (San Diego, CA). Convulxin was from Centerchem (Norwark, CT). PAR1/PAR4-peptide was from Bachem (Bubendorf, Switzerland). Fibronectin-free fibrinogen was from Enzyme Research Laboratory (South Bend, IN). DNA ligation Kit was from Enzymax (Lexington, KY). Fura-2/acetoxymethyl ester (AM) was from Molecular Probes (Eugene, OR). Complete, EDTA-free protease inhibitor cocktail was from Roche (Indianapolis, IL). Acid citrate dextrose (ACD) blood collection tubes were from BD diagnostics (Sparks, MD). 70% glutaraldehyde was from Electron Microscopy Sciences (Hatfield, PA). Other reagents used were at least laboratory grade.

Rabbit anti-GIT1 antibody was from Bethyl Laboratory (Montgomery, TX). Mouse monoclonal antibodies against protein GIT1, Hic-5, Rac1, Cdc42, and FITC-anti-CD62P antibody and PE-anti-LAMP1 antibody were from BD Biosciences (San Jose, CA). Mouse anti-RhoA antibody was from Cytoskeleton (Denver, CO). Rabbit anti-GIT1 antibody and rabbit anti-phospho-myosin light chain antibody were from Santa Cruz (Dalla, TX). Rabbit anti-β-PIX antibody and rabbit anti-Integrin β3 antibody were from Cell Signaling (Danvers, MA). Rabbit anti-Arf1/3 antibody, rabbit anti-Sema3F antibody and mouse anti-actin antibody were from Sigma (St. Louis, MO). Mouse anti-p-selectin antibody, rabbit anti-VMAT2 antibody, rabbit anti-vWF antibody and mouse anti-fibronectin antibody were from Abcam (Cambrige, MA). FITC-anti-CD41/CD61 antibody and PE-Jon/A antibody were from Emfret Analytics (Eibelstadt, Germany). Rabbit anti-fibrinogen antibody was from Dako (Glostrup, Denmark). Rabbit anti-vitronectin antibody was from Molecular Innovation (Novi, MI). Rabbit anti-murine

PF4 antibody was from R&D Systems (Pittsburgh, PA). Mouse anti-phospho-tyrosine antibody (4G10 clone platinum) and anti-FAK antibody were from Millipore (Darmstadt, Germany). Rabbit Anti-Arf6 serum was homemade as described before [414]. Rabbit anti-RabGDI polyclonal antibody was generated by Dr. Tara Rutledge in our laboratory using recombinant RabGDIα as antigen.

Generation of Arf6$^{flox/flox}$/PF4-Cre^{+} Mice (Arf6 KO Mice)

All animal procedures were done under and approved protocol (884M2005) from the University of Kentucky Institutional Animal Care and Use Committee (IACUC)

Embryos of Arf6$^{flox/flox}$ mouse [474] were a generous gift from Dr. Kanaho (University of Tsukuba, Tsukuda, Japan) and were recovered under contract with the Jackson laboratory (Bar Harbor, ME). The animals were crossed with a PF4-Cre^{+} strain [475] which was a generous gift from Dr. Radek Skoda, (University Hospital Basel, Switzerland). Polymerase Chain Reaction (PCR)-based genotyping was performed using genomic DNA isolated from tail tip of individual mouse. For Arf6 gene, the PCR analysis was carried out using following primer set:

forward primer, 5'-GACCCCATGAGTGTTGTCAC-3',

reverse primer, 5'-GGGATACATAGAGAAACCTTGTCTCAGG-3'.

The PCR yields a 240 bp DNA product for WT allele and a 270 bp one for floxed allele.

The PCR analysis for the Cre transgene was carried out using the following primer set:

for the wild type allele,

forward primer 5'-CCCATACAGCACACCTTTTG-3',

reverse primer 5'-GAAACAACAGGCCCAGAAGC-3';

for the Cre allele,

forward primer 5'-CCCATACAGCACACCTTTTG-3',

reverse primer 5'-TGCACAGTCAGCAGGTT-3'.

PCR yielding a 450 bp DNA product indicates the presence of PF4-Cre gene.

PCR conditions for both Arf6 gene and PF4-Cre gene are: 94°C for 10 min for 1 cycle, followed by 30 cycles of 94°C for 1 min, 54°C for 45 sec and 68°C for 30 sec, and lastly 68°C for 7 min for 1 cycle.

Platelet specific Arf6 KO mice (Arf6$^{flox/flox}$/PF4-Cre^{+}) were generated by crossing two above murine strains. WT littermates (Arf6$^{flox/flox}$/PF4-Cre^{-}) were used as control.

Generation of GIT1$^{flox/flox}$/PF4-Cre^{+} Mice (GIT1 KO Mice)

GIT1$^{flox/flox}$ mice [467] were a generous gift from Dr. Premont (Duke University). PCR analysis was carried out using the following primer set:

forward primer: 5'-CCCAGAATGTCAAGCTGGTT-3',

reverse primer: 5'-GATCAGCCACCTCTGCCAAG-3'.

Using same PCR conditions for Arf6 gene, WT allele gives a 360 bp DNA product and floxed allele gives a 380 bp DNA product. PCR products were determined by DNA agarose (containing Ethidium Bromide) electrophoresis.

Platelet specific GIT1 KO mice (GIT1$^{flox/flox}$/PF4-Cre^{+}) were generated by crossing GIT1$^{flox/flox}$/PF4-Cre^{-} mice with GIT1$^{flox/flox}$/PF4-Cre^{+} mice. WT littermates (GIT1$^{flox/flox}$/PF4-Cre^{-}) were used as control.

Generation of Integrin β3 KO Mice

Integrin β3 KO mice [476] were a generous gift from Dr. Zhenyu Li (University of Kentucky). PCR analysis was carried out using the following primer set:

forward primer: 5'-CTTAGACACCTGCTACGGGC-3',

reverse WT: 5'-CCTGCCTGAGGCTGAGTG-3',

reverse Neo: 5'-CACGAGACTAGTGAGACGTG-3'.

Using same PCR conditions for Arf6 gene, WT allele give a 446 bp DNA product and neo allele gives a 538 bp DNA product.

Genomic DNA Isolation from Mouse Tail Tip

Mouse tail tips (3-5 mm) were digested overnight (O/N) at 55°C in 400 µl tail lysis buffer (50 µM Tris/HCl, pH 7.5, 100 µM EDTA, 100 µM NaCl, and 1% SDS after autoclave) containing 20 µl 10 mg/ml proteinase K., Protein/peptide was precipitated by mixing with 200 µl saturated 6 M NaCl and centrifuged at 13,000 x g for 30 min at room temperature (RT). The supernatant containing DNA was harvested and mixed with equal volume of 100% ethanol. After incubation for 10 min at RT, DNA was pellet down by centrifuge at 13,000 x g for 10 min. DNA pellets were washed once with 1 ml 70% ethanol and then dried in a vacuum centrifuge for around 20 min. Genomic DNA was finally dissolved in 100 µl autoclaved ddH_2O.

Whole Blood Count

Animals were euthanatized by CO_2 asphyxiation just prior to harvest blood. Using sodium citrate as anticoagulant, mouse blood was harvest *via* heart puncture by 26G needles attached to a syringe which contains 100 µl mixture of 3.8% sodium citrate, 2 U/ml apyrase and 100 ng/ml PGI_2. Whole blood counting was performed using Hemavet (DREW Scientific, Erba Diagnostics, Inc.). Concentrations of blood cells (red blood cells, white blood cells, and platelets) were determined by back-calculation according to anticoagulant dilution factor. Also, the platelet size was recorded. Analysis of both blood count and platelet size was performed using Sigmaplot 12.0 (Systat software Inc.).

Preparation of Mouse Platelets

Animals were euthanatized by CO_2 asphyxiation just prior to harvest blood. Using sodium citrate as anticoagulant, mouse blood was harvest via heart puncture with a 26G needle attached to a syringe which contains 100 µl mixture of 3.8% sodium citrate, 2 U/ml apyrase and 100 ng/ml PGI_2. Blood was diluted in HEPES Tyrode buffer (pH 6.5, 20 mM HEPES/KOH, 128 mM NaCl, 2.8 mM KCl, 1 mM $MgCl_2$, 5 mM D-glucose, 12 mM $NaHCO_3$, 0.4 mM NaH_2PO_4) in 1:1 ratio in the presence of 0.2 U/ml apyrase and 10 ng/ml PGI_2 and then pooled in a 15 ml conical tube. Whole blood was subjected to centrifugation at 215 x g for 10 min at RT and the supernatant (platelet rich plasma, PRP) was recovered. To remove residual red blood cells and white blood cells, PRP was subjected to centrifugation again at 215 x g for 10 min at RT. To recover the platelets, the PRP was subjected to centrifugation at 675 x g for 10 min at RT. Platelet pellet was resuspended in HEPES Tyrode buffer (pH 6.5) containing 0.2 U/mL apyrase, 10 ng/mL PGI_2 and 1 mM EGTA. After a second centrifugation step at 675 x g for 10 min at RT, platelet pellets were resuspended in HEPES Tyrode buffer (pH 7.4) supplemented with 1 mM $CaCl_2$. Platelet concentration was measured using Z2 Counter (Beckman Coulter, Inc., Miami, FL) and adjusted with HEPES Tyrode buffer (pH 7.4) to the concentrations indicated. Platelets were incubated at RT for 30 min before use.

Preparation of Fresh Human Platelets

Fresh whole blood was drawn from informed healthy donor using acid citrate dextrose (ACD) collection tubes (BD Biosciences). Whole blood was diluted in HEPES Tyrode

buffer (pH 6.5) with 1:1 ratio in the presence of 0.2 U/ml apyrase and 10 ng/ml PGI_2. After centrifugation at 215 x g for 20 min at RT, the supernatant (PRP) was harvested. To remove residual red blood cells and white blood cells, PRP was subjected to centrifugation at 155 x g for 10 min at RT. Platelets were recovered from the resulting PRP by centrifugation at 750 x g for 13 min at RT, and then resuspended in HEPES Tyrode buffer (pH 6.5) in the presence of 0.2 U/ml apyrase, 10 ng/ml PGI_2 and 1 mM EGTA. Platelets suspensions were subjected to centrifugation again at 750 x g for 13 min at RT and the resulting pellet was resuspended in HEPES Tyrode Buffer (pH 7.4) supplemented with 1 mM $CaCl_2$. Platelet concentrations were determined by using Z2 Counter (Beckman Coulter, Inc., Miami, FL) and adjusted to 4 X 10^8/ml with HEPES Tyrode buffer (pH 7.4), unless otherwise indicated. Platelets were incubated at RT for 30 min before use.

Preparation of Banked Human Platelets

Human platelet rich plasma (PRP) was obtained from the Kentucky Blood Center. The PRP was pooled in a 50 ml conical tube and pre-incubated with 0.2 U/ml apyrase and 10 ng/ml PGI_2 for 10 min. To remove residual red blood cells (RBC) and white blood cells (WBC), PRP was subjected to centrifugation at 155 x g for 13 min at RT. After this step, the platelets were treated as described in the previous “preparation of fresh human platelets” section.

Platelet Aggregometry and ATP Release Measurements

Washed platelets were freshly prepared and adjusted to the desired concentration with HEPES Tyrode buffer (pH 7.4) containing 1 mM $CaCl_2$. Platelets suspensions (250 or 500 µl) in a siliconized glass cuvette (Chrono-log, Havertown, PA) was warmed to 37°C under stirring conditions (1200 rpm) for 2-3 min in a Model 460Vs Lumi-dual aggregometer (Chrono-log). After pretreatment with the indicated reagents, platelets were stimulated with the indicated agonists to initiate platelet aggregation. A model 810 Aggro/Link computer interface and Aggro/Link software (Chrono-log) were used to monitor platelet aggregation traces. Buffer alone had been used to set baselines.

ATP release from platelets dense-core granules was monitored using CHRONO-LUME reagents (Chrono-log) while measuring platelet aggregation. CHRONO-LUME reagents containing luciferin and luciferase were pre-incubated with platelet suspension (1 to

20 ratio) for 1 min prior to addition of agonists. Luminescence traces were recorded using the same interface and software as platelet aggregation. Graphs of platelet aggregation and ATP release was processed and assembled into figures using Adobe Photoshop CS5 (Adobe Inc. San Jose, CA).

Flow Cytometry Analysis

Washed platelets from WT and KO mice were freshly prepared and adjusted to 5 X 10^7/ml in HEPES Tyrode buffer (pH 7.4) in the presence of 1 mM $CaCl_2$. Platelets (20 µl) were either kept resting or stimulated with 0.1 U/ml thrombin for 1 min. The reactions were stopped by adding a 2 fold excess of hirudin. Samples were then incubated with fluorophore-conjugated antibodies (2.5 µl each) for 15 min at RT. FITC-anti-CD41/CD61 antibody, PE-Jon/A antibody, FITC-anti-CD62 antibody and PE-anti-LAMP1 antibody were used to measure surface levels of total integrin αIIbβ3, active integrin αIIbβ3, P-selectin, and LAMP1, respectively. The mixture was transferred to polystyrene Falcon™ tubes (BD Biosciences, San Jose, CA) containing 200 µl HEPES Tyrode buffer (pH 6.5). Unlabeled platelets were used as background controls. Samples were analyzed at the flow cytometry facility at University of Kentucky. Flow cytometry analysis was performed using FACScan™ flow cytometer (BD Biosciences). Platelet populations were determined by combination of forward light scattering (FSC) and side light scattering (SSC). CellQuest™ (BD Biosciences) was used to monitor platelet fluorescent intensities. A total of 10,000 platelet events were analyzed and the geometric mean of the fluorescent intensity were determined and analyzed using the SigmaPlot 12.0 statistical routines.

Electron Microscopy

Washed platelets from WT and KO mouse were prepared and adjusted to 4 X 10^8/ml. Platelets (500 µl) were either kept resting or stimulated with 0.1 U/ml thrombin for 3 min, followed by fixation using equal volume of 0.1% glutaraldehyde in White's Saline [solution A (20X, 2.4 M NaCl, 0.1 M KCl, 46 mM $MgSO_4.7H_2O$, 64 mM $Ca(NO_3).H_2O$), solution B (20X , 130 mM $NaHCO_3$, 8.4 mM Na_2HPO_4 , 3.8 mM KH_2PO_4 , 0.01% Phenol Red]. After fixation in a 37°C metal block for 15 min, platelets were subjected to centrifugation at low speed (3000 x g) for 2 min. Platelet pellet were resuspended in cold 3% glutaraldehyde in White's saline, followed by incubation at 4°C for 1 h. After

3X washing with White's saline, platelet pellets were recovered by centrifugation at maximum speed (13,000 x g) and then resuspended in 300 µl 1% O_sO_4 (osmium tetroxide, to make 4.5 ml add 2.25 ml O_sO_4, 225 µl White's A, 225 µl White's B, 2 ml ddH_2O) for 30~60 min on ice. The pellets are then washed three times with 1 ml White's saline, followed by another two washes with 1 ml ddH_2O. The samples were dehydrated with a series of ethanol washes (50%, 70%, 90%, 100% absolute ethanol), 5 min each, followed by two washes with propylene oxide. The samples were infiltrated with 1 ml of 1:1 propylene oxide: Spurr's resin (18 g VCD/ERL 4221, 14 g DER, 48 g NSA, well mixed with a wooden spatula without generating air bubbles; add 0.6 g DMAE and mix by stir for 20 min) and incubated overnight on a rotator at RT. The platelets were recovered by centrifugation and the platelet pellet was resuspend in new Spurr's resin followed by incubation on rotator for 1 hr at RT. This was repeated 4-5 times. Finally the samples were embedded in Spurr's resin (~200 µl volume) and allowed to polymerize for 48 hr at 60°C. Polymerized blocks were sectioned using a Reichert-Jung Ultracut E. microtome and mounted onto copper grids. Following counterstaining with uranyl acetate and lead citrate (if necessary for contrast), sample grids were viewed on a Hitachi H-7000 transmission electron microscope and images were obtained with Gatan digital micrograph software (Pleasanton, CA).

Platelet Adhesion

Washed platelets from WT and KO mice were freshly prepared and adjusted to 5 X 10^8/ml in HEPES Tyrode buffer (pH 7.4). Washed platelets were then incubated for 30 min at 37°C with 7 µM calcein AM (BD Bioscience) in the presence of 0.2% of pluronic F-127 (Invitrogen). After centrifugation, platelets were resuspended in double the original volume of HEPES Tyrode buffer (pH 7.4, the platelet final concentration is 2.5 X 10^8/ml) in the presence of 1 mM Ca^{2+}. 50 µl labeled platelets were then seeded on 96-well opaque plate which was pre-coated with either 50 µg/ml human fibrinogen or 5% BSA (background control) in Tris buffer (25 mM Tris, 137 mM NaCl, pH 7.4). After incubation for 30 min at 37°C, the plates were washed twice with HEPES Tyrode Buffer (pH 7.4) and adherent platelets were measured using a fluorescence plate reader (SpectraMax M5, Molecular Devices, Sunnyvale, CA) with excitation/emission/cutoff

at 485/538/515, respectively. Standard curves, to calculate cell binding, were generated using platelets of serial fixed numbers from each genotype.

Platelet Spreading

Fibrinogen-coated slides were prepared by incubating 50 µg/ml human fibrinogen (Enzyme Research Laboratory) in PBS onto Nunc Lab-Tek II 16 well chamber slide (Thermo Scientific) overnight at 4°C. Washed platelets (2 X 10^7/ml) were prepared from mouse blood and resuspended in HEPES Tyrode buffer (pH 7.4) containing 1 mM $CaCl_2$. Before seeding on fibrinogen-coated slide, washed platelet suspensions (100 µl) were supplemented with 1 µl platelet-poor plasma (PPP). The slides were incubated at 37°C for the indicated times. The unbound platelets were removed by washing and the adherent platelets were fixed with 4% paraformaldehyde overnight at 4°C.

For TRITC-phalloidin staining, platelets were permeabilized with permeabilization buffer (0.1 M Tris, 0.1% Triton X-100, 0.15 M NaCl, 0.01 M EGTA, 5 mM, $MgCl_2$, pH 7.4) for 30 min at Room Temperature (RT). After washing with PBS, platelets were stained with 0.1 µM TRITC-phalloidin (Sigma, St Louis, MO) in permeabilization buffer for 30 min at RT. After 3 washes with 1X PBS, platelets were mounted with VectorShield preservative (Vector Laboratories) on a Lab-Tek coverglass. Coverglasses were sealed with nail polish and samples were visualized immediately or kept at 4°C prior to image processing. Images were visualized using Nikon Eclipse E600 microscope (Nikon, Melville, NY) with a 100X/1.40 numeric aperture DIC H oil objective lens (Nikon). Images were taken using a Zeiss camera (AxioCam MR, Germany) and they were processed using Zen 2011 software (blue edition, Zeiss) and Quantified with Image J (V1.47, NIH).

Clot Retraction

Washed platelets from WT and KO mice were prepared and adjusted to 3 X 10^8/ml in HEPES Tyrode buffer (pH 7.4). During platelet preparation, cuvettes with 100 µl 10% sodium dodecyl sulfate polyacrylamide gel (SDS PAGE) at the bottom were prepared and soaked in HEPES Tyrode buffer (pH 7.4). Platelets were then supplemented with 0.5 mg/ml human fibrinogen (Sigma) and 1 mM $CaCl_2$. Immediately after mixing platelets with 0.1 U/ml thrombin in the presence or absence of the indicated treatment, platelets were transferred to the cuvettes and clot retraction was started.

Images were taken at the indicated time points. Clot volume was measured using Image J v1.47 and normalized to the initial volume. Analysis was done using SigmaPlot 12.0.

Endocytosis of Biotinylated-fibrinogen *In vivo*

Biotin-fibrinogen was synthesized based on manufacture's protocol using EZ-Link™ sulfo-NHS-Biotinylation Kit (Thermo Scientific) and human fibrinogen (Sigma) in PBS. WT and KO mice were anaesthetized by isoflurane and then injected with 200 µl 5 mg/ml labeled fibrinogen via retro-orbital plexus using 27 1/2 G needle. After 24 hours, mice were euthanized and platelets were harvest as described above. Platelet lysates were prepared and subjected to western blot with streptavidin-alkaline phosphatase conjugates.

Preparation of GST-GBDs (Activated GTPase Binding Domains)

pGEG-5X/GGA3 (Arf6 binding domain, VHS-GAT) was a generous gift from Dr. Julie G. Donaldson (NIH). pGEX-KG/mousePAK3 (Rac/Cdc42 binding domain, RBD) was kindly provided by Dr. Douglas A. Andres (University of Kentucky, Lexington, KY). These prokaryotic expression plasmids were transformed into the Rosetta™(DE) *E coli* and the positive clones were cultured in 25 ml LB/amp/cam media (100 µg/ml ampicillin and 35 µg/ml chloramphenicol) O/N at 37°C. The bacteria were harvested by centrifugation at 4,500x g for 15 min and the pellets were resuspended in 1L of fresh LB/amp/cam media. Protein expression was induced, when the OD_{600} of the culture reached 1, with 1 mM IPTG for 4 hours. The cells were harvested and processed immediately or kept frozen at -80^{o}C. The cells were resuspended in TBS (Tris-buffer-saline, 50 mM Tris/HCl, 147 mM NaCl) containing 1 mM dithiothreitol (DTT) and 1X protease inhibitors (20 µg/mL Leupeptin, 20 µg/mL Chymostatin, 20 µg/mL Pepstatin A, 10 µg/mL Antipain). The cells were then disrupted by passing the suspensions through a French Press three times at 12,000 psi. The lysates were cleared by centrifugation at 100,000 x g for 1 hour at 4°C. The supernatants were further clarified by passage through a 0.2 µm nylon filter. After mixing with glycerol (10% final, v/v), the supernatants were aliquot and stored at -80°C. The ratio of supernatant to glutathione beads (v to v) was determined by glutathione agarose beads binding assay. Different amount of clarified lysates were incubated with 10 µL beads for 1 hour at

4 °C. The beads complex was then washed three times with HEPES wash buffer and the bound proteins were eluted by 2X SDS sample buffer with 5 min boiling. The samples were analyzed by SDS-PAGE and subsequently stained by Coomassie blue. The sample with the saturation binding was selected to determine the optimal ratio for the following pull-down assay.

Small GTPase Pulldown Assay

Washed platelets (4 X 10^8/ml, 500 µl), which were kept resting or stimulated by agonists for indicated times in the aggregometer, were lysed with equal volumes of 2X ice-cold lysis buffer (pH 7.4, 20 mM HEPES/KOH, 128 mM NaCl, 9 mM $MgCl_2$, 2% Triton X-100, 0.2% SDS, 1% sodium deoxycholic acid, 20% Glycerol, and 2 mM benzamidine) containing 2X EDTA-free protease inhibitors cocktail. Platelet lysates were either processed immediately or stored at -80°C. For assay, the samples were thawed, transferred to Eppendorf tubes, and clarified by centrifugation at 15,000 x g for 5 min at 4°C. Sixty microliters of supernatant were used as input and 850 µl was incubated for 45 min at 4°C with a specific effector binding domain bound to glutathione-agarose. Specifically, all six Arf isoforms [477, 478] were recovered on GST-human $GGA3_{VHS\text{-}GAT\ (1\text{-}133)}$, Rac and Cdc42 [479] were recovered on GST-mouse $PAK3_{RBD(397\text{-}518)}$. The bound material was recovered by centrifugation at 500 x g for 12 sec and washed 3 times with wash buffer (pH7.4, 20 mM HEPES, 128 mM NaCl, 2 mM $MgCl_2$, 1% Triton X-100, 10% Glycerol). Input was prepared by adding 40 µl 5X sample buffer (62.6 mM Tris, pH6.8, 2% SDS, 5% Glycerol, 2% β-mercaptoethanol, 0.01% Bromophenol blue). Bead-bound material was dissolved in 80 µl 2X sample buffer. Samples were immediately used or kept at -20°C after boiling at 95°C for 5 min.

Separation of Supernatant (Detergent-soluble) and Pellet (Detergent-insoluble)

Washed human platelets (500 µl, 4 X 10^8/ml) was pretreated with indicated inhibitors and then were either kept resting or stimulated with agonists for 5 min in the aggregometer. The reactions were terminated with 2X ice-cold lysis buffer (HEPE/KOH, pH7.4, 2% Triton-X 100 or 2% NP-40, 127 mM NaCl, 5 mM $MgCl_2$, 20% glycerol, 2 mM sodium orthovanadate, 2 mM benzamidine) containing 2X protease inhibitor cocktail. After incubation on ice for at least 30 min, the platelet lysates were clarified by centrifugation at 15,000x g for 10 min to remove detergent-insoluble material (e.g.

actin cytoskeleton and lipid rafts). The pellet were dissolved in 100 µl 1X sample buffer and 80 µl supernatant (detergent soluble fractions) was treated with 1X sample buffer.

Immunoprecipitation/Co-immunoprecipitation

Washed platelets (500 µl, 4 X 10^8/ml) were prepared as described above and kept at resting or stimulated with agonists for the indicated time at 37°C in the aggregometer. Platelets were pretreated with indicated inhibitors prior to stimulation. The reactions were terminated by adding equal volume of 2X ice-cold immunoprecipitation buffer (20 mM HEPE/KOH, pH 7.4, 128 mM NaCl, 9 mM $MgCl_2$, 2% Triton-X 100, 20% glycerol, 2 mM sodium orthovanadate, 2 mM benzamidine, 2 mM EGTA) containing 2X protease inhibitor cocktail and phosphatases inhibitor cocktail. Platelets lysates were incubated on ice for at least 30 min and then clarified by centrifugation at 15,000 X g for 10 min at 4°C. The supernatant (80 µl) was used as input and 850 µl of the remaining supernatant were first incubated with 10 µl protein-A agarose for 30 min at 4°C to remove non-specific binding proteins. The resulting supernatants were then incubated with the indicated antibodies bound to protein-A agarose for 2 hours at 4°C. The bound proteins were recovered by centrifugation and washed 3 times with 1X ice-cold IP lysis buffer. The bound proteins were eluted by addition of 1X sample buffer. Input was also prepared in 1X sample buffer. All samples were then boiled for 5 min and subjected to western blotting.

Western Blotting and Quantification

Protein samples were boiled at 95°C for 5 min and then loaded into sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE). After separation, the proteins were transferred electrophorectically to Immobilon-P polyvinylidene fluoride membrane (PVDF, Milipore, Bedford, MA) using a constant voltage (100 V) for 1 hour. PVDF membranes were blocked with blocking buffer [5% non-fat dried milk in TBST (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5% Tween-20)] for 1 hour at RT. Membranes were then incubated with appropriately diluted primary antibody in blocking buffer overnight (O/N) at 4°C. After wash for 3X 5 min with TBST, membrane was incubated with 1 to 10,000 diluted -secondary antibodies in blocking buffer for 45 min at RT. After another 3X 5 min washes with TBST, membranes were incubated with substrate and processed for imaging.

For HRP-conjugated secondary antibody, enhanced chemiluminescence (ECL) substrate was used. After incubation with substrate, membrane was scanned in ChemiDoc™ MP system (BioRad, Life Science Research, Hercules, CA) and signal was quantified using Image Lab Software.

For alkaline phosphatases-conjugated secondary antibody, enhanced chemofluorescence (ECF) substrate (GE healthcare, Piscataway, NJ) was used. Membrane was placed on a transparent film (3M, St. Paul, MN) and incubated with substrate. The fluorescent signal was detected using Typhoon™ 9400 (Amersham Biosciences) or Fluorescent imager (GE Healthcare). Quantification was performed using ImageQuantTL 5.2 software (Amersham Biosciences).

Measurement of F-actin in Platelets

F-actin content in platelets has been measured by a spectrofluorometer-based assay system. Resting and stimulated platelets (2×10^7) were fixed and permeabilized by an equal volume of F-actin staining buffer (10 mM PIPES, pH 6.9, 20 mM KH_2PO_4, 5 mM EGTA, 2 mM $MgCl_2$, 0.2% Triton X-100, 3% glutaraldehyde, 2 µM TRITC-phalloidin) with constant rotation for 1 hour at RT. F-actin fraction was collected by centrifuge at 15,000 x g for 5 min and washed once with F-actin wash buffer (10 mM PIPES, pH 6.9, 20 mM KH_2PO_4, 5 mM EGTA, 2 mM $MgCl_2$, 0.1% Saponin). Bound TRITC-phalloidin was extracted by incubating the pellet with 300 µl methanol at RT for 1 hour. Freed phalloidin was separated by centrifuge at 15,000 x g for 5 min. The fluorescence intensities of samples were measured using a microplate spectrofluorometer SpectramMax GeminiXS (Molecular Devices, Sunnyvale, CA) and analyzed with SoftMax Pro software (Molecular Devices).

***Ex vivo* FITC-fibrinogen Uptake Assay**

Washed WT and KO platelets were resuspended at concentrations of 5×10^8/ml in HPEPS Tyrode Buffer containing 1% BSA and 1 mM $CaCl_2$. Platelets (100 µl) were incubated with 0.05 mg/ml FITC-fibrinogen for 1 hour at 37°C. Platelets were subjected to centrifugation at low speed to avoid activation and resuspended in an equal volume of platelet poor plasma (PPP) or HEPES Tyrode Buffer containing BSA and $CaCl_2$. Platelets were then incubated for 3 hours at 37°C and fixed with 2 % paraformaldehyde at 4°C overnight. Platelets were recovered by centrifugation and

resuspended in 15 µl HEPES Tyrode buffer. Microscopic slides with 5 µl platelets mixed with 1 µl Trypan blue (stock 4%) were prepared and visualized using Nikon Eclipse E600 microscope (Nikon, Melville, NY) with a 100X/1.40 numeric aperture DIC H oil objective lens (Nikon). Images were taken using Zeiss camera (AxioCam MR, Germany). Images were processed using Zen 2011 (blue edition, Zeiss). As a metric of FITC-uptake, the number of FITC-positive granules per platelet was counted.

Tail Bleeding Assay

Mice from WT and KO groups (4-6 weeks of age) were anaesthetized by intraperitoneal injection with (Fort Dodge SOURCE, 75 mg/kg). The tail was transected 3 mm from the tip and immediately immersed in 37°C saline. The time from transection to bleeding cessation was recorded. Mice were watched for an additional minute to monitor re-bleeding events. The recordings were terminated at 10 min if prolonged bleeding occurred and the bleeding was stopped manually. The data was analyzed using the Logrank test.

$FeCl_3$-induced Carotid Artery Injury Model

Mice from age-matched WT and KO groups were anaesthetized by intraperitoneal injection with Avertin (0.2 g/kg). Mice were placed on a heating pad with tape on the legs and thread on the teeth to maintain their position. The left carotid artery was surgically exposed and the ultrasound probe (Transonic probe 0.5P) was place underneath. The artery was immersed with saline during the entire recording period. Flow in the artery was recorded using the TS420 Transonic System. A stable baseline signal was recorded for 3 min prior to placing a $FeCl_3$-soaked filter paper (for 3 min) on the site supported by a plastic wedge. After removal of the filter paper, flow was measured until it ceased and the time was recorded. Mouse was monitored for an additional minute to look for unstable thrombus formation. The recordings were terminated by 30 min. Mice were euthanatized at the end of experiments. The data was analyzed using the Logrank test.

Study Approval

All animal work was approved by the Institutional Animal Care and Use Committee at the University of Kentucky. Since banked platelet units were anonymized, there is no need for approval by the Institutional Review Board.

Statistics

The data from bleeding time and occlusion assays were analyzed using the Logrank test. The data from secretion assays and FACS-based experiments were analyzed by two-way ANOVA. A one-tailed Student's t-test was used to analyze the properties of platelets from KO mice. In all cases, the p values are indicated.

Chapter Three: Role of Arf6 in Murine Platelets

Introduction

Endocytic trafficking of platelet surface receptors plays a role in the accumulation of granule cargo (*i.e.* fibrinogen and VEGF) and thus could contribute to hemostasis, angiogenesis, or inflammation. However, the mechanisms of platelet endocytosis are poorly understood. The small GTP-binding protein, ADP-ribosylation factor 6 (Arf6), regulates integrin trafficking in nucleated cells; therefore, we posited that Arf6 functions similarly in platelets. To address this, we generated platelet-specific, Arf6 knockout mice. Consistent with our hypothesis that Arf6 controls integrin αIIbβ3-trafficking and thus uptake of fibrinogen Arf6$^{-/-}$ platelets had a fibrinogen storage defect, though other cargo were unaffected. Additionally, platelets from Arf6$^{-/-}$ mice injected with biotinylated-fibrinogen, showed lower accumulation of the modified protein than did WT mice. Resting and activated αIIbβ3 levels, measured by FACS, were unchanged in Arf6$^{-/-}$ platelets. Arf6$^{-/-}$ platelets had normal agonist-induced aggregation and ATP release; however, they showed faster clot retraction and enhanced spreading, which appears due to altered αIIbβ3 trafficking since myosin light chain phosphorylation and Rac1 activation, in response to thrombin, were unaffected. Arf6$^{-/-}$ mice showed no hemostasis defect in tail-bleeding or $FeCl_3$–induced carotid injury assays. This is the first report, using animal models, to identify a role for Arf6 in integrin αIIbβ3 trafficking in platelets.

Generation of Platelet-specific, Arf6 KO Mice

Mouse embryos with a floxed Arf6 gene were kindly provided by Dr. Yasunori Kanaho at University of Tsukuba in Japan [474] and recovered by the Jackson Laboratory (Bar Harbor, ME). Platelet-specific Arf6 KO mice were generated by crossing Arf6-gene floxed mice with PF4-Cre mice that were regularly used by our laboratory to generate mouse strains with specific, protein-deficiencies in platelets [35]. The strategy of generating the mice is shown in Figure 1A. For experiments, mice from an Arf6-floxed/Cre^{-} (referred as WT hereafter) and Arf6-floxed/Cre^{+} (referred as KO hereafter) cross were used. The genotypes of all mice were determined by PCR. Isolated genomic DNA from tail tips was used for PCR

Figure 1

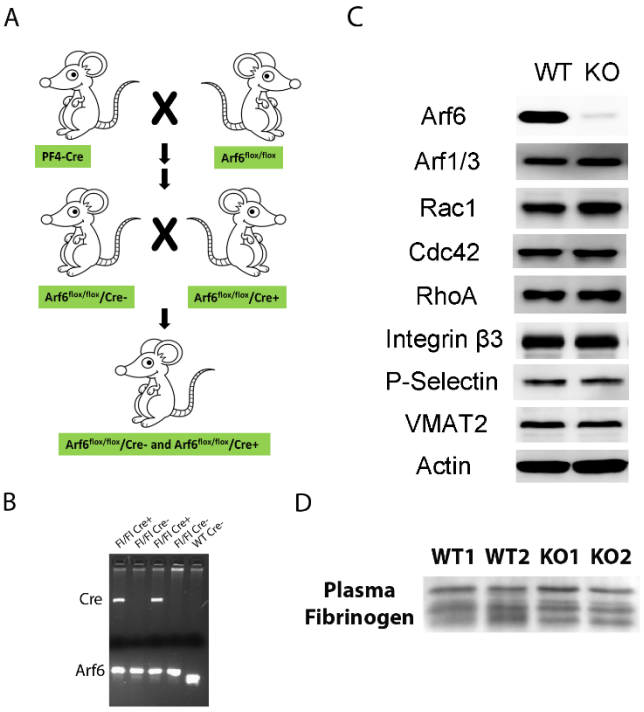

A
PF4-Cre
Arf6flox/flox
Arf6flox/flox/Cre-
Arf6flox/flox/Cre+
Arf6flox/flox/Cre- and Arf6flox/flox/Cre+
C
WT KO
Arf6
Arf1/3
Rac1
Cdc42
RhoA
Integrin β3
P-Selectin
VMAT2
Actin
B
Fl/Fl Cre+
Fl/Fl Cre-
Fl/Fl Cre+
Fl/Fl Cre-
WT Cre-
Cre
Arf6
D
WT1 WT2 KO1 KO2
Plasma Fibrinogen

Figure 1 Generation of Platelet-specific, Arf6 KO Mice. (A) Strategy of generating platelet-specific, Arf6 KO mice. Arf6 gene-floxed mice were first crossed with PF4-Cre mice. For experiments, mice were generated by intercross of $Arf6^{flox/flox}$/PF4-Cre^{+} and $Arf6^{flox/flox}$/PF4-Cre^{-}. **(B)** DNA electrophoresis of PCR products of Arf6 gene and Cre gene. PCR production was amplified using the primer and condition described in method section. A WT control, the one on the right, was used as control. Data is representative of at least three independent experiments. **(C)** Protein profile of Arf6 WT and KO platelets. Washed platelets (1 X 10^7) from WT and KO were loaded. Indicated proteins were probed by western blotting using corresponding antibodies. Actin was used as a loading control. Data is representative of at least three independent experiments. **(D)** Levels of plasma fibrinogen in Arf6 WT and KO mice. Equal amount of plasma from WT and KO mice were used to measure fibrinogen levels in plasma by western blotting using anti-fibrinogen antibody. Each lane represents plasma from a single mouse.

amplification using primers mentioned in Chapter 2. Figure 1B is a representative image of the electrophoretic patterns of the PCR products. Each lane represents a single mouse. As expected, the PCR product of the floxed Arf6 gene is bigger than the WT allele due to the insertion of flox sites. The PCR product for the PF4-Cre transgene is also shown. Various proteins in WT and KO platelets were measured by western blotting. As shown in Figure 1C, Arf6 protein levels were reduced by ~95% in platelets from Arf6 KO mice. The expression levels of other proteins were not affected: Arf1/3, Rho family members (Rac1, Cdc42, and RhoA), integrin β3 subunit, and granule membrane markers (P-Selectin and VMAT2, representing α-granule and dense-granule, respectively).

Arf6 KO Platelets were Defective in Uptake of Fibrinogen

Platelets contain a diverse set of proteins in their granules. At least some are not synthesized by platelets or by their precursor cells, megakaryocytes. In fact, these proteins are derived from plasma, and are internalized and stored in platelet granules (mostly alpha granules). These proteins include fibrinogen, immunoglobulin (lgG), laminin, *etc*. Uptake of these proteins by platelets is mediated by either receptor-mediated endocytosis, *e.g.* fibrinogen, or fluid phase endocytosis, *e.g.* lgG. Therefore, the levels of these proteins in platelets are indicative of the cargo endocytosis. Arf6 is an important element of the endocytic machinery in several different cell types [294, 316, 317, 319]. To determine if Arf6 plays a role in platelet endocytosis, we measured the levels of several granule cargo, *e.g.* fibrinogen, fibronectin, vitronectin, von Willebrand Factor (vWF), platelet factor 4 (PF4), Sema3F, and lgG. Among them, fibrinogen is known to be internalized through an αIIbβ3 integrin-mediated process. As a control for the analysis, fibrinogen levels were severely decreased in platelets from β3 knockout mice (Figure 2A). Interestingly (and consistent with our hypothesis), fibrinogen levels in Arf6 KO platelets were ~50% lower compared to WT (Figure 2B). To exclude the possibility that the decreased fibrinogen levels were due to lower levels of fibrinogen in plasma, plasma fibrinogen was measured and was shown to be comparable between WT and KO (Figure 1D). These data suggest that Arf6 KO platelets are defective in fibrinogen storage and/or

Figure 2

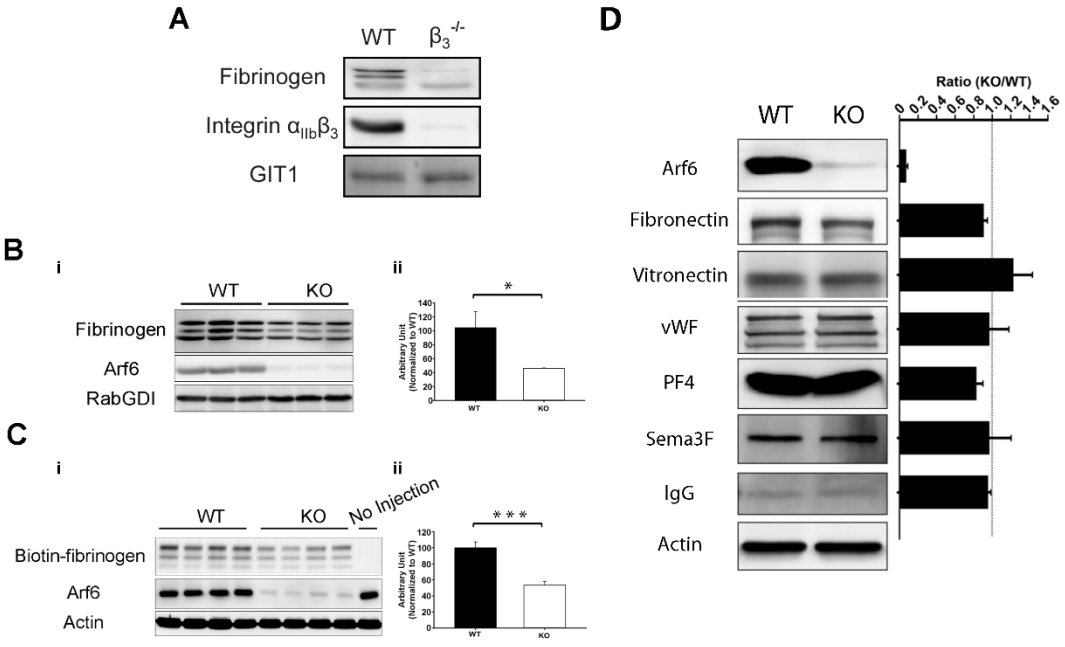

A
WT
β3-/-
Fibrinogen
Integrin αIIbβ3
GIT1
B
i
WT
KO
Fibrinogen
Arf6
RabGDI
ii
*
Arbitrary Unit (Normalized to WT)
WT
KO
C
i
WT
KO
No Injection
Biotin-fibrinogen
Arf6
Actin
ii
***
Arbitrary Unit (Normalized to WT)
WT
KO
D
WT
KO
Ratio (KO/WT)
0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
Arf6
Fibronectin
Vitronectin
vWF
PF4
Sema3F
IgG
Actin

Figure 2 Arf6 KO platelets had storage defect on fibrinogen, but not other cargo. (A) Comparison of fibrinogen levels between WT and $\beta3^{-/-}$ KO platelets. Platelets (1 X 10^7) were loaded each lane and indicated proteins were probed using corresponding antibodies. GIT1 was used as a loading control. Blots shown are representative of at least two independent experiments. **(B)** Endogenous fibrinogen levels in WT and KO platelets were determined by western blotting. Platelets (1 X 10^7) were loaded each lane and indicated proteins were probed using corresponding antibodies. Each lane represents platelets from a single mouse. RabGDI was used as a loading control. Quantification of fibrinogen levels was performed using ImageQuantTL and analyzed by SigmaPlot 12.0. **(C)** Comparison of biotinylated fibrinogen levels between WT and KO platelets. Platelets were harvested 24 hours post retro-orbital injection of biotinylated fibrinogen. Each lane represents platelets from a single mouse. Platelets (1 X 10^7) were loaded in each lane and indicated proteins were probed using corresponding antibodies. Actin was used as a loading control. A mouse with no injection was included as a control. Quantification of biotinylated fibrinogen levels was performed using ImageQuantTL and analyzed by SigmaPlot 12.0. **(D)** Comparison of different cargo in WT and KO platelets. Indicated cargo were measured by western blotting using the corresponding antibodies. Actin was used as a loading control. Quantification was performed using ImageQuantTL and ratio of KO to WT was calculated. The dash line represents ratio of 1 (KO/WT). Blots shown are representative of at least two independent experiments.

uptake. To further test this hypothesis, WT and KO mice were injected with biotinylated fibrinogen and platelets were harvested after 24 hours. As shown in Figure 2C, biotin- conjugated fibrinogen levels in KO platelets were ~50% less relative to WT. It should be noted that under these assay conditions, the level of biotinylated fibrinogen in plasma is similar between WT and KO as the same amount of biotinylated fibrinogen was injected.

It has been reported that both megakaryocytes and platelets can take up biotin-fibrinogen from plasma in an αIIbβ3-dependent manner [29]. At this point, we were not able to define which cell was defective in endocytosis of biotin-fibrinogen, although it was very likely that both could be defective. Regardless, these data indicated that Arf6 plays a role in αIIbβ3 integrin-mediated fibrinogen uptake in platelets and/or megakaryocyte. To determine if Arf6 had a global role on cargo storage and uptake, the levels of other proteins were determined. Surprisingly, none of the other proteins tested were significantly affected by Arf6 knockdown as shown in Figure 2D, including fibronectin (ligand of α5 integrin), vitronectin (ligand of αv integrin), Sema3F (ligand of neuropilin), and IgG (target of fluid phase endocytosis). These data suggest that Arf6 specifically regulates fibrinogen uptake by platelets or megakaryocytes.

To further test whether Arf6 KO platelets are defective in fibrinogen uptake *ex vivo,* platelets were incubated with FITC-fibrinogen and examined by epi-fluorescence microscopy (Figure 3A). Clearly, Arf6 KO platelets have lower signal intensity compared to WT. Quantification data in Figure 3B shown that Arf6 KO platelets have more cells that contain fewer FITC-labelled punctae per platelet, consistent with a lower uptake of FITC-fibrinogen. Taken together these *in vivo* and *ex vivo* results demonstrate that Arf6 KO platelets are specifically defective in uptake of fibrinogen, but not the other cargo examined.

Arf6 KO Platelets Had Normal Morphology

Since fibrinogen is an abundant cargo protein in platelet α-granules, we next examined whether Arf6 deficiency causes any morphology defect. Using EM, platelet morphology in resting state and thrombin-stimulated state was examined (Figure 4).

Figure 3

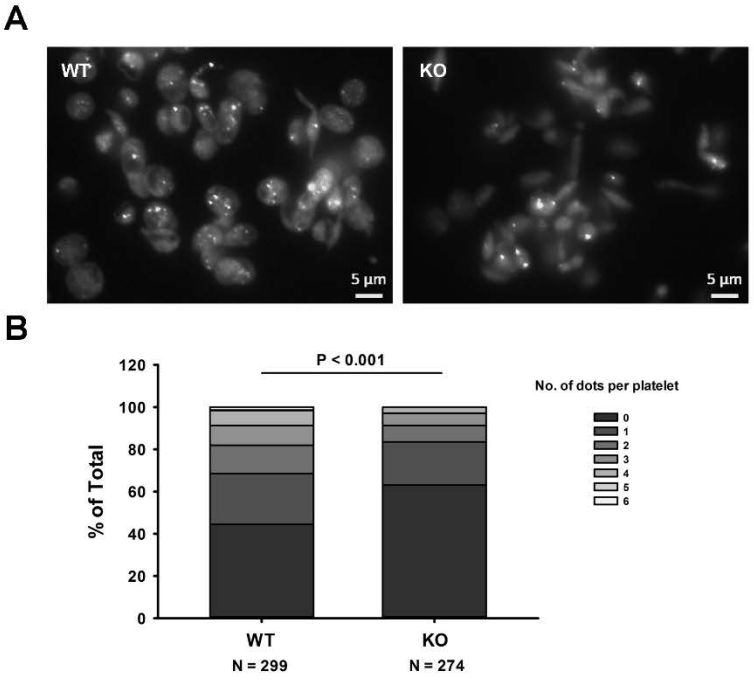

A
WT
5 µm
KO
5 µm
B
P < 0.001
No. of dots per platelet
0
1
2
3
4
5
6
% of Total
120
100
80
60
40
20
0
WT
N = 299
KO
N = 274

Figure 3 Uptake of FITC-fibrinogen *ex vivo* was impaired in Arf6 KO platelets. (A) 5 X 10^8/ml washed platelets were incubated with FITC-fibrinogen (0.05 mg/ml) for 1 hour at 37°C. After washing away the extracellular FITC-fibrinogen, platelets were continued to incubate with plasma for another 2 hour. Platelets were fixed with 2% paraformaldehyde over night at 4°C and subjected to fluorescence microscope. The surface signal was quenched by Trypan blue (0.1%) and representative images of area of interested were presented. Images shown are representative of three independent experiments. **(B)** Quantification of A. The number of FITC-positive puncta was manually determined and platelets were grouped based on that number. The percentage of each group to total was calculated and plot in bar graph. Statistics significant was determined using student t test.

Figure 4

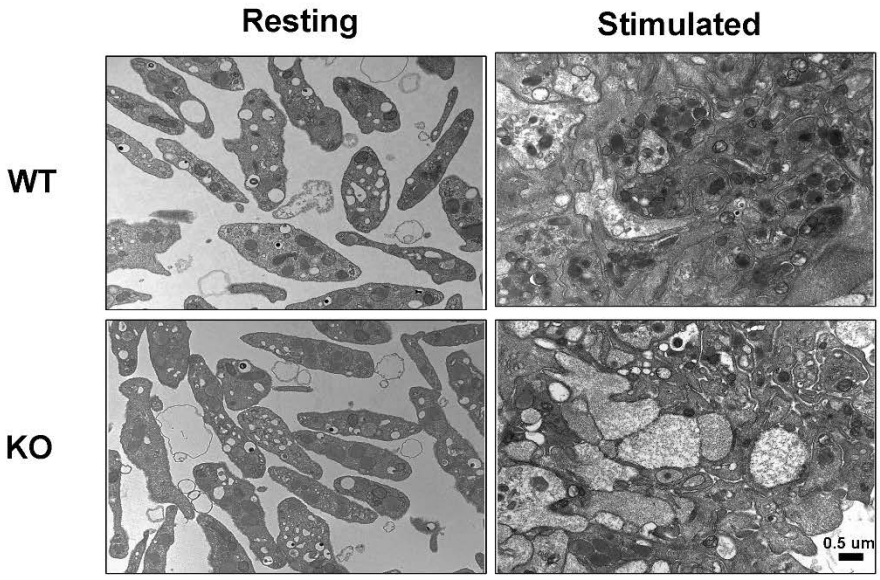


Figure 4 Arf6 KO platelets had normal morphology. Washed platelets from WT and KO mouse were prepared in HEPES tyrode buffer (pH 7.4) and adjusted to 4 X 10^8/ml. Resting or thrombin-stimulated platelets were preceded for electron microscope analysis (see Chapter 2). Images were taken using a Hitachi H-7000 transmission electron microscope. All images were same in scale.

Table 2 Characteristics of Blood Cells from WT and KO

	WT (n=26)	KO (n=26)	P
RBC (M/μL)	10.63 ± 2.32	9.59 ± 2.36	0.061
WBC (K/μL)	8.19 ± 1.90	7.54 ± 1.24	0.117
Platelet (K/μL)	978.7 ± 416.3	893.9 ± 305.8	0.602
Platelet Size (fL)	4.25 ± 0.45	4.03 ± 0.37	0.021

Though there was no overt change in the morphology of the platelet granules, the Arf6 KO platelets were slightly smaller which was consistent with whole blood counting data (Table 2). This observation was perhaps expected given that platelets from fibrinogen-deficient mice have normally appearing α-granules [480]. These data demonstrated that Arf6-deficiency does not cause any dramatic change in overall platelet and platelet granule morphology.

Arf6 KO Platelets Had Normal Surface Expression Levels of Integrin αIIbβ3

Several reports including the data presented here (Figure 2A) show that fibrinogen uptake by platelets is mediated by integrin αIIbβ3 [29]. Therefore, reduced fibrinogen levels in Arf6 KO platelets suggest that Arf6 may be involved in endocytosis of and/or subsequent trafficking of integrin αIIbβ3 in platelets. In nucleated cells Arf6 plays an active role in both endocytosis and subsequent trafficking and recycling pathways of integrins [216, 317, 319].

Defects in any of these pathways could lead to a fibrinogen-storage defect. If Arf6 participates in the initial steps of the endocytosis process, higher surface levels of αIIbβ3 integrin might be expected because the integrin is not internalized. Conversely, if Arf6 is involved in a recycling process, lower surface levels of αIIbβ3 integrin might be expected because the integrin is not returned to the surface. Alternatively, if αIIbβ3 integrin trafficking routes are altered by loss of Arf6, there may be no significant difference in surface integrin levels. To distinguish these possibilities, the surface levels of αIIbβ3 integrin were measured by flow cytometry. Washed WT and KO mouse platelets, either kept resting or stimulated with thrombin, were labeled for total and activated αIIbβ3 integrin using anti-CD41/61 antibody (Figure 5A) and Jon/A antibody (Figure 5B), respectively. Surprisingly, WT and KO platelets do not show any significant difference in either total or activated αIIbβ3 integrins. This data is consistent with an alteration in the αIIbβ3 integrin trafficking route and not a defect in internalization from or recycling to the plasma membrane. However, it is also possible that the FACS assay is not sufficiently sensitive to distinguish the small difference that is actually present between WT and KO platelets.

Figure 5

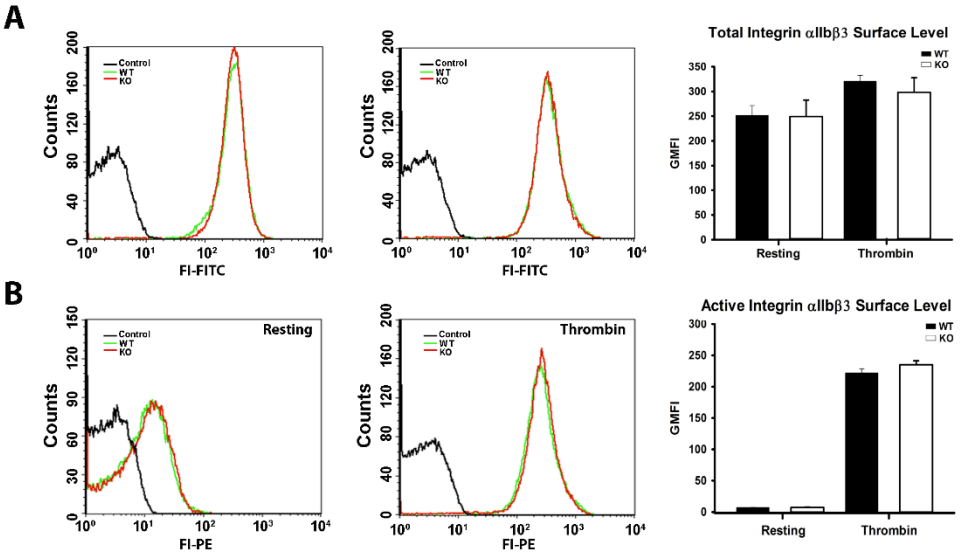

A
Counts
Control
WT
KO
FI-FITC
Total Integrin αIIbβ3 Surface Level
GMFI
WT
KO
Resting
Thrombin
B
Resting
Thrombin
FI-PE
Active Integrin αIIbβ3 Surface Level

Figure 5 Arf6 KO platelets did not show any defect on the surface levels of total integrin αIIbβ3 and active integrin αIIbβ3 in both resting and thrombin-stimulated states. (A) Comparison of total surface levels of integrin αIIbβ3 using FITC-CD41/CD61 antibody between WT (green) and KO (red) platelets in resting and stimulated states. No labeled platelets (black) were used as background control. **(B)** Comparison of surface level of active integrin αIIbβ3 using PE-Jon/A antibody between WT (green) and KO (red) platelets in resting and stimulated states. No labeled platelets (black) were used as background control. i to iii represents resting state, thrombin-stimulated state, and quantification of Geo mean of fluorescent intensity (GMFI), respectively. Data shown are representative of at least two experiments.

Integrin αIIbβ3 is the major receptor mediating platelet aggregation *ex vivo*. The surface level of activated αIIbβ3 is correlated with the degree of platelet aggregation. Consistent with the flow cytometry data, KO platelets, in response to various agonists, did not show significant defects in aggregation when compared to WT (Figure 6). Interestingly, Arf6 KO platelets had no defect in ATP release as monitored by lumi-aggregometer. This is distinct from the observations reported previously [353] where Arf6 is suggested be essential for dense-granule secretion, as measured by ATP release.

Arf6 Depletion Enhanced Platelet Spreading on Fibrinogen-Coated Surfaces and Clot Retraction

Although there were no significant defects in the surface levels of integrin αIIbβ3, we wondered whether the dynamics of integrin trafficking were affected in Arf6 KO platelets since platelet uptake of fibrinogen from plasma is a dynamic process both *in vivo* and *ex vivo*. As possible metrics of such a process, we first looked at the static adhesion of platelets on fibrinogen-coated surfaces. As shown in Figure 7A, there is no significant difference in the number of adhered platelets between WT and KO after 30 min incubation on fibrinogen-coated surfaces.

As an additional metric of integrin trafficking, the rate of platelet spreading on fibrinogen-coated surfaces was probed by measuring platelet surface area versus time. Inhibition of integrin β1 trafficking by Arf6 depletion in HeLa cells results in diminished cell spreading on fibronectin, integrin β1's substrate [317]. However, manipulation of Arf6-GTP levels by over-expression of a GAP, increased axonal growth, which is a sense of polarized spreading [220]. Arf6 KO platelets had larger surface areas as early as 60 min into the incubation period, compared to WT (Figure 7B). At 90 and 120 min, Arf6 KO platelets were significantly larger than WT (Figure 7B-D). These data indicate that Arf6 KO platelets spread significantly faster on fibrinogen-coated surfaces than WT controls. A possible explanation for these results is that integrin αIIbβ3 is more effectively expressed on the platelet surface thereby increasing the efficacy of the platelet contacts with fibrinogen. If depletion of Arf6 leads to this effect, as our results suggest, it implies that Arf6 controls integrin αIIbβ3 turnover/trafficking at the cell surface. In neurons, Arf6 is involved in rapid, Rab4-mediated short-loop trafficking of

Figure 6

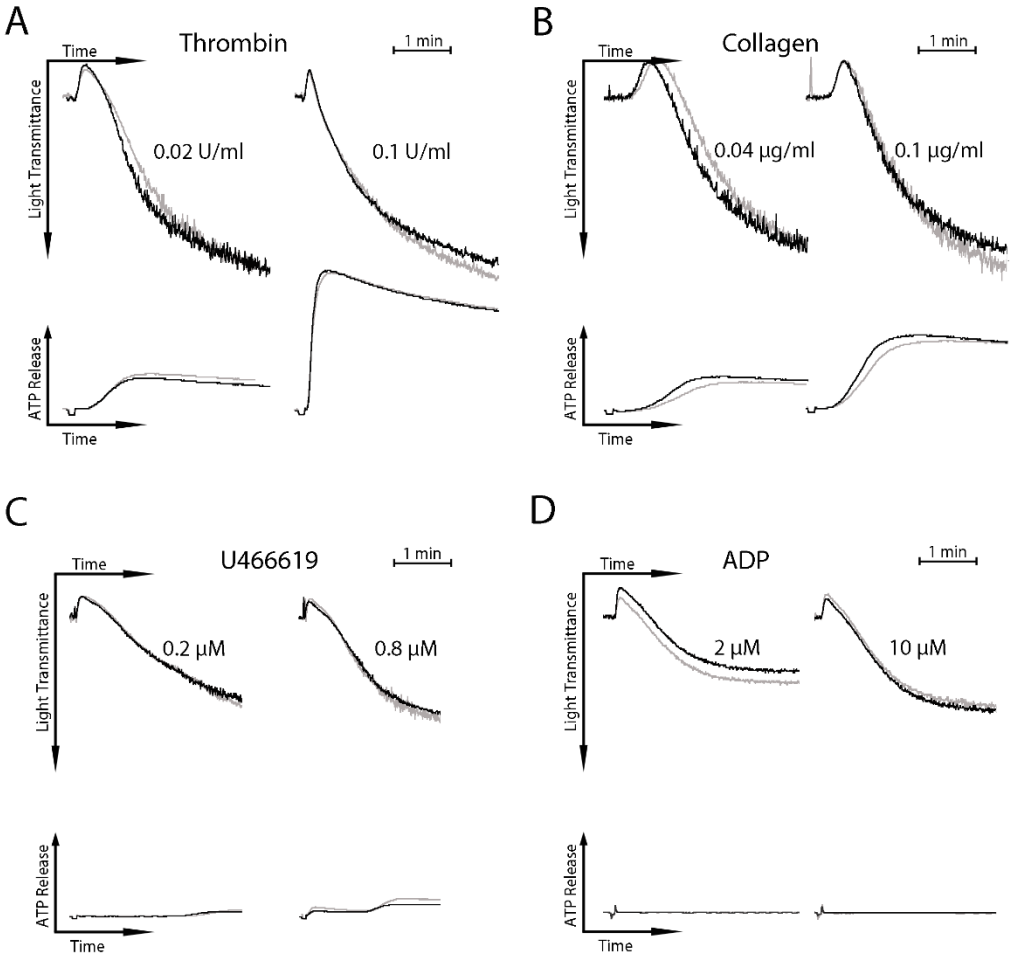

A
Thrombin
1 min
Time
Light Transmittance
0.02 U/ml
0.1 U/ml
ATP Release
Time
B
Collagen
1 min
Time
Light Transmittance
0.04 µg/ml
0.1 µg/ml
ATP Release
Time
C
U466619
1 min
Time
Light Transmittance
0.2 µM
0.8 µM
ATP Release
Time
D
ADP
1 min
Time
Light Transmittance
2 µM
10 µM
ATP Release
Time

Figure 6 Arf6 KO platelets had no aggregation defect. Washed platelets from WT (black) and KO (grey) mice were freshly prepared and adjusted to 2.5 X 10^8/ml in HEPES Tyrode's buffer. Thrombin **(A)**, Collagen **(B)**, U46619 **(C)**, and ADP **(D)** were used to stimulate platelet aggregation in stirring condition at 37°C in aggregometer. The concentrations of agonists were indicated. ATP release was monitored using CHRONO-LUME reagent (Chrono-log) while platelet aggregation was performed. Data shown are representative of at least three experiments. Data was organized using Adobe Photoshop CS5.

Figure 7

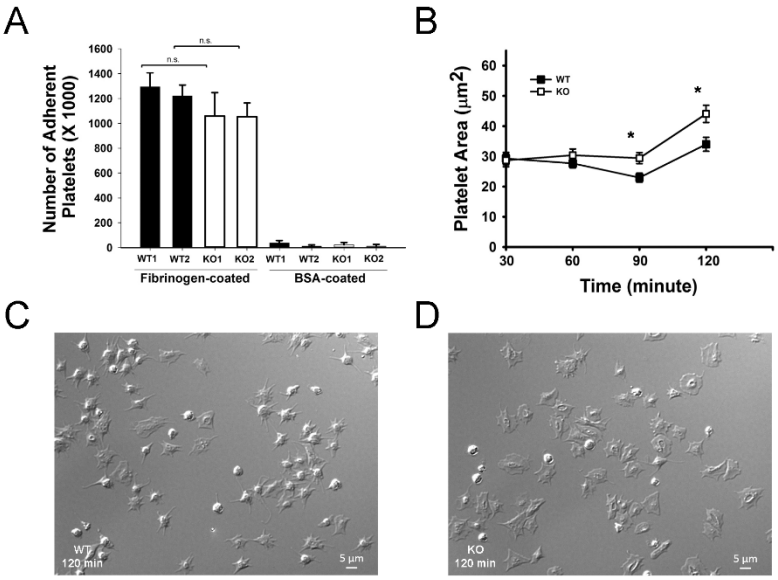

A
Number of Adherent Platelets (X 1000)
1600
1400
1200
1000
800
600
400
200
0
n.s.
n.s.
WT1
WT2
KO1
KO2
WT1
WT2
KO1
KO2
Fibrinogen-coated
BSA-coated
B
Platelet Area (μm2)
60
50
40
30
20
10
0
WT
KO
*
*
30
60
90
120
Time (minute)
C
WT
120 min
5 μm
D
KO
120 min
5 μm

Figure 7 Arf6 KO platelets had enhanced spreading but normal static adhesion. (A) Quantification of static platelet adhesion on fibrinogen- and BSA-coated surface. Washed calcein-labelled platelets (2.5 X 10^8/ml) from WT and KO mouse were incubated on fibrinogen- or BSA-coated surface for 30 min at 37°C. Using plate reader with excitation/emission at 485/538 nm, the number of adhered platelets was measured referral to the standard curve with fixed number of platelets from each strain. Each bar represents platelets from a single mouse. Quantification analysis was performed using SigmaPlot 12.0. **(B)** Quantification of surface area of platelets spread on fibrinogen-coated surface at indicated time points. Washed platelets from WT and KO mouse were adjusted to 2.0 X 10^7/ml and supplemented with 1 mM Ca^{2+}. Platelets were then incubated on fibrinogen-coated surface for indicated amount of time and fixed with 4% paraformaldehyde. DIC images were taken using Nikon Eclipse E600 microscope (Nikon, Melville, NY) with a 100X/1.40 numeric aperture DIC H oil objective lense (Nikon). Images were taken using Zeiss camera (AxioCam MR, Germany). Image was processed by Zen 2011 (blue edition, Zeiss) and Quantified by image J (V1.47, NIH). **(C-D)** Representative DIC images of WT (C) and KO (D) platelet spreading at 120 min point. Spreading data are representative of at least three independent experiments.

integrin α9β1, which is important for controlling integrin-dependent axon growth. Overexpression of ACAP1, a GAP protein for Arf6, inactivated Arf6, which in turn increased the recycling of integrin α9β1 and increased axon growth [220]. Our data could be considered consistent with such a phenotype. Loss of Arf6 (like inactivation of Arf6 in neurons) might increase integrin αIIbβ3 recycling (or divert it to an alternate route) and thereby enhance platelet spreading. Following this line of reasoning, we performed another assay that is a potential metric of integrin αIIbβ3 function, platelet clot retraction. Consistent with the spreading data above, Arf6 KO platelets had faster clot retraction times (Figure 8). Fibrinogen is added exogenously to these assays and is in excess. The increased clot retraction supports the contention that loss of Arf6 increases the efficacy of integrin αIIbβ3 engagement with substrate. Since downstream signaling by integrin αIIbβ3 plays an important role in clot retraction [481], myosin light chain phosphorylation (Figure 9B), tyrosine phosphorylation profiles (Figure 9A), and Rac1 activation (Figure 10) were examined. Surprisingly, we could not detect any overt differences between WT and KO. Consistently, F-actin formation was also not affected in Arf6 KO platelets following thrombin stimulation (Figure 11). These data exclude the possibility that the enhanced phenotypes (clot retraction and spreading) were due to up-regulation of some signaling step that is downstream of integrin αIIbβ3. However, as noted with the FACS analysis, these assays may not be sufficiently sensitive to determine a small difference that actually exists between WT and KO platelets. Together, the clot retraction and spreading data demonstrate that loss of Arf6 affects normal platelet-matrix contacts and alters their efficacy. The FACS data, despite its caveats, is not consistent with a wholesale change in integrin αIIbβ3 surface expression and the signaling analysis confirms that there is no overt change in the steps thought to be downstream of integrin αIIbβ3 engagement. Based on Arf6's role in other cell types, *i.e.* neurons, these data are best explained by Arf6 playing a role in the dynamics or routing of integrin αIIbβ3 trafficking in the platelets.

Inhibiting Dynamin-dependent Endocytosis Enhanced Platelet Clot Retraction

As an attempt to confirm our conclusions about Arf6's role, we sought to disrupt integrin αIIbβ3 trafficking, pharmacologically at a different step. Morphological

Figure 8

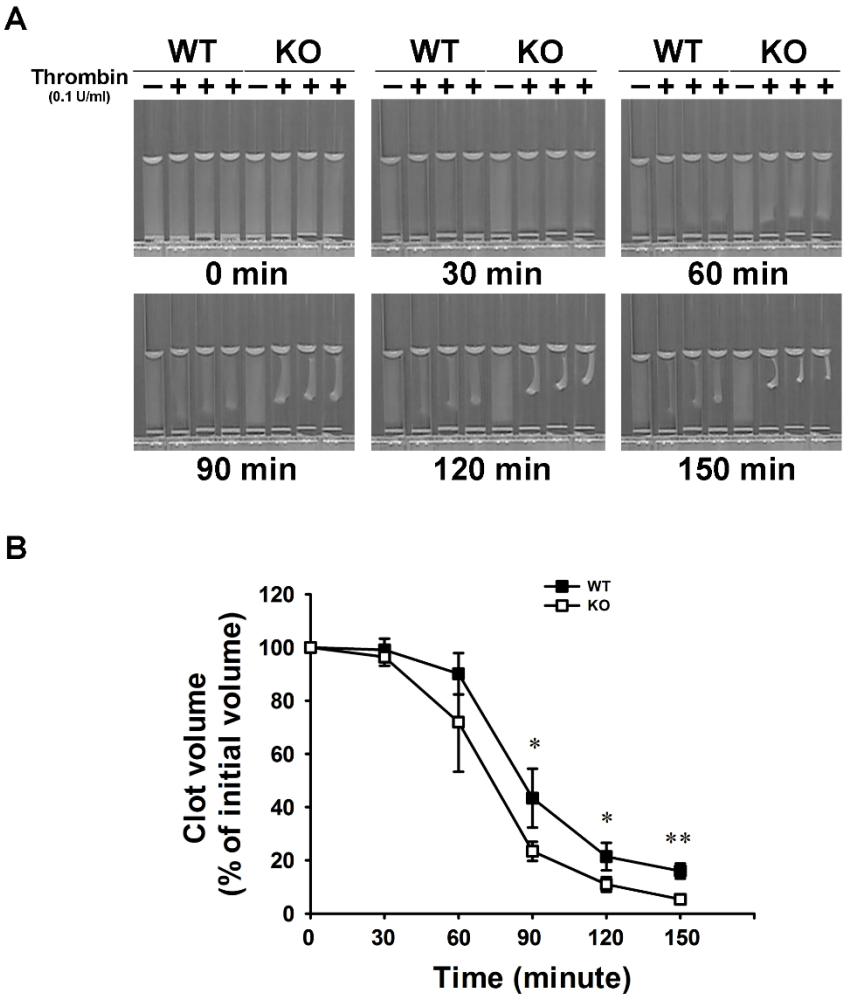

A
WT
KO
WT
KO
WT
KO
Thrombin
(0.1 U/ml)
– + + + – + + +
0 min
30 min
60 min
90 min
120 min
150 min
B
WT
KO
Clot volume
(% of initial volume)
120
100
80
60
40
20
0
0
30
60
90
120
150
Time (minute)
*
*
**

Figure 8 Arf6 KO platelets had enhanced platelet clot retraction. (A) Washed platelets from WT and KO mice were adjusted to 3 X 10^8/ml and supplemented with 0.5 mg/ml human fibrinogen and 1 mM Ca^{2+}. Clot retraction was initiated in cuvette with 0.1 U/ml thrombin. Images were taken at the indicated time points. **(B)** Quantification of clot retraction. Clot size in (A) were measured using image J v1.48 and percentage of clot size to time 0 were determined and then analyzed using SigmaPlot 12.0. Clot retraction data are presentative of at least five independent experiments.

Figure 9

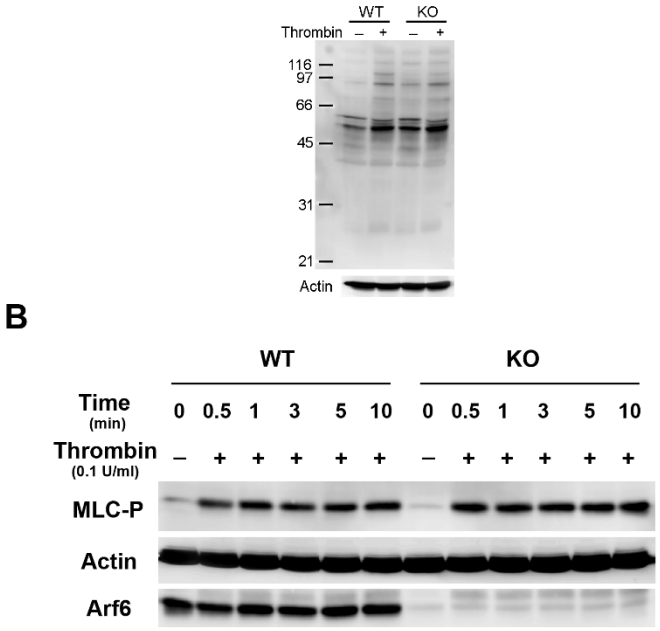

A
WT
KO
Thrombin
– + – +
116
97
66
45
31
21
Actin
B
WT
KO
Time
(min)
0 0.5 1 3 5 10 0 0.5 1 3 5 10
Thrombin
(0.1 U/ml)
– + + + + + – + + + + +
MLC-P
Actin
Arf6

Figure 9 Arf6 KO platelets did not show any defect on global tyrosine phosphorylation and myosin light chain phosphorylation upon thrombin stimulation. **(A)** Washed platelets from WT and KO mice were prepared at 4 X 10^8/ml in HEPES Tyrode's buffer (pH 7.4) and kept resting or stimulated with 0.1 U/ml thrombin for 5 min. The reaction was stopped by addition of SDS-PAGE sample buffer containing both protease inhibitor cocktail and phosphatase inhibitor cocktail. The lysates were subjected to western blotting for the global tyrosine-phosphorylation profile. Actin was used as a loading control. Data are representative of at least two independent experiments. **(B)** Washed platelets from WT and KO mice were prepared at 4 X 10^8/ml in HEPES Tyrode's buffer (pH 7.4) and kept resting or stimulated with 0.1 U/ml thrombin for the indicated times. The reaction was stopped by addition of SDS-PAGE sample buffer containing both protease inhibitor cocktail and phosphatase inhibitor cocktail. The lysates were subjected to western blotting for MLC-P profile. Actin was used as a loading control. Blots shown are representative of at least three experiments.

Figure 10

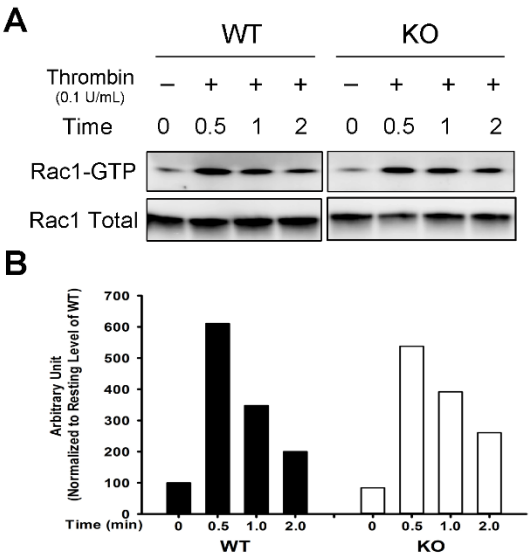

A
WT
KO
Thrombin
(0.1 U/mL)
– + + + – + + +
Time
0 0.5 1 2 0 0.5 1 2
Rac1-GTP
Rac1 Total
B
Arbitrary Unit
(Normalized to Resting Level of WT)
700
600
500
400
300
200
100
0
Time (min)
0 0.5 1.0 2.0
WT
0 0.5 1.0 2.0
KO

Figure 10 Arf6 KO platelets did not show defect on Rac1 activation. (A) Rac1 activation upon thrombin stimulation. Washed platelets from WT and KO mice were prepared at 4 X 10^8/ml in HEPES Tyrode's buffer (pH 7.4) and kept resting or stimulated with 0.1 U/ml thrombin for the indicated times. The reaction was stopped by addition of 2 X GTPases-pulldown lysis buffer containing both protease inhibitor cocktail. Rac1-GTP was pulled down based on the methods described in method section. The pellets (Rac1-GTP) and the supernatant (total Rac1) was subjected to western blotting. **(B)** Quantification of A. Quantification of Rac1-GTP and total Rac1 level was performed using ImageQuantTL and the ratio of Rac1-GTP/Total Rac1 was calculated and plotted in bar graph. Blots shown are representative of at least two experiments.

Figure 11

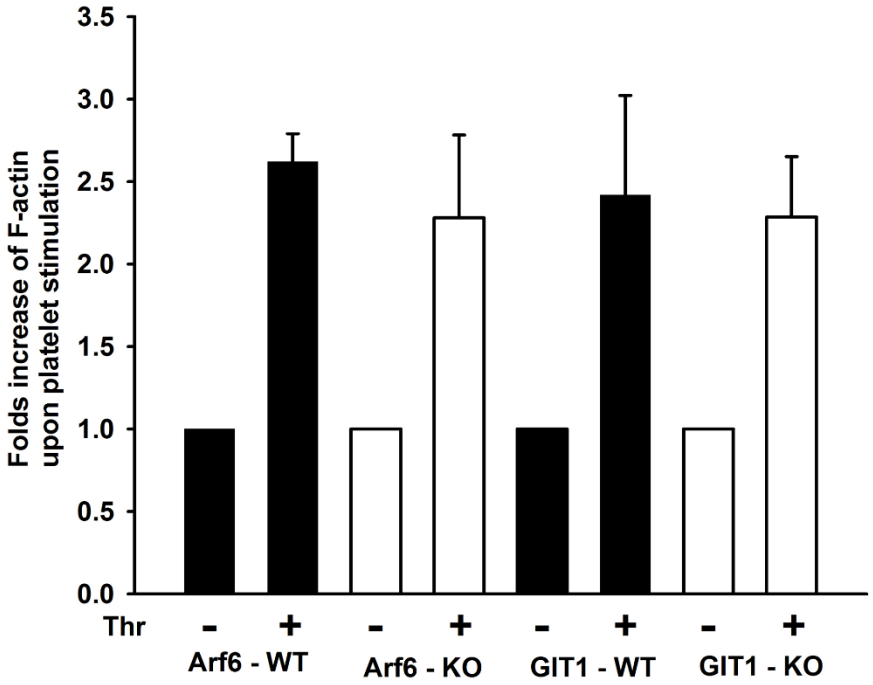

Folds increase of F-actin upon platelet stimulation
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Thr
-
+
-
+
-
+
-
+
Arf6 - WT
Arf6 - KO
GIT1 - WT
GIT1 - KO

Figure 11 Arf6 KO platelets and GIT1 KO platelets had normal F-actin content in resting and thrombin stimulated platelets. Platelets (2 X 10^7) were either kept resting or stimulated with 0.1 U/ml thrombin for 5 min. F-actin level in platelets were measured according to the method section. F-actin levels in thrombin-stimulated platelets were normalized to its corresponding resting states. Data is representative of two independent experiments.

analysis has suggested that endocytosis of integrins in platelets is clathrin- and dynamin-mediated process [233]. Therefore, disrupting integrin αIIbβ3 endocytosis by inhibiting dynamin activity could recapitulate the phenotype of the Arf6 KO platelets. To test this hypothesis, a dynamin inhibitor, dynasore, was used. As shown in Figure 12, the presence of dynasore promotes platelets clot retraction, which suggests that dynamin-mediated endocytosis plays a role in clot retraction. It is likely that this enhanced clot retraction is due to inhibited endocytic trafficking of integrin αIIbβ3 by dynasore. However, we could not exclude other possibilities at this point, for example, endocytic trafficking of other receptors, like P2Y receptors, could also be inhibited by dynasore treatment [52].

Arf6 KO Mice Had no Defect in Arterial Thrombosis and Hemostasis

Given the effects on spreading and clot retractions we next wanted to determine whether loss of Arf6 in platelets caused any overt effects on hemostasis and thrombosis. Hemostasis was monitored using a tail-bleeding-time assay and thrombosis was monitored using a $FeCl_3$-induced carotid artery injury model. It should be noted that in both assay systems, defective hemostasis and/thrombosis is readily detectible, but enhancements are more difficult to document. Compared to WT, we did not detect any defect in tail-bleeding or in $FeCl_3$-induced, occlusive thrombosis in the Arf6 KO mice (Figure 13). These data suggest that although Arf6 depletion caused enhanced spreading and clot retraction probably via regulating integrin αIIbβ3 trafficking in platelets, this effect may not sufficient to grossly influence thrombus formation and hemostasis in vivo.

Conclusions

In this chapter Arf6'roles in platelets has been further defined using platelet-specific, Arf6 knockout mice. Although depletion of Arf6 in mouse platelets did not affect platelet morphology, aggregation, and ATP release, Arf6 knockout did affect the loading of fibrinogen, but not other cargo examined. This phenotype suggests that integrin αIIbβ3-mediated uptake of fibrinogen by platelets is affected and indicating a role for Arf6 in the process. Enhanced spreading and clot retraction of Arf6 KO platelets suggests that Arf6 may play a role in the endocytic trafficking of integrin αIIbβ3, which is supported by the effects of the endocytosis inhibitor, dynasore, on

clot retraction. Given the 'hyperactive' phenotype of Arf6 KO platelets, Arf6 KO mouse might have hyper thrombotic phenotype, however that was not detectible in the assay systems that we used. More sensitive systems will be needed to fully appreciate the physiological relevance of the effects of Arf6.

Figure 12

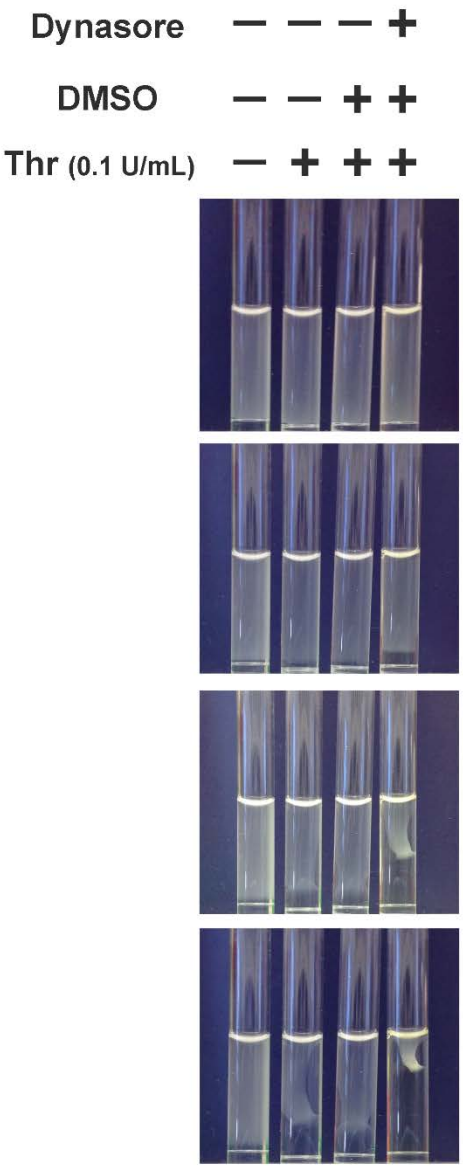


Figure 12 Dynasore enhanced mice platelets clot retraction. Washed mouse platelets were prepared in HEPES Tyrode's buffer (pH 7.4) at 4.0 X 10^8/ml. Platelets were supplemented with 1 mM Ca^{2+} and 0.5 mg/ml fibrinogen. Clot retraction was initiated by 0.1 U/ml thrombin in the presence or absence of 80 μM dynasore. The images were taken at the indicated times. Data is representative of at least two independent experiments.

Figure 13

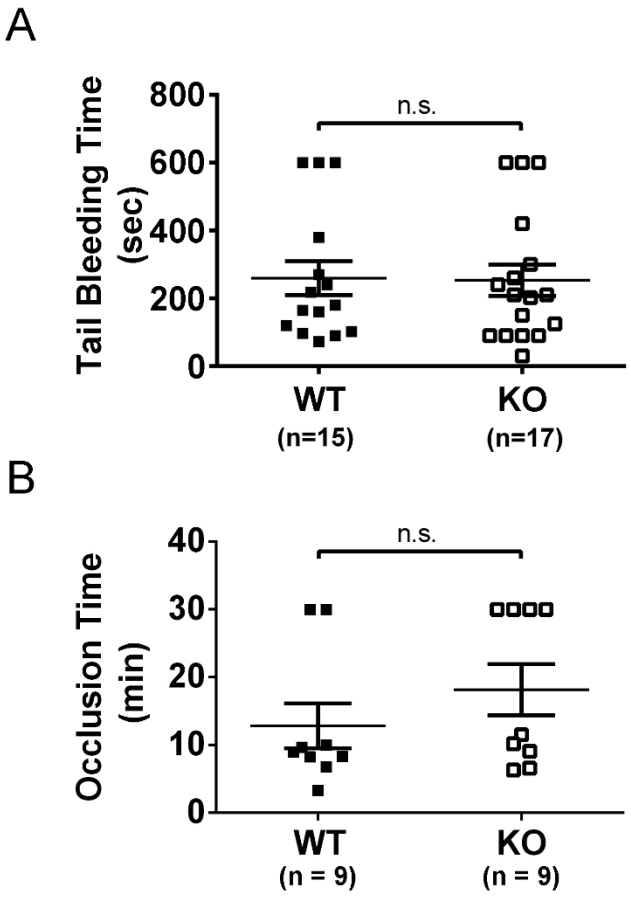

A
Tail Bleeding Time (sec)
800
600
400
200
0
n.s.
WT
(n=15)
KO
(n=17)
B
Occlusion Time (min)
40
30
20
10
0
n.s.
WT
(n = 9)
KO
(n = 9)

Figure 13 Arf6 KO mice did not have any significant defect in thrombosis formation and hemostasis. (A) Tail bleeding assay. Mice from WT and KO group at 4-6 weeks age were anaesthetized by intraperitoneal injection with Ketamine (75 mg/kg). The tail tip was transected by 3 mm and immediately immersed in 37°C saline. The time from transection to bleeding cessation was recorded. Mouse was monitored for an additional minute to look for re-bleeding event. The recording was terminated by 10 min. For the prolonged bleeding mice, bleeding was stopped manually. The data was analyzed using Logrank test. **(B)** $FeCl_3$-induced Carotid Artery Injury model. Mice from WT and KO group at 8-12 weeks old were used. Fresh 4% $FeCl_3$ applied on left carotid artery for 3 min was used to induce an injury. The time from removal of $FeCl_3$ to blood flow cessation was recorded. Mouse was monitored for an additional minute to look for unstable thrombus formation. The recording was terminated by 30 min. Mice were sacrificed at the end of experiments. The data was analyzed by Logrank test.

Chapter Four: Potential Role for GIT1 in Human Platelets

Introduction

Our previous work [414, 417] and the studies described in the previous chapter demonstrate that Arf6 is an important contributor to platelet function, particularly in integrin αIIbβ3-mediated functions. We and other groups [353] have shown that resting platelets maintain relatively high level of Arf6-GTP and that upon platelet activation, Arf6-GTP level decreased dramatically. Interestingly, blocking integrin αIIbβ3 outside-in signaling partially recovers Arf6-GTP levels, a phenotype that was also observed when β3 KO platelets were used. This suggests that there are two waves of signaling regulating Arf6 in platelets. A first wave that is initiated by agonist stimulation and a second wave that appears to require integrin αIIbβ3.

As other Ras-like, small GTPase, Arf6 nucleotide states are regulated by two groups of proteins, GEFs and GAPs. However, it is still unknown which of the regulators present in platelets are responsible for controlling Arf6. Recent, comprehensive proteomics analysis [352] has identified several potential Arf6-specific GEFs and GAPs in platelets, including the GIT1 protein. For αIIbβ3 integrin-regulated loss of Arf6-GTP, the simplest explanation is the presence of an Arf6 GAP, whose activity is activated following integrin, outside-in signaling. Compared to other potential Arf6 regulators identified so far, the GIT1 protein has some unique features that make it a candidate for this scenario.

GIT1 is a multi-domain protein, containing an N-terminal ArfGAP domain that is specific for Arf6 [422-424]. GIT1 has a C-terminal, paxillin-binding site (PBS), which allows it to bind paxillin, a focal adhesion protein, and thus to associate with integrins. GIT1 can be phosphorylated by kinases in focal adhesions, *e.g.* FAK and Src [437, 443, 449, 450]. Thus, it was not surprising when GIT1 was found to be tyrosine-phosphorylated downstream of αIIbβ3 integrin outside-in signaling in platelets [425]. Based on this observation, we hypothesized that GIT1 is a key Arf6GAP downstream αIIbβ3 integrin outside-in signaling, and thus is important for function.

A GIT1-containing Complex Translocated to a Detergent-insoluble Fraction upon Platelet Activation

GIT1 could form complexes through interactions with several other proteins, including β-PIX. It has been shown that GIT1-containing complexes cycle between three subcellular localizations, cytoplasm, peripheral area and FAs. The first thing we sought to determine in platelets was whether GIT1-containing complexes changed locale upon platelet activation and become associated with a cellular compartment that is associated with αIIbβ3. For this, we performed a time-course experiment in which washed human platelets were stimulated with thrombin and the reactions stopped by addition of Triton X-100-containg lysis buffer at the indicated times. Detergent-insoluble fractions (pellet) and detergent-soluble fractions (supernatant) were separated by centrifugation and analyzed by western blotting. RabGDI is a cytosolic protein that was used as a marker for fractionation efficacy. As shown in Figure 14, there was no detectable RabGDI in the Triton X-100 insoluble, pellet fraction. It should be noted that most proteins tested, except integrin and actin, are soluble in resting platelets. Upon platelet activation by around 60 seconds, GIT1, β-PIX, FAK, Rac1, Arf6 and the integrin β3 subunit all redistributed into the pellet fraction which may be a αIIbβ3 integrin-containing actin cytoskeleton structure.

Translocation of GIT1-containing Complexes to the Detergent-insoluble Fraction Depended on αIIbβ3 Integrin Outside-in Signaling and Arf6 Activity

Next, we wondered if blocking integrin αIIbβ3 outside-in signaling would affect translocation of the GIT1-containing complex. As shown in Figure 15, pretreatment of washed platelets with RGDS peptide, an inhibitor of integrin αIIbβ3 binding to substrate *i.e.* fibrinogen, completely blocked translocation of GIT1-related proteins to the Triton X-100 insoluble fraction, suggesting that integrin αIIbβ3 outside-in signaling is important for this complex translocation and supporting our hypothesis that GIT1 is a signaling component downstream αIIbβ3 outside-in signaling. Myristoylated-Arf6 N-terminal peptide (mryArf6p), an inhibitor, which blocks interactions between Arfs and GAPS and that was previously used to block the loss of Arf6-GTP in platelets [414], was used to determine if Arf6 was important for complex translocation. As shown in Figure

Figure 14

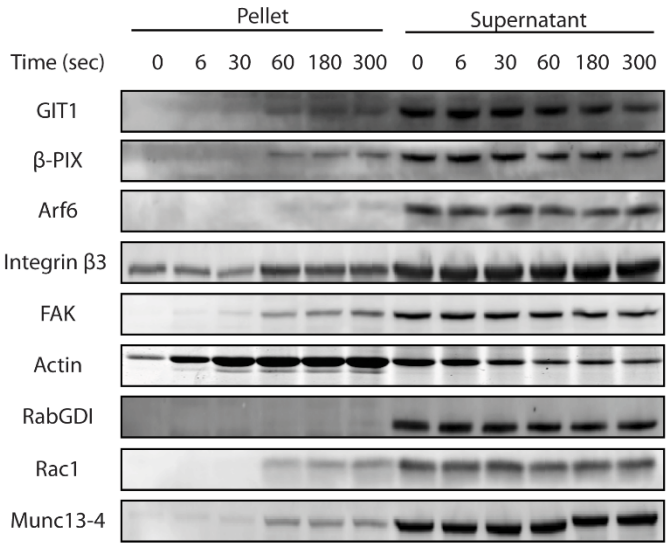

Pellet
Supernatant
Time (sec)
0 6 30 60 180 300 0 6 30 60 180 300
GIT1
β-PIX
Arf6
Integrin β3
FAK
Actin
RabGDI
Rac1
Munc13-4

Figure 14 Translocation of GIT1-containing complex upon platelet stimulation is time-dependent. Washed platelets (6×10^8 platelets/mL) were pre-incubated for 2 min in an aggregometer then stimulated with 0.1 U/mL thrombin for the indicated times. Platelets were lysed in buffer containing 2% NP-40. The soluble and the insoluble fractions were separated by centrifugation. Indicated proteins were detected by western blotting using the corresponding antibodies. The pellets represent 4 times the platelet equivalents in the corresponding supernatant fractions. Data is representative of at least three independent experiments.

Figure 15

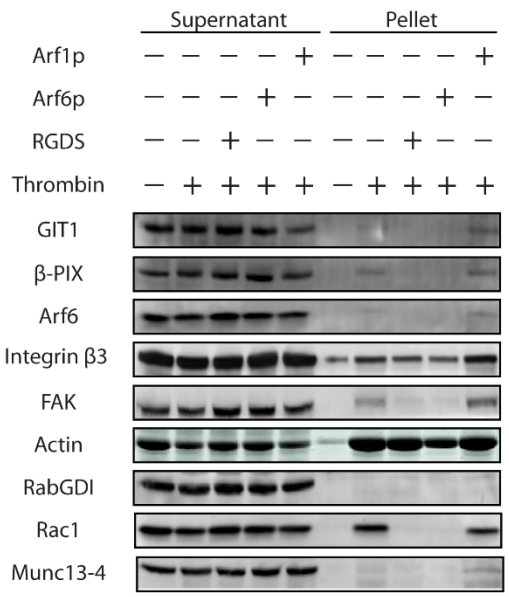

Supernatant
Pellet
Arf1p
Arf6p
RGDS
Thrombin
GIT1
β-PIX
Arf6
Integrin β3
FAK
Actin
RabGDI
Rac1
Munc13-4

Figure 15 Translocation of GIT1-containing complex upon platelet stimulation was dependent on Outside-in signaling- and Arf6 activity. Washed platelets (6×10^8 platelets/mL) were pre-incubated with or without 250 µM RGDS peptide, 10 µM MyrArf6 peptide, 10 µM sMyrArf6 peptide as indicated for 3 min in an aggregometer then stimulated with 0.1 U/mL thrombin for 3 min. Platelets were lysed in buffer containing 2% NP-40. The soluble and the insoluble fractions were separated by centrifugation. Indicated proteins were detected by western blotting using corresponding antibodies. The pellets represented 15 times the platelet equivalents in the corresponding supernatant fractions. Data is representative of at least three independent experiments.

15, pretreatment of washed platelets with myrArf6p, but not myrArf1p, completely abolished the translocation, suggesting that Arf6 activity is important. It should also be noted that pretreatment with myrArf6p decreased F-actin level in pellet fraction, which is consistent with previous observations [414]. There are several possible explanations for this effect. One possibility is that myrArf6p affects the activation of Rho family members as shown previously [414], which in turn diminish PAK activation, GIT1 activation, and complex translocation. Another possibility is that Arf6p affects integrin αIIbβ3 activation, and thus abolishes its outside-in signaling and GIT1 translocation. Indeed, treatment of mryArf6 peptide decreased both active and total integrin αIIbβ3 levels on plasma membrane of human platelets in response to thrombin (Choi WS, unpublished data). Regardless, GIT1 translocation appears to require Arf6 activity, suggesting a positive feedback loop in platelets that regulates Arf6.

GIT1 Phosphorylation Depended on Integrin αIIbβ3 Outside-in Signaling and Arf6 Activity

GIT1 phosphorylation is an important mechanism for controlling GIT1 cellular activities [450, 451], affecting GIT1's ArfGAP activity, cellular localization, and its interactions with paxillin. Previously, it was shown that tyrosine phosphorylation of GIT1 in platelets is affected by different kinases [425]. In Figure 16, GIT1 phosphorylation is blocked by pretreatment with RGDS peptide, which is consistent with previous results [425], suggesting that GIT1 tyrosine phosphorylation requires integrin αIIbβ3 outside-in signaling. GIT1 always forms a complex with β-PIX and Hic-5 (a member of paxillin family present in platelets) regardless of the state of the platelets (consistent with previous observations; [425]). Interestingly, integrin β3 levels in GIT1-containing complexes increased upon platelet activation but this increase was not affected by RGDS peptide treatment. These data would suggest that the GIT1-containing complex is recruited to the integrins before αIIbβ3 outside-in signaling and translocation to detergent-insoluble fraction. GIT1 phosphorylation in platelets also requires Arf6 activity since pretreatment of washed platelets with myrArf6p, but not scrambled Arf6p (scArf6p), abolished it. It also appears that Arf6

Figure 16

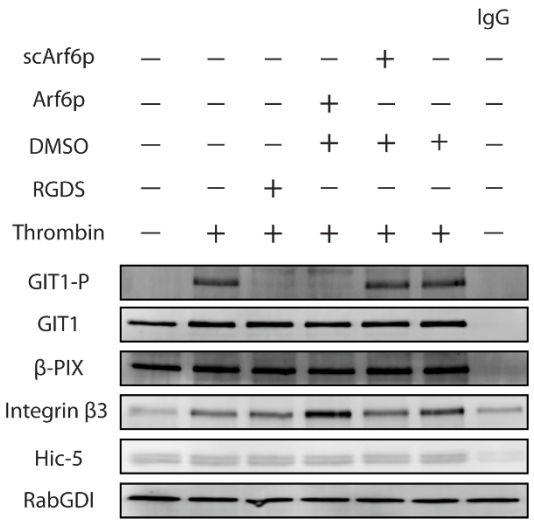

IgG
scArf6p — — — — + — —
Arf6p — — — + — — —
DMSO — — — + + + —
RGDS — — + — — — —
Thrombin — + + + + + —
GIT1-P
GIT1
β-PIX
Integrin β3
Hic-5
RabGDI

Figure 16 GIT1 phosphorylation upon thrombin stimulation depends on outside-in signaling and Arf6 activity. Washed platelets (4×10^8 platelets/mL) were pre-incubated with or without 250 µM RGDS peptide, 10 µM MyrArf6 peptide, 10 µM scMyrArf6 peptide as indicated for 3 min in an aggregometer then stimulated with 0.1 U/mL thrombin for 3 min. Platelets were lysed in buffer containing 2% Triton X-100 and phosphatase inhibitor cocktail. Co-immunoprecipitation was performed using anti-GIT1 antibody. The proteins in the complex were probed using corresponding antibodies. Data is representative of two independent experiments.

activity is not required for the association of GIT1/β-PIX/Hic-5 complex with integrin β3 (Figure 16). Instead, myrArf6p increased the levels of integrin β3 subunit in the complex, which might be due to recruitment of GIT1 by Arf6-GTP to integrin. Taken together, these data show that GIT1 phosphorylation requires both integrin αIIbβ3 outside-in signaling and Arf6 activity, but is not required for the association of the GIT1-containing complex with the integrin β3 subunit.

GIT1 Phosphorylation Required the Actin Cytoskeleton

Since GIT1-containing complexes eventually are translocated to Triton X-100-insoluble fractions (which could be F-actin complexes and/or lipid rafts), we wondered if GIT1 phosphorylation is affected by the actin cytoskeleton. As shown in Figure 17, both latrunculin A (latA) and cytochalasin D (cytoD), inhibitors of actin polymerization, almost completely inhibit GIT1 phosphorylation without disrupting GIT1-containing complex formation or recruitment of GIT1 complexes to integrin β3 subunit. It has been known that latA and cytoD do not inhibit platelet aggregation, suggesting that actin polymerization is not essential for integrin αIIbβ3 activation [482, 483]; thus actin polymerization following integrin αIIbβ3 outside-in signaling appears to be important for GIT1 phosphorylation but not its associations with β-PIX and Hic-5 and integrin.

Summary: It appears that, GIT1 complex is dynamically regulated in platelets as shown in model (Figure 18). In resting platelets, GIT1 is present in cytoplasm and forms complexes with β-PIX and Hic-5. Upon platelet activation, GIT1 complexes are recruited to integrins, which is not dependent on integrin αIIbβ3 outside-in signaling, Arf6 activity, and actin cytoskeleton network. Following integrin αIIbβ3 outside-in signaling, GIT1 gets phosphorylated very likely by Src and/or FAK. This step appears to require Arf6 activity and intact actin polymerization. It is proposed that GIT1's Arf6 GAP activity is enhanced by phosphorylation, resulting in integrin αIIbβ3-mediated loss of Arf6-GTP and leading to the recruitment of whole protein complex to a detergent-insoluble fraction.

Generation of Platelet-specific GIT1 KO Mice

The work in Figure 14-17 in this chapter was all done with human platelets. To better understand the physiological role of GIT1 protein in platelets, we created platelet-

Figure 17

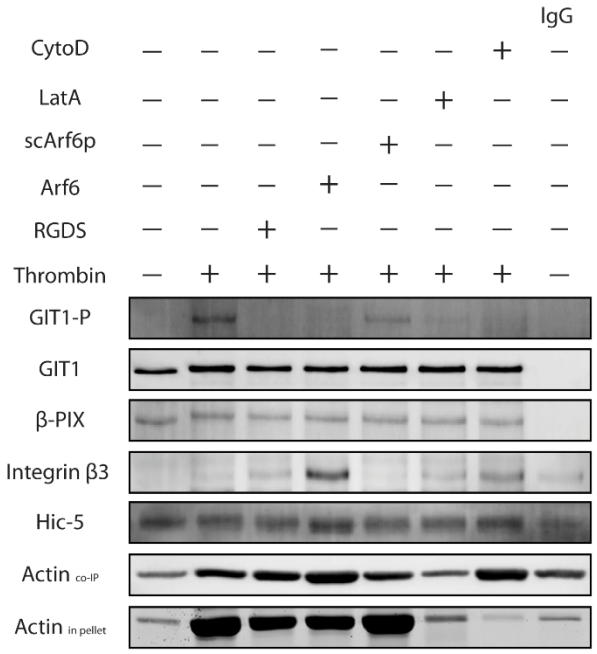

IgG
CytoD – – – – – – + –
LatA – – – – – + – –
scArf6p – – – – + – – –
Arf6 – – – + – – – –
RGDS – – + – – – – –
Thrombin – + + + + + + –
GIT1-P
GIT1
β-PIX
Integrin β3
Hic-5
Actin co-IP
Actin in pellet

Figure 17 GIT1 phosphorylation upon thrombin stimulation needed intact actin polymerization. Washed platelets (4×10^8 platelets/mL) were pre-incubated with or without latrunculin A (LatA) or cytochalasin D (CytoD) as indicated for 3 min in an aggregometer then stimulated with 0.1 U/mL thrombin for 3 min. Platelets were lysed in buffer containing 2% Triton X-100 and phosphatase inhibitor cocktail. Co-immunoprecipitation was performed using anti-GIT1 antibody. The proteins in the complex were probed using corresponding antibodies. Data is representative of two independent experiments.

Figure 18

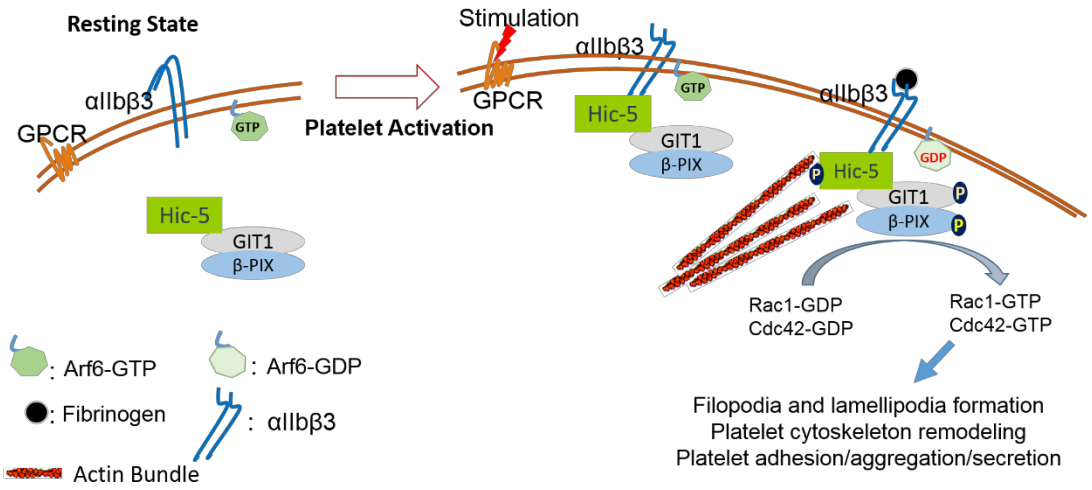

Resting State
αIIbβ3
GPCR
GTP
Platelet Activation
Stimulation
GPCR
αIIbβ3
GTP
Hic-5
GIT1
β-PIX
αIIbβ3
GDP
Hic-5
GIT1
β-PIX
Hic-5
GIT1
β-PIX
: Arf6-GTP
: Arf6-GDP
: Fibrinogen
: αIIbβ3
Actin Bundle
Rac1-GDP
Cdc42-GDP
Rac1-GTP
Cdc42-GTP
Filopodia and lamellipodia formation
Platelet cytoskeleton remodeling
Platelet adhesion/aggregation/secretion

Figure 18 Model of GIT1 regulation in human platelets. In the cytoplasm of resting platelets, GIT1 forms a complex with β-PIX and Hic-5, a member of paxillin family. Upon platelet activation, GIT1/β-PIX/Hic-5 complex is recruited to integrin cytoplasmic tail, which is independent of integrin αIIbβ3 outside-in signaling and Arf6 activity. Following integrin αIIbβ3 outside-in signaling by engaging extracellular fibrinogen, GIT1 is tyrosine-phosphorylated which requires Arf6 activity and intact actin polymerization. Phosphorylated GIT1 mediates second-wave of Arf6-GTP loss and activated GEF activity of β-PIX, probably caused by phosphorylation, leads to Rac1 and Cdc42 activation and subsequent further platelet activation, like platelet adhesion, aggregation and secretion.

specific GIT1 KO mice (referred as GIT1 KO). GIT1 KO mice were generated by crossing floxed GIT1 mice, a generous gift from Dr. Richard Premont (Duke University, Durham, NC), with PF4-Cre mice. Similar to Arf6 KO mice, the target mice for experiments were offspring of $GIT1^{flox/flox}/Cre^{-}$ and $GIT1^{flox/flox}/Cre^{+}$. In initial studies, we had a difficult time confirming the loss of GIT1 in platelets by western blotting. One reason was that the commercially-available anti-GIT1 antibodies had various efficacies in detecting mouse

GIT1. Only one out of three antibodies that we tried was able to detect the mouse GIT1 protein. The second, more important reason for our problems was that the expression level of GIT1 protein in mouse platelets is very low. As shown in Figure 19A, two sets of mouse platelet lysates were tested. Given the same platelet numbers loaded, GIT1 protein levels in mouse platelets were significantly lower compared to human samples. With the help from Dr. Premont, it was noticed, in Figure 19B, that GIT2 levels were much lower (the big arrow) in human platelets while GIT1 levels were much lower in mouse platelets (the small arrow). Therefore, it is likely that GIT2 is the dominant isoform in mouse platelets, while GIT1 is the dominant one in human platelets. Given our experience with isoforms of secretory components in platelets, the dominant one by mass is most often the dominant isoform functionally.

GIT1 Deficiency in Mouse Platelets Did Not Inhibit the Loss of Arf6-GTP upon Platelet Activation

To test if GIT1 behaves as a critical Arf6GAP protein in platelets, particularly downstream of integrin αIIbβ3 outside-in signaling, Arf6-GTP pulldown assays were performed using platelets from the GIT1 KO mice and WT controls. As shown in Figure 20, in WT platelets (black bar), Arf6-GTP levels decreased dramatically upon thrombin stimulation as seen in human platelets [417]. Interestingly, GIT1 KO platelets (white bar) had a similar pattern of Arf6-GTP loss, suggesting that GIT1 is not an essential GAP protein for Arf6 in mouse platelets.

MyrArf6 Peptide Did Not Block Mouse Platelet Aggregation, for Both WT and Arf6 KO Platelets

From the above observation, it seems that the regulation of Arf6 in mouse platelets is probably different from that in human platelets. In fact, a myristoylated Arf6 peptide (the N-terminal of Arf6), which was previously shown to inhibit Arf6-GTP loss and

Figure 19

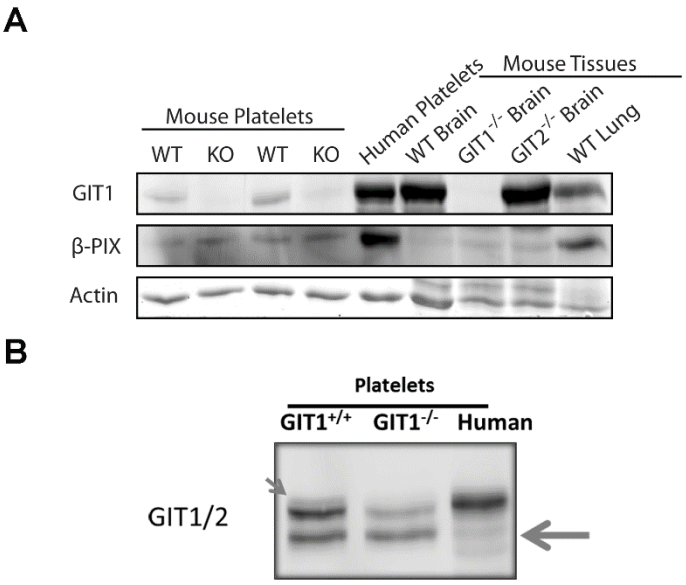
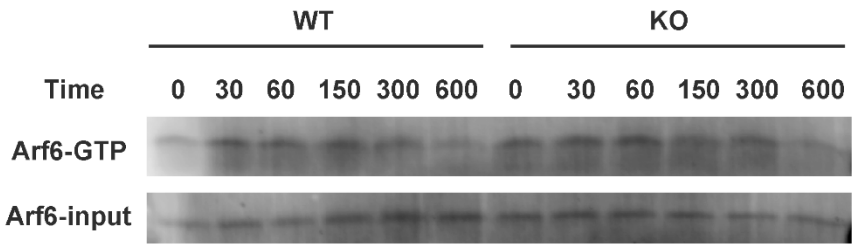

A
Mouse Platelets
Mouse Tissues
WT
KO
WT
KO
Human Platelets
WT Brain
GIT1-/- Brain
GIT2-/- Brain
WT Lung
GIT1
β-PIX
Actin
B
Platelets
GIT1+/+
GIT1-/-
Human
GIT1/2

Figure 19 Mouse platelet contained much less GIT1 protein than human. (A-B) Equal numbers (1.6 X 10^7) of washed WT or KO MP and HP were loaded. 100 μg of brain tissues or lung tissue were loaded in each sample. For mouse platelet samples, each lane represents platelets from a single mouse. Indicated protein were detected by WB. Actin was stained by Coomassie blue as loading control. In B, the big arrow points to GIT2 and the small arrow points to GIT1.

Figure 20

A


WT
KO
Time
0 30 60 150 300 600 0 30 60 150 300 600
Arf6-GTP
Arf6-input

B

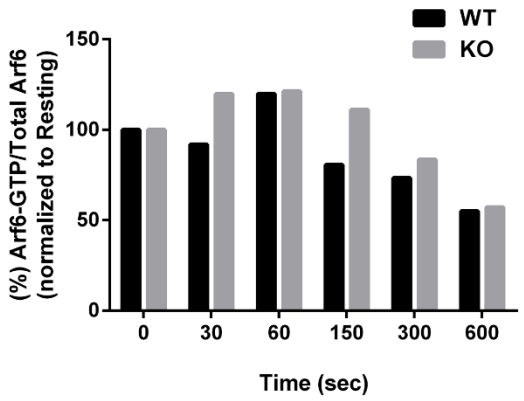

WT
KO
(%) Arf6-GTP/Total Arf6
(normalized to Resting)
150
100
50
0
0 30 60 150 300 600
Time (sec)

Figure 20 GIT1 depletion in mouse platelets did not affect Arf6-GTP dynamics upon thrombin stimulation. (A-B) Washed platelets from WT and KO mice were prepared at 5 X 10^8/ml in HEPES Tyrode's buffer (pH 7.4) and kept resting or stimulated with 0.1 U/ml thrombin for the indicated times. The reaction was stopped by addition of 2 X GTPases-pulldown lysis buffer containing protease inhibitor cocktail. Arf6-GTP was pulled down based on the methods described in method section. The pellets (Arf6-GTP) and the supernatant (total Arf6) was subjected to western blotting. Quantification of the levels of Arf6-GTP and total Arf6l was performed using ImageQuantTL. The ratio of Arf6-GTP to total Arf6 was calculated and plotted in bar graph. Data is representative of at least two independent experiments.

human platelet aggregation (studies here, [414] and the Bristol group), actually did not inhibit mouse platelet aggregation. As shown in Figure 21A, myrAf6 peptide inhibits human platelet aggregation and ATP release in dose-dependent manner, which is consistent with previous observation. However, this peptide did not inhibit mouse platelet aggregation or ATP release (Figure 21B). Interestingly, Arf6 deletion in mouse platelets had no effect on myrArf6 peptide-pretreated mouse platelet aggregation and

ATP release (Figure 21C). These results suggested that the regulation of Arf6 in mice is different from that in humans.

GIT1 KO Platelets Had no Defect in Fibrinogen Levels or Clot Retraction

Since Arf6 KO platelets are defective in fibrinogen storage and have enhanced clot retraction, the fibrinogen levels and clot retraction were also determined in GIT1 KO platelets. As shown in Figure 22, fibrinogen levels were not significantly lower in GIT1 KO platelets compared to WT. As shown in Figure 23, GIT1 KO platelets had similar clot retraction profiles as WT. Taken together, it appears that the GIT1 protein is not important in mouse platelets, at least for the functions that were tested.

Summary: Apparently GIT1 is differentially expressed and used in human platelets versus in mouse platelets. This is yet another example in which mouse platelets and human platelets differ [484]. In human platelets, cellular localization and phosphorylation of GIT1 are regulated by integrin αIIbβ3 outside-in signaling and Arf6 activity, suggesting that GIT1 could behave as Arf6GAP protein in the second wave signaling that was observed by Karim *et al*. However, it seems that GIT1 is not the dominant isoform for Arf6 regulation in mouse platelets since GIT1 depletion had no effect on the loss of Arf6-GTP upon platelet activation. This would suggest that GIT2 may be the critical isoform controlling Arf6 in mouse platelets. This would be consistent with expression/function data in platelets where the dominant isoform by mass is usually the functionally relevant one: however, this point will need further investigation.

Figure 21

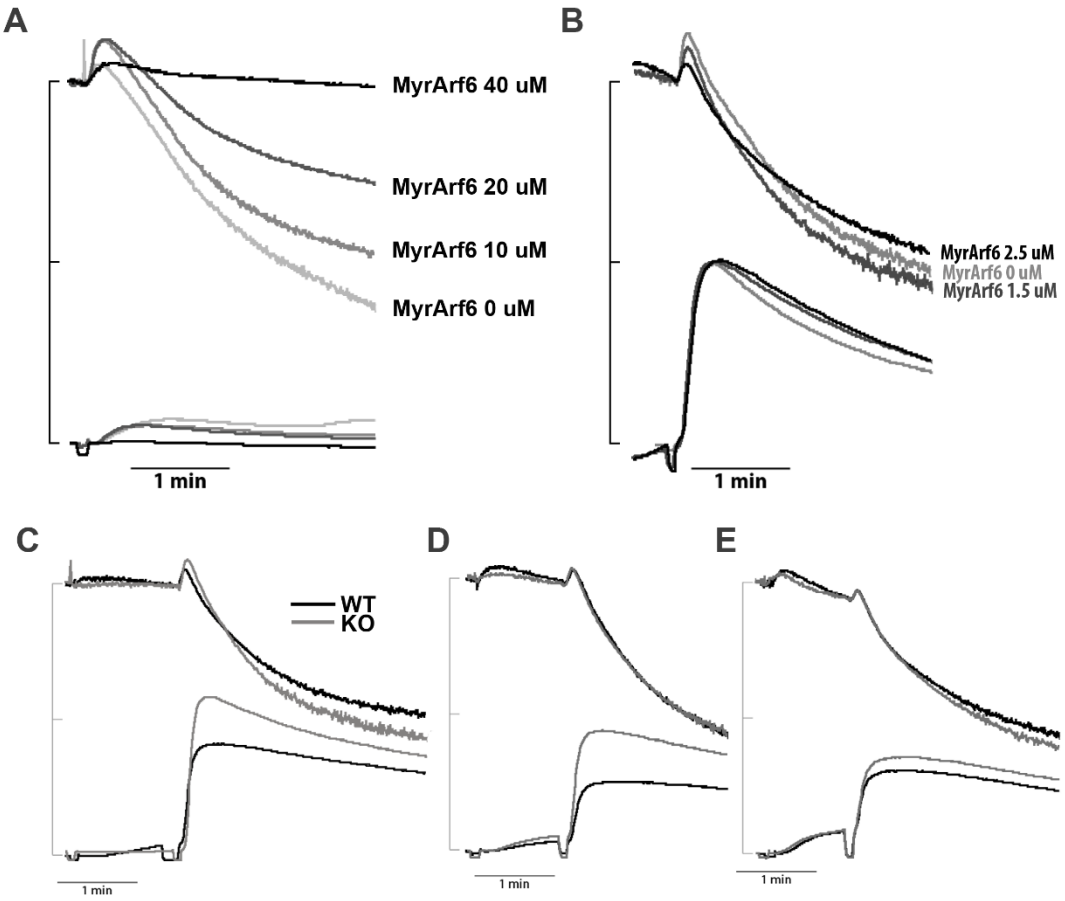

A
MyrArf6 40 uM
MyrArf6 20 uM
MyrArf6 10 uM
MyrArf6 0 uM
1 min
B
MyrArf6 2.5 uM
MyrArf6 0 uM
MyrArf6 1.5 uM
1 min
C
WT
KO
1 min
D
1 min
E
1 min

Figure 21 MyrArf6 peptide did not block mouse platelet aggregation, for both WT and Arf6$^{-/-}$ platelets. Washed platelets were prepared in HEPES-Tyrode's buffer (pH 7.4) containing 1 mM Ca^{2+} and adjusted to 2.5 X 10^8/ml. Platelet aggregation was initiated by addition of 0.1 U/ml thrombin. ATP release was monitored using CHRONO-LUME reagent (Chrono-log) while platelet aggregation was performed. Data was organized using Adobe Photoshop CS5. **(A)** MyrArf6 peptide inhibits fresh human platelet aggregation in dose-dependent manner. Washed Platelets were pre-incubated with myrArf6 peptide for 3 min in aggregometer in the presence of stirring at 37°C. **(B)** MyrArf6 peptide did not block WT mouse platelet aggregation. Washed platelets were pre-incubated with myrArf6 peptide for 1 min in the presence of stirring at 37°C. **(C, D, E)** Arf6 KO platelets have same aggregation profile as WT platelets in the response of myrArf6 peptide pretreatment. Wash WT and KO platelets were pre-incubated with myrArf6 peptide (**C**, 0 μM, **D**, 1.5 μM, and **E**, 2.5 μM) for 1 min in the presence of stirring at 37°C. Data is representative of at least three independent experiments.

Figure 22

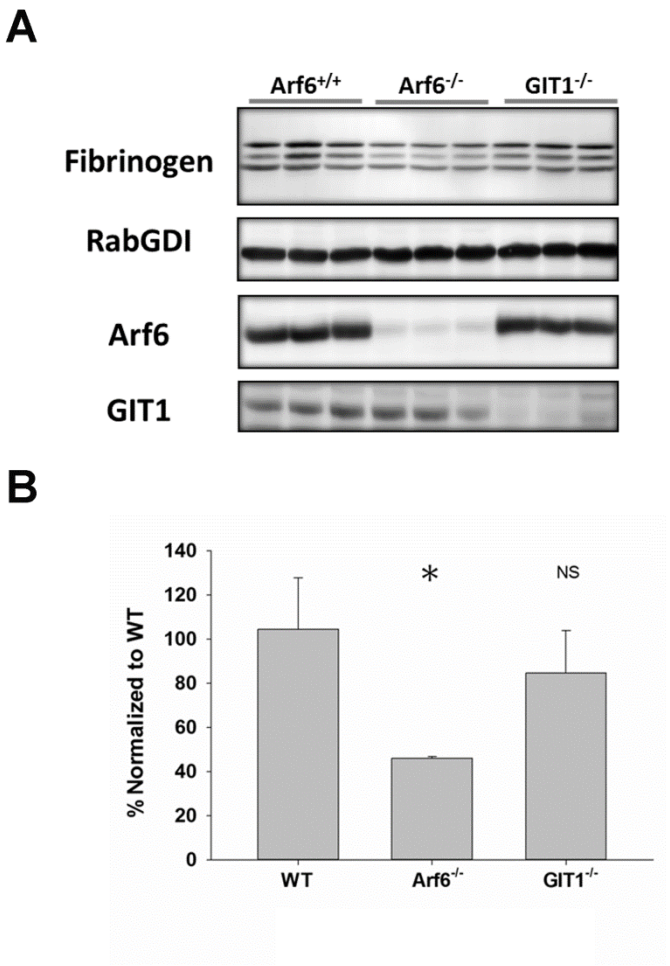

A
Arf6+/+
Arf6-/-
GIT1-/-
Fibrinogen
RabGDI
Arf6
GIT1
B
% Normalized to WT
140
120
100
80
60
40
20
0
*
NS
WT
Arf6-/-
GIT1-/-

Figure 22 Platelets lacking in GIT1 had normal fibrinogen storage. (A) Comparison of endogenous fibrinogen levels among WT, Arf6 KO, and GIT1 KO platelets by western blotting. Mouse platelet lysates of WT, Arf6 KO, and GIT1 KO were prepared. 1 X 10^7 platelets were loaded each lane and indicated proteins were probed using corresponding antibodies. Each lane represents platelets from a single mouse. RabGDI was used as a loading control. **(B)** Quantification of fibrinogen levels in **(A)** was performed using ImageQuantTL and analyzed by SigmaPlot 12.0.

Figure 23

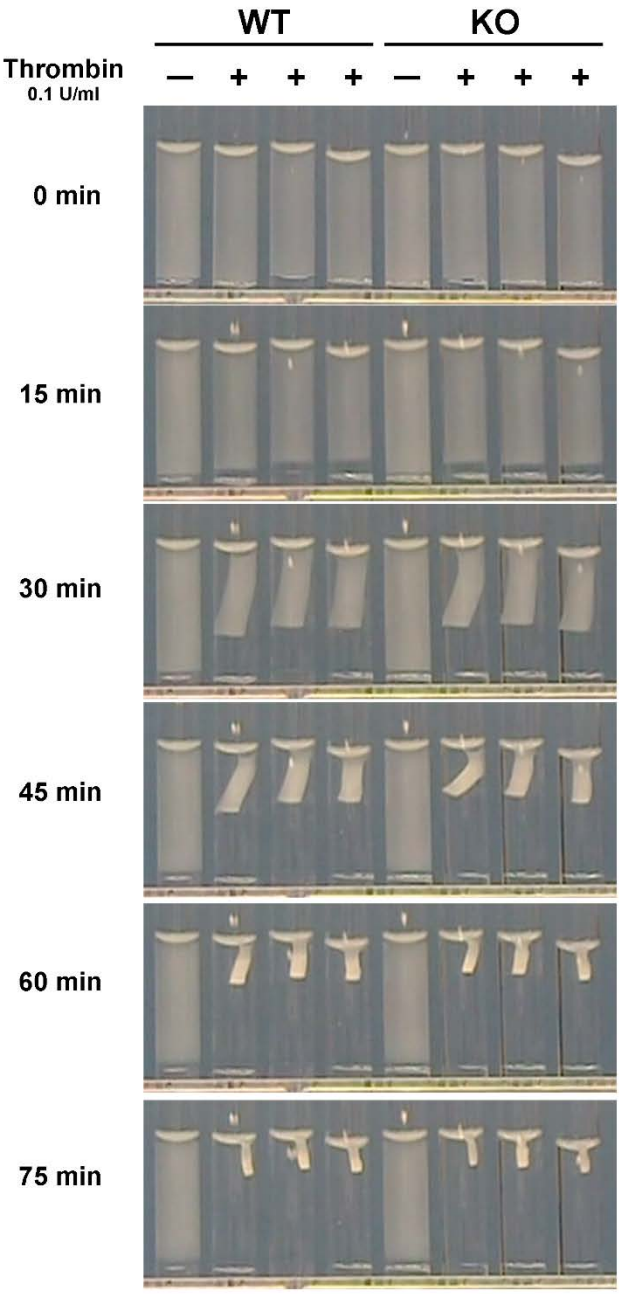


Figure 23 GIT1 deficient in mouse platelets did not affect clot retraction. Washed WT and GIT1 KO mouse platelets were prepared in HEPES Tyrode's buffer (pH 7.4) at 4.0 X 10^8/ml. Platelets were supplemented with 1 mM Ca^{2+} and 0.5 mg/ml fibrinogen. Clot retraction was initiated by 0.1 U/ml thrombin. The images were taken at the indicated times. Data is representative of at least two independent experiments.

Chapter Five: Arf6 and Endocytosis in Platelet Function

In the work presented, we have continued to explore Arf6's role in platelets, using biochemical assays and genetic tools: Arf6 KO and GIT1 KO mice. Depletion of Arf6 in mouse platelets does not affect the expression levels of other proteins, including Arf1/3, Rho family, integrin β3 subunit, and granule marker proteins. Consistent with that, no overt morphology defect is noticed on Arf6 KO platelets, though there is a slight decrease in platelet size. Interestingly, Arf6 KO platelets are defective in fibrinogen storage and uptake, but not in other cargo. Using flow cytometry, no detectible defect is observed on the surface levels of integrin αIIbβ3, total or activated. Consistently, platelet aggregation and ATP release are not affected in response to various agonists. Surprisingly, Arf6 deficiency enhances platelet spreading on fibrinogen and clot retraction but these defects do not cause obvious alterations in thrombus formation or hemostasis.

Mechanisms for the Defective Fibrinogen Storage in Arf6 KO Platelets

It is well-established that platelets and megakaryocytes do not synthesize fibrinogen [485] and that fibrinogen that is stored in platelets comes via a receptor-mediated endocytosis process with integrin αIIbβ3 as the receptor [29, 486]. This concept is confirmed in Figure 2A, where β3 KO platelets contain almost no fibrinogen. Based on Arf6 regulation of integrin trafficking [216, 316-319] and fibrinogen trafficking pathway in platelets [23], a model of Arf6-involved integrin αIIbβ3 (or fibrinogen) endocytic trafficking is proposed (Figure 24). Potentially, Arf6 could play a role in every step of endocytic trafficking of integrin αIIbβ3.

As shown in Figure 1C and 1D, platelet integrin αIIbβ3 levels and plasma fibrinogen levels are normal in Arf6 KO mice, suggesting that the fibrinogen storage defect is due to defective endocytic-trafficking of fibrinogen in Arf6 KO platelets. According to the proposed model in Figure 24, the defective fibrinogen storage (~50% reduction) in Arf6 KO platelets could be due to the mechanisms discussed below.

First, the defective fibrinogen storage in Arf6 KO platelets could be because of less fibrinogen been endocytosed. In this scenario, there are two possibilities. One possibility is that binding of fibrinogen to integrin αIIbβ3 is less stable in Arf6 KO platelets, resulting in less efficient endocytosis of fibrinogen. The other one is that

Figure 24

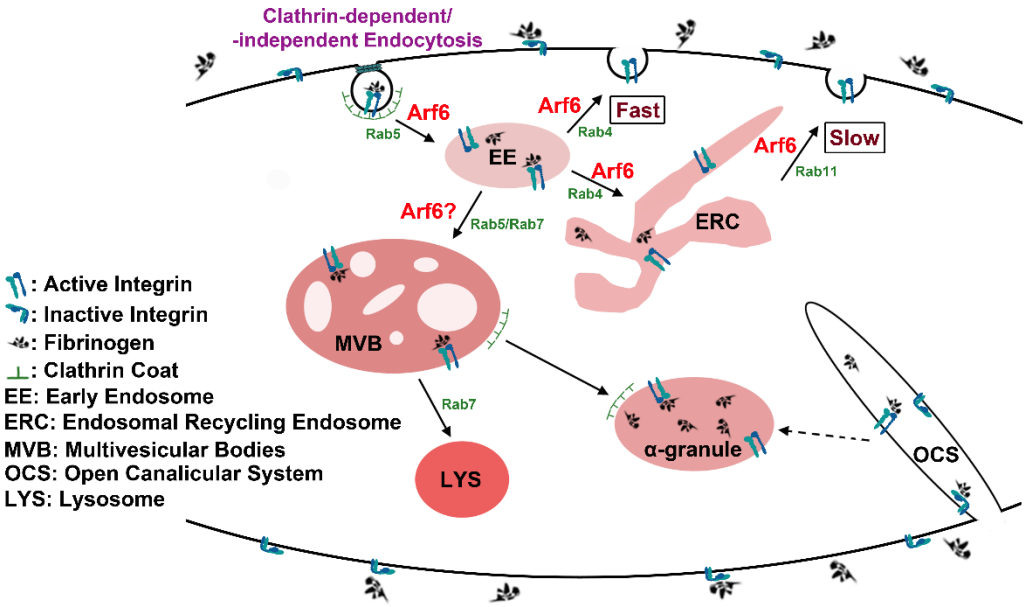

Clathrin-dependent/
-independent Endocytosis
Arf6
Rab5
EE
Arf6
Rab4
Fast
Arf6
Rab4
Arf6
Rab11
Slow
ERC
Arf6?
Rab5/Rab7
MVB
Rab7
LYS
α-granule
OCS
: Active Integrin
: Inactive Integrin
: Fibrinogen
: Clathrin Coat
EE: Early Endosome
ERC: Endosomal Recycling Endosome
MVB: Multivesicular Bodies
OCS: Open Canalicular System
LYS: Lysosome

Figure 24 Model for Arf6's roles in integrin αIIbβ3 trafficking and fibrinogen storage in platelets. According to the roles of Arf6 in integrin trafficking in nucleated cells, Arf6 may play a role in various steps in integrin αIIbβ3 in platelets. There are major two endocytic recycling pathways for integrins, Rab4-mediated fast-loop via early endosomes and Rab11-mediated slow-loop via endosomal recycling endosomes. Arf6 may be important in either recycling process or in endocytosis process. Since multivesicular bodies (MVBs) are an intermediate stage in the formation of platelet α-granules, Arf6 may also regulate the integrin transition from early endosome to MVBs, resulting the storage of fibrinogen within α-granules. In addition to the endocytosis from plasma membrane, endocytosis from open canalicular system (OCS) might also play a role for integrin trafficking.

depletion of Arf6 causes defective endocytosis of fibrinogen-occupied integrin αIIbβ3. There haven't been any reports that Arf6 regulates integrin activation, thus it is very unlikely that Arf6 deletion causes reduction in binding affinity of integrin αIIbβ3 to its ligand, fibrinogen. However, it has been shown that endocytic trafficking of active and inactive β1 integrins in cancer cells is differentially regulated [316] and that active αIIbβ3 integrins in human platelets are preferentially endocytosed [240]. We have hypothesized that Arf6 depletion leads to decreased endocytosis of fibrinogen-occupied integrin αIIbβ3, thus deceased fibrinogen deposit within platelets. However, I did not notice any difference on the surface levels of active and inactive integrin αIIbβ3 between WT and Arf6 KO platelets (Figure 5), which could be due to the insufficient sensitivity of the assay. Regardless of which possibilities, measuring the FITC-fibrinogen uptake using washed platelets over the time, particularly the early time points, should be able to tell if the endocytosis of fibrinogen-occupied integrin is lower or not in Arf6 KO platelets. Moreover, the first possibility (binding affinity) could be experimentally test by measuring the surface levels of FITC-fibrinogen over the time.

Second, the defective fibrinogen storage in Arf6 KO platelets could be because that endocytosed fibrinogen gets degraded in Arf6 KO platelets rather than be delivered to α-granules as in WT platelets, resulting in less fibrinogen deposit within Arf6 KO platelets. It has been demonstrated by immunofluorescence microscopy that endocytosed fibrinogen is not accumulated in late endosomes or lysosomes in human platelets [487], suggesting that degradation in lysosomes is not the major destination for endocytosed fibrinogen. If endocytosed fibrinogen gets degraded in Arf6 KO platelets, fibrinogen fragments should be shown up by western blotting in low molecular weight area in Arf6 KO platelets. If this is the case, it would suggest, according to the model, that Arf6 deletion leads to delivering fibrinogen to lysosomes for degradation rather than to α-granules or recycling compartments and that Arf6 may be involved in the transition from MVBs to α-granules, a novel role for Arf6 in integrin αIIbβ3 trafficking in platelets.

Lastly, the defective fibrinogen storage in Arf6 KO platelets could be equally possibly due to altered integrin αIIbβ3 recycling. If fibrinogen endocytosis and fibrinogen

degradation are not affected in Arf6 KO platelets, one of the possibilities is that endocytosed fibrinogen is recycling back to the surface, which is exacerbated by Arf6 depletion. Arf6 has been suggested to be involved in two recycling pathways of integrins (Figure 24), Rab4-mediated fast-recycling pathway and Rab11-mediated slow-recycling pathway. In platelets, Arf6 probably is required for slow-recycling process and deletion of Arf6 pushes recycling of fibrinogen-occupied integrin αIIbβ3 to the fast-loop, resulting in less fibrinogen deposited within platelets. It is also possible that in platelets Arf6 mediates the trafficking of integrin from endosomes to multivesicular bodies and deletion of Arf6 cause decreased accumulation of fibrinogen to α-granules.

Potential Role of Arf6 in Integrin αIIbβ3 Endocytic trafficking in Platelets

Arf6 regulation of integrin trafficking has been observed in different cells [216, 220] and is important for cell migration, neurite outgrowth and tumor metastasis. It appears that Arf6 could participate in different routes and different steps of integrin trafficking depending on the cell systems, thus it may be difficult to generalize too much.

Our previous studies showed that Arf6-GTP accounts for ~10% of the total Arf6 in resting platelets [414, 417]. How do the resting platelets maintain this high amount of Arf6-GTP? It was suggested that Arf6 is locked in GTP state by its GEF protein, cytohesin2, which is important to sequester the release of dense-granules [353]. However, given the fact that Arf6 is indispensable for fibrinogen storage in platelets, it is reasonable to speculate that the high ratio of Arf6-GTP/Arf6-GDP in resting platelets is consequence of constitutive trafficking of integrin αIIbβ3 between the plasma membrane and endocytic compartments.

In platelets, there is EM evidences suggesting that the trafficking of integrin αIIbβ3 is mediated by clathrin-coated vesicles. An integrin αIIbβ3-specific antibody, c7E3 fab/abciximab, was found to be co-localized with clathrin [233]. Fibrinogen, labeled with gold beads, was also shown co-localized with integrin αIIbβ3 [488]. Consistently, in primary megakaryocytes and megakaryocytic differentiating human leukemic K562 cells, uptake of fibrinogen is dependent on integrin αIIbβ3 and clathrin [489].

Arf6 could participate in the clathrin-coat assembly for endocytosis of integrin αIIbβ3. It is well-known that PI(4,5)P2 is one of the most important lipids regulating clathrin-mediated endocytosis [292]. Arf6 could induce local accumulation of PI(4,5)P2 by activating lipid modification enzymes, such as PLD and PIP5K. Therefore, Arf6 could mediate endocytosis of integrin αIIbβ3 by facilitating the recruitment of clathrin coats through PI(4,5)P2 (Figure 24). Indeed, Arf6 and PI(4,5)P2 have been shown to play roles in clathrin coat recruitment in synaptic vesicle preparations [259] and onto liposome [293].

Arf6 could also be involved in the fission of integrin αIIbβ3-containing vesicles by regulating dynamin activity. Arf6 has been shown to regulate the dynamin-dependent endocytosis of P2Y receptor in human platelets [54]. Arf6 was proposed to recruit NM23-H1, a nucleoside diphosphate kinase which functions as a GTP source for dynamin, and thus regulate dynamin-dependent fission of coated vesicles during P2Y endocytosis in platelets. Similarly, it has been shown that endocytosis of E-cadherin in epithelial cells also requires Arf6-mediated recruitment of NM23-H1, which in turn facilitates the dynamin-dependent fission [294].

It is equally possible that Arf6 is involved in other steps of integrin αIIbβ3 endocytic trafficking (Figure 24). For example, Arf6 could regulate the formation of actin cytoskeleton or microtubule, which is responsible for moving of the integrin αIIbβ3-containing vesicles. All these possibilities require further investigation.

Other Factors may Affect Integrin αIIbβ3 Endocytic Trafficking in Platelets

Clathrin-mediated endocytosis is a complex, spatiotemporally-coordinated process involving many essential factors [490]. Some novel players have been found to be specifically involved in integrin αIIbβ3 endocytosis. In human platelets, the Src family members, Fyn and Lck, were shown to be co-localized with clathrin-coated vesicles [235], suggesting their role in endocytosis. Indeed, the surface levels of integrin αIIb subunit, measured by flow cytometry, was slightly higher in $fyn^{-/-}lyn^{-/-}$ platelets compared to WT, although it was not statistically significant [236]. These $fyn^{-/-}lyn^{-/-}$ platelets were defective in platelet aggregation and ATP release in response to CRP (collagen-related peptide) and defective thrombus formation on collagen fibers under

flow condition, and had enhanced spreading on fibrinogen [236]. However, it is not clear whether these platelets had a fibrinogen storage defect.

DAB-2 (disabled-2) is another potential regulator of integrin αIIbβ3 endocytosis. It is an adapter protein involved in multiple cellular activities. It binds to the NPXY motif of integrin β1 through its N-terminal phosphotyrosine binding (PTB) domain [198, 491]. Integrin β3 contains two NPXY/NPXY-like motifs in its cytoplasmic tail, which interacts with numerous proteins, including DAB2 [492, 493]. DAB2 could potentially mediate the trafficking of clathrin-coated vesicle through binding to myosin VI [494]. Knockdown of DAB2 in megakaryocytes caused ~50% reduction of fibrinogen uptake [489] and DAB2$^{-/-}$ platelets have ~40% reduction in fibrinogen content [237]. These platelets are defective in platelet spreading, clot retraction, aggregation in response to low concentration of thrombin [237].

It seems that Integrin αIIbβ3 trafficking occurs not only in resting platelets as discussed above but also in activated platelets in response to extracellular stimuli. It was found that ADP or TRAP (thrombin receptor activation peptide) stimulation significantly increases integrin αIIbβ3 internalization in an actin-dependent manner [240]. Also, direct activation of integrin αIIbβ3 with LIBS antibody leads to a ~8-fold increase in the extent of integrin αIIbβ3 internalization. These results demonstrate that the endocytic magnitude (rate of integrin αIIbβ3 uptake) is different between resting platelets and activated platelets, implying that the molecular mechanisms regulating the endocytosis of αIIbβ3 might be different in these two states. It seems exceedingly possible that there are more regulators to be identified which can affect the endocytosis of integrin αIIbβ3.

In addition, there are many interesting questions that remain to be answered. There are two major conformational states of integrin αIIbβ3 in platelets, inactive and activated. Is the endocytic trafficking of these two populations regulated in a same manner? It has been suggested that the endocytic trafficking of inactive and activated β1 integrins is differentially regulated [316]. Therefore, it is possible that αIIbβ3 trafficking is different depending on which conformation it is in. Also, after the clathrin-coated pits are formed, how is the vesicle transported? It has been implicated that actin cytoskeleton could be involved since pre-treatment of platelets with

Cytochalasin E blocks the internalization of integrin αIIbβ3 in both resting and activated platelets. Therefore it would be interesting to look for actin-related proteins, like myosin isoforms, which could bind to β3 subunit [495], and, moesin, which was reported to regulate trafficking of nascent clathrin-coated vesicles through interaction with PI(4,5)P2 [496]. Moreover, it was found that, in CHO cells, the constitutive endocytosis of expressed recombinant integrin αIIbβ3 was not affected by deletion of the cytoplasmic domain of β3, suggesting a potential role of α subunits in this process [202]. However, internalization of fibrinogen-coated particles was affected by this deletion, probably due to the compromised affinity of this mutant integrin for fibrinogen. It has been well-known from in vivo mutagenesis studies that tyrosine-containing, sorting motifs are signals for concentration of transmembrane receptors into coated pits and are both necessary and sufficient for efficient constitutive endocytosis. So, what is the trigger for the constitutive endocytosis of integrin αIIbβ3, particularly the inactive conformation? This question becomes more interesting in platelets, given the fact that they are not adherent to any surface but are circulating in blood as singular entities.

Potential Hyper-thrombotic Phenotype in Arf6 KO mice

Flow cytometry (Figure 7) data shows that the surface levels of integrin αIIbβ3 were not affected in the Arf6 KO platelets. Given the fact that FACS assay is a static measure that reflects the steady state levels of αIIbβ3, it may not report on population of αIIbβ3 that is rapidly recycling on and off the plasma membrane. It is possible that any change in dynamic αIIbβ3 trafficking is too small to be detected by the assay. Further experiments are needed to measure small, dynamic changes in integrins on the platelet surface. Since Arf6 could affect the endocytic trafficking of integrin αIIbβ3, Arf6 depletion was expected to affect platelet functions that are related to integrin αIIbβ3. Indeed, Arf6 deficiency enhanced platelet spreading on fibrinogen-coated surfaces and promoted platelet clot retraction (Figure 7 and 8). Because Arf6 depletion did not appear to affect platelet signaling, as shown in Figure 9-11, the phenotype observed is probably due to some alteration in the surface availability of integrin αIIbβ3. αIIbβ3 could recycle more efficiently in the KO platelets and thus be more available to make contacts with fibrinogen. Despite the effects on spreading and

clot retraction, deletion of Arf6 did not have an obvious effect on thrombosis or hemostasis, in the two arterial thrombosis assays used (Figure 13). Based on the spreading and clot retraction phenotype, one might expect that the Arf6 KO mice could be hyper-thrombotic. Such a phenotype is more difficult to detect in our thrombosis/hemostasis assays. It seems possible that Arf6 KO mice should show increased hemostasis and perhaps decreased embolization given the increased spreading and clot retraction that their platelets exhibit *ex vivo* in our assays. Thus it will require some additional experimentation and refinement of assay systems to fully evaluate the importance of Arf6 function in vivo.

Arf6 Regulation in Platelets

Our previous work has shown that Arf6 is regulated by two waves of platelet signaling and many well-known signaling components contribute to the process (Karim 2008). Since Arf6 has low level of intrinsic GTPase and exchange activities, the switch between the two different nucleotide states requires the GAP and GEF proteins. Recently, using proteomics, several potential regulators were identified in platelets, including ASAP1/2, GIT1/2, ACAP1/2 and cytohesin 2 [497]. This suggests that the control of Arf6 in platelets could be complex and that Arf6 could participate in several aspects of platelet activation and function. Cytohesin 2, as substrate of protein kinase C, has been shown to be functionally relevant to platelet secretion [353]. We hypothesized that GIT1 is an important regulator for Arf6 downstream integrin αIIbβ3 signaling pathway since phosphorylation of GIT1 is regulated by outside-in signaling of integrin αIIbβ3 and our data using human platelets is at least consistent with that hypothesis. Unfortunately, the mouse system could not be used to probe the role of GIT1 in platelets. Arf6GTP levels were not altered in GIT1 KO mouse platelets upon thrombin stimulation (Figure 20). Our further analysis showed that GIT1 was not the dominant GIT isoform in mice (as it is in humans). Interestingly, GIT2 may be the dominant isoform in mouse platelets and thus affect Arf6, downstream of integrin αIIbβ3, as GIT1 appears to do in human platelets. More experimentation and additional KO mouse strains will be require to resolve this point.

Does Fibrinogen Storage-deficiency in Platelets Affect Platelet Function?

Fibrinogen is synthesized by hepatocytes and released into the plasma. Fibrinogen deficiency in mouse and human causes a bleeding disorder [498, 499]. Using fibrinogen knockout mice, it has been suggested that fibrinogen may also contribute to wound healing [500], metastatic potential, atherogenesis, and affect the intensity of inflammation in arthritis. Interestingly, it has been shown that fibrinogen is also required for P-selectin expression in platelets [480]. It seems that the engagement of the C-terminus of the fibrinogen γ chain and β3 integrin, not through the RGDS binding site, plays an important role in this synthesis since platelets from FgγΔ5 (fibrinogen lacking the 5 carboxyl-terminal γ chain amino acid) mice had similar deficiency in P-selectin expression as the one from fibrinogen deficient mice [480]. As shown in Figure 1, Arf6 KO platelets have normal P-selectin levels compared to control, although there is around 50% reduction in fibrinogen content in Arf6 KO platelets. It is possible that the engagement of fibrinogen and β3 on platelet surface generates sufficient signal to drive *de novo* synthesis of P-selectin. It is also possible that the internalized fibrinogen is needed to maintain P-selectin levels in platelets. Therefore, it would be worthwhile to determine whether the P-selectin levels are affected by completely blocking the trafficking of β3 integrins. Fibrinogen is one of the major cargo proteins in platelet α-granules and platelets contain approximately 3% of the total circulating pool of fibrinogen [501]. By electron microscopy, it seems that fibrinogen deficient platelets have normal morphology and α-granule biogenesis [480], suggesting fibrinogen is not required for maintaining platelet morphology. Based on these data, it is not completely clear if platelet-derived fibrinogen is functional important for platelets or platelet-involved physiological processes.

The Significance of Platelet Endocytosis

It has been known for decades that certain cargo molecules in platelet granules are internalized and packed in platelets through either receptor-mediated endocytosis (*e.g.* fibrinogen), or pinocytosis (*e.g.* immunoglobulin, albumin). Autotaxin, also called secreted lysophospholipase D, could be another molecule that is internalized by platelets since platelets contain undetectable autotaxin mRNA but contain readily detectible levels of autotaxin protein [140]. It has been shown that platelet-derived

autotaxin binds to β3 integrin, increasing local LPA level and promoting the metastasis of breast cancer cells to bone [140]. It has been hypothesized that repacking the cargo content into platelets by endocytosis is important for platelets to regain their function after transfusion [502, 503]. As shown in Figure 23, inhibition of dynamin-mediated endocytosis by dynasore, which inhibits dynamin-mediated fission process, enhances platelet clot retraction, mimicking the phenotype of Arf6 KO platelets. Therefore, endocytosis is a key mechanism for modulating platelet granule content and thus may be important for platelets functions.

In conclusion, we show for the first time by using mouse model that Arf6 is involved in integrin αIIbβ3 trafficking in platelets. Controlling the trafficking of integrin αIIbβ3 could be an angle to regulate platelet activity and be a therapeutic approach for cardiovascular disease. Also, regulating platelet endocytosis, thus the granule content in platelets, will benefit the treatment for other diseases, like cancer.

Appendices

ABBREVIATIONS

ACD	Acid citrate dextrose
ADAP	Arf GAP with dual PH domain-containing
ADHD	Attention-deficit hyperactivity disorder
ADP	Adenosine diphosphate
Arf	ADP-ribosylation factor
Arl	Arf-like
ARNO	Arf nucleotide-binding site opener
ASAP	Arf GAP containing SH3, ankyrin repeat and PH domains
ATP	Adenosine triphosphate
BRAG	Brefeldin-resistant Arf GEF
CHO	Chinese hamster ovary
DAB-2	Disabled-2
DAG	Diacylglycerol
EC	Endothelial cells
ECM	Extracellular matrix
EE	Early endosome
EFA	Exchange factor for Arf
EGF	Epithelial growth factor
EM	Electron microscope
EphA2	Erythropoietin-producing hepatocellular carcinoma-A2
ER	Endoplasmic reticulum
ERC	Endocytic recycling compartment
FA	Focal adhesion
FAK	Focal adhesion kinase

GAP	GTPase-activating protein
GDP	Guanosine diphosphate
GEF	Guanine nucleotide exchange factor
GIT	G protein-coupled receptor kinase interacting protein
GPCR	G protein-coupled receptor
GPI	Glycosylphosphatidylinositol
GPVI	Glycoprotein VI
GSK	G protein-coupled receptor kinase
GT	Glanzmann thrombasthenia
GTP	Guanosine triphosphate
HGF	Hepatocyte growth factor
IP	Immunoprecipitation
IP3	Inositol triphosphate
JIP3	JNK-interacting protein 3
LD	Leucine-rich domain
LPA	Lysophosphatidic acid
MAPK	Mitogen-activated protein kinase
MHC	Major histocompatibility complex
MVB	Multivesicular bodies
NDP	Nucleoside diphosphate
NETs	Neutrophil extracellular DNA traps
NO	Nitric Oxide
OCS	Open Canalicular System
PA	Phosphatidic acid
PAK	p21-activated kinase
PAR	Protease-activated receptor

PBS	Paxillin-binding site
PDGF	Platelet-derived grow factor
PF4	Platelet factor 4
PGI2	Prostaglandin I2
PGI2	Prostacyclin I2
PH	Pleckstrin homology
PI(4,5)P2	Phosphatidylinositol 4,5-bisphosphate
PI3K	Phosphoinositide 3-kinase
PIP5K	Phosphatidylinositol 4-phosphate 5 kinase
PIX	PAK interacting exchange factor
PKC	Protein Kinase C
PKC	Protein Kinase C
PKD	Protein kinase D
PLC	Phospholipase C
PLD	Phospholipase D
PNRC	Perinuclear recycling compartment
PPP	Platelet poor plasma
PRP	Platelet rich plasma
RBC	Red blood cells
RGDS	Arginine-glycine-asparagine-serine acid
Robo4	Roundabout homology 4
S1P	Sphingosine-1-phosphate
SFK	Src family kinase
SH2	Src homology 2
SHD	Spa-homology domain
SNARE	Soluble N-ethylmaleimide-sensitive fusion protein

	attachment receptor
SNPs	Single nucleotide polymorphism
TCIPA	Tumor cell induced platelet aggregation
TF	Tissue factor
TfR	Transferrin receptor
TP	Thromboxane A2 receptor
TRAP	Thrombin receptor activation peptide
TXA2	Thromboxane A2
VAMP8	Vesicle-associated membrane protein 8
VEGF	Vascular endothelial growth factor
vWF	von Willebrand Factor
WAVE	WASP family veroprolin homolog
WBC	White blood cells
WRC	WAVE regulatory complex

Reference

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Awards and Honors

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Yunjie Huang, Yasunori Kanaho, Sidney W. Whiteheart. Arf6 Plays a Role in Fibrinogen Uptake in Resting Platelets. Poster presentation delivered at Departmental Retreat in Carrollton, KY, May, 2013

Yunjie Huang, Sidney W. Whiteheart. Integrin-mediated outside-in signaling controls Arf6 and platelet activation via GIT1/β-PIX in humans but not in mice. Poster presentation delivered at 14th Biennial Midwest platelet conference in Case Western Reserve University at Cleveland, OH, October, 2012, at 15th Annual Gill Heart Institute Cardiovascular Research Day in Lexington, KY, October, 2012 and at Departmental Retreat in Carrollton, KY, June, 2012

Yunjie Huang, Sidney W. Whiteheart. Integrin-mediated outside-in signaling controls Arf6 and platelet activation via GIT1/β-PIX. Poster presentation delivered at 14th Annual Gill Heart Institute Cardiovascular Research Day in Lexington, KY, October, 2011 and at Departmental Retreat in Corbin, KY, May, 2011

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