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Ubiquitin-Proteasome System Modulates Platelet Function

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PLATELET FUNCTION

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DEDICATION

This thesis is dedicated to My father Subash Gupta,

My mother Rita Gupta, My mother-in-law Meenu Sharma,

My wife Arishya Sharma, and My son Aryan Gupta.

A very special thanks to all of you for your constant love,

support, encouragement and

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ABSTRACT OF THE DISSERTATION

Ubiquitin-proteasome system modulates
platelet function

By Nilaksh Gupta

Atherothrombotic diseases are responsible for more than 25% of all deaths worldwide. Anti-platelet drugs are the mainstay treatment because of the direct involvement of platelets in the initiation and propagation of thrombosis. However, the currently available anti-platelet drugs, such as antagonists of platelet receptors or of effector systems participating in platelet activation, have their own limitations. A new mode of affecting platelet reactivity may prove to offer unique advantages in a host of clinical settings.

Proteasome inhibitors are in clinical use to treat hematologic cancers, but also reduce thrombosis. Whether the proteasome participates in platelet activation or function is opaque since little is known of the proteasome in these terminally differentiated cells. Therefore, I investigated the role of proteasome-mediated proteolysis on platelet function (AIM 1). I find platelets displayed all

three primary proteasome protease activities, which MG132 and bortezomib (Velcade®) inhibited. Proteasome substrates are marked by ubiquitin, and platelets contained a functional ubiquitination system that modified the proteome by mono- and poly-ubiquitination. Proteasome inhibition suppressed platelet aggregation by low thrombin concentrations and ristocetin-stimulated agglutination through the GPIb-IX-V complex. Proteasome inhibitor MG132 reduced stimulated spreading and clot retraction. The effects of proteasome inhibitors were not confined to a single receptor as MG132 and bortezomib suppressed thrombin-, ADP-, and LPS-stimulated microparticle shedding. Systemic MG132 strongly suppressed formation of occlusive, platelet-rich thrombi in $FeCl_3$-damaged carotid arteries. Transfusion of platelets treated *ex vivo* with MG132 and washed prior to transfusion into thrombocytopenic mice also reduced carotid artery thrombosis.

The inhibition of the proteasome quells the ultimate step of ubiquitin-mediated protein degradation pathway. Proteasome-mediated degradation is the final common step, however, multiple layers of regulated processes are involved upstream of this degradative machine that determines whether to target a protein for degradation or not. Platelets express a number of deubiquitinases that reverse protein ubiquitination, but their potential function in platelets is unstudied. So, I investigated the role of deubiquitinase enzymes in modulating platelet reactivity (Aim 2). I show platelets express deubiquitinase activity and specific inhibitor of the proteasome-associated deubiquitinases (b-AP15) as well as general deubiquitinase inhibitors (PYR41 and PR619) increased mono- and poly-

ubiquitination of platelet proteins. Deubiquitinase inhibition strongly suppressed αIIbβ3 activation, degranulation, platelet aggregation and adhesion/ spreading in response to diverse platelet agonists. This inhibition also blocked downstream signaling from platelet receptors by inhibiting agonist-induced Akt phosphorylation and intracellular calcium release. Inhibition of platelet deubiquitinase activity strongly suppressed formation of platelet-rich occlusive thrombi in $FeCl_3$-damaged murine carotid arteries and prevented *in vitro* thrombus formation on collagen-coated surfaces at high shear rates.

Overall, this study uncovers the role of ubiquitin-proteasome system in regulating platelet reactivity and thrombosis.

Contents

List of Abbreviations

ADP	Adenosine diphosphate
Akt	AK-8 thymoma (or transformed)
APC	Activated protein C
BSS	Bernard Soulier Syndrome
cAMP	Cyclic adenosine monophosphate
cGMP	Cyclic guanosine monophosphate
CVD	Cardiovascular disease
DAG	1,2-diacyl-glycerol
DIC	Differential interference contrast
DMSO	Dimethylsulfoxide
DTS	Dense tubular system
DUB	Deubiquitinase
FlnA	Filamin A
gp VI	Glycoprotein VI
GPCR	G-protein coupled receptor
GPIb-IX-V	Glycoprotein Ib-IX-V
MI	Myocardial infarction
NO	Nitric oxide
OCS	Open canalicular system
PAR	Protease activated receptor
PG_{I2}	Prostaglandin I_2
PI3K	Phosphoinositide 3-kinase
PIP2	Phosphatidylinositol 1, 4-bisphosphate
PIP3	Phosphatidylinositol 3,4,5-trisphosphate
PLCβ	Phospholipase C beta
PLCγ2	Phospholipase C gamma 2
PRP	Platelet rich plasma
TFPI	Tissue factor pathway inhibitor
TIRF	Total internal reflection fluorescence
TxA_2	Thromboxane A_2
Ub	Ubiquitin
UBD	Ubiquitin binding domain
UFD	Ubiquitin fold domain
UPS	Ubiquitin-proteasome system
αIIbβ3	Integrin alpha IIb beta 3

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CHAPTER I

Introduction

1.1 Overview of platelet functions and roles

Thrombotic cardiovascular diseases (CVD), predominantly manifested as myocardial infarction and ischemic stroke, remain the single most common cause of death and disability in the developed world. CVD is responsible for ~30% of all deaths worldwide (Alwan 2011), with arterial thrombosis as the main underlying cause. In the United States alone, more than 2000 patients die each day as a direct result of myocardial infarction and ischemic stroke caused by arterial thrombosis (Go, Mozaffarian et al. 2014).

The initial trigger for arterial thrombosis, formation of occlusive thrombi within the lumen of arteries (Engelmann and Massberg 2013), is the disruption of atherosclerotic plaque that exposes its thrombogenic molecules to the arterial circulation. When released, these molecules cause concomitant platelet activation and fibrin formation leading to platelet-rich occlusive thrombi (Ruggeri 2002; Mackman 2008). This occurs often with fatal consequences e.g. thrombotic occlusion of coronary artery that results in acute myocardial infarction and

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occlusive thrombi in cerebral artery that results in ischemic stroke (Michelson 2010).

Platelets are small, disc-shaped anuclear cells that originate from bone marrow precursors, megakaryocytes, and circulate in blood as sentinels of vascular integrity. Platelet aggregation is a hallmark of hemostasis and a contributing factor in pathologic thrombosis (Italiano, Lecine et al. 1999; Hartwig and Italiano 2003; Ruggeri and Mendolicchio 2007). In the event of injury to the vessel wall, platelets adhere to the exposed subendothelial proteins and polymers to undergo rapid aggregation to form the hemostatic plug, the first response to stop bleeding. However, platelets cannot distinguish between physiological wounds in the vessel lining and pathogenic lesions on diseased atherosclerotic vessels. This contributes to atherothrombosis (thrombosis at sites of atherosclerotic plaque disruption), which stops adequate blood supply to downstream tissues or organs (Fuster, Badimon et al. 1992; Ruggeri 2000).

Owing to the vital role of platelets in athrothrombosis, anti-platelet agents, e.g. aspirin and clopidogrel, are widely prescribed for individuals at high risk of arterial thrombosis (Michelson 2010). However, the downside is an increased risk of bleeding, which limits use of such agents (Fisher and Loscalzo 2011). Identifying new therapeutic approaches that prevent thrombosis without undermining underlying hemostasis seems imperative, but is restricted by our insufficient understanding of molecular events that enhance platelet adhesion, and activation and thus prevents identification of new targets to control platelet

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deposition. Therefore, uncovering of new pathways and advancements in the understanding of molecular events that regulate platelet deposition and activation in thrombus propagation are sought and are likely to provide new therapeutic targets and insight for the improvement of existing anti-platelet agents.

1.1.1 Role of platelets in hemostasis and thrombosis

Blood platelets are all-important for maintaining hemostasis, the cessation of bleeding. The critical role of platelets in hemostasis is highlighted by disease states that affect platelet number and/or function. For example, disorders resulting in abnormally low platelet count, thrombocytopenia, or genetic conditions that impair platelet function, such as Bernard-Soulier syndrome (BSS, quantitative or qualitative defects in the platelet GPIb-IX-V complex) results in increased bleeding (Lanza 2006; Cox, Price et al. 2011). The mammalian hemostatic system preserves the integrity of the high pressure circulatory system by balancing processes that maintain blood fluidity under physiological conditions with those that prevent hemorrhage after penetrating injuries to the vessel wall. Maintenance of blood fluidity rests on an intact vessel wall lined by endothelial cells (Fig.1.1) that maintains a quiescent inert surface through a series of regulatory pathways that prevent platelet activation and keeps the coagulation cascade in check (Pinsky, Broekman et al. 2002; Michelson 2010; Lippi, Franchini et al. 2011).

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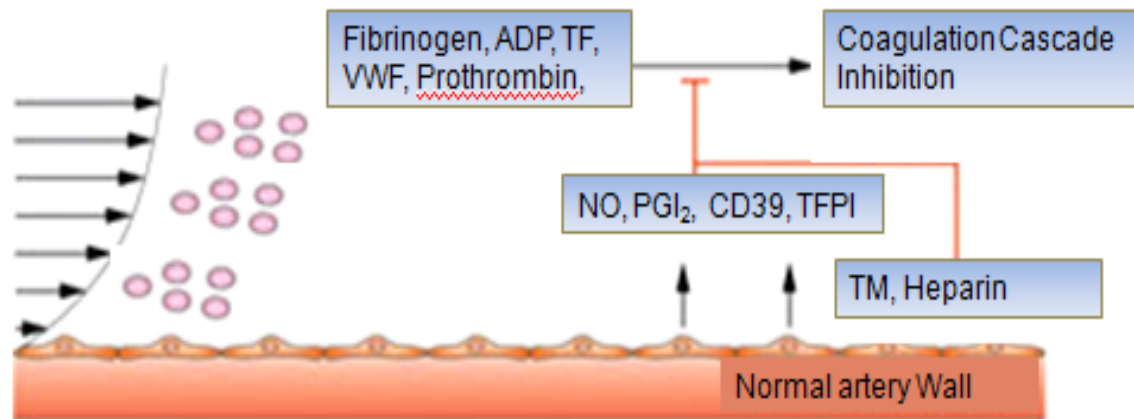
Figure 1.1 Vascular endothelium keeps platelets quiescent and coagulation under check

Vascular endothelium keeps platelet quiescent by releasing: Ecto-ADPase (CD39) that degrades extracellular adenosine diphosphate (ADP, platelet agonist), prostaglandin I_2 (PGI_2) and nitric oxide ($NO^.$), both of these compounds are potent inhibitors of platelet activation as they stimulate an increase in the intracellular levels of cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP), respectively. Vascular endothelium keeps coagulation under check by releasing: Thrombomodulin (TM), which via activated protein C (APC), inhibits factor (F) Va and FVIIIa and tissue factor pathway inhibitor (TFPI), which inhibits FXa and the TF/FVIIa complex. Heparin acts as a cofactor for anti-thrombin, which inhibits thrombin, FXa, FXIa and FIXa [figure modified from (Otsuka, Finn et al. 2012)].

However, disruption or fissuring of an unstable atherosclerotic plaque compromises endothelial integrity and triggers instant platelet recruitment and activation to the exposed thrombogenic subendothelial matrix proteins (Fig.1.2) (Schulz and Massberg 2012).

Recruitment of platelets at the site of the ruptured plaque is an immediate response. This involves interactions between platelet-cell surface receptors and exposed subendothelial matrix proteins including fibrillar collagen (type I and III) and von Willebrand factor (VWF) (Savage, Almus-Jacobs et al. 1998; Massberg, Gawaz et al. 2003; Denis and Wagner 2007). Platelet recruitment via individual receptor-ligand interactions depends on the extent of vascular damage, as well as prevailing rheological conditions. Importantly, shear rates up to 10,000 s^{-1} have been observed in the coronary artery occluded by 50% and even up to 50,000 s^{-1} in severe stenosis (Strony, Beaudoin et al. 1993; Mailhac, Badimon et al. 1994) VS normal shear flow.

Under conditions of rapid blood flow, a feature of arterioles and stenotic arteries, VWF recruits circulating platelets by reversible binding of its A1 domain with multiple GPIb-IX-V receptors. Platelet GPIb-IX-V is essential for the initial recruitment of platelets on the vascular lesion under high shear conditions (Savage, Saldivar et al. 1996; Goto, Ikeda et al. 1998; Ruggeri 2001; Ruggeri and Mendolicchio 2007). The VWF-GPIbα bond displays rapid on-off rates that facilitates platelet translocation to the vessel wall, but it does not support stable adhesion and requires the contribution of additional interactions between

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platelets and other matrix macromolecules (Savage, Almus-Jacobs et al. 1998). Stable platelet adhesion occurs through engagement of platelet glycoprotein VI (gpVI) to collagen (Nieswandt, Brakebusch et al. 2001; Furie and Furie 2006) that triggers downstream signaling leading to platelet activation.

This is critical for subsequent platelet activation as platelet stimulation upon engagement with gpVI causes synthesis and release of secondary platelet agonists, most notably thromboxane A_2 (TxA_2) and ADP, as well as locally generated thrombin, further amplifying platelet activation in an autocrine or paracrine fashion by stimulating their respective receptors on platelets (Share 1976; Offermanns 2006). Inside-out signaling from both gpVI and GPIb-IX-V receptors induces a change in the activation status of β1 integrins {α2β1 [a Collagen receptor], α5β1 [a fibronectin receptor] (Beumer, MJ et al. 1994), and α6β1 [a laminin receptor] (Ruggeri 2009)} and β3 integrin, αIIbβ3 (binding VWF, fibrinogen and fibronectin), from low affinity ligand binding states to high affinity states (Shattil, Kim et al. 2010). Of all the integrins, αIIbβ3 is the dominant integrin present on platelet surface, and high affinity adhesive interactions between αIIbβ3 and adhesive proteins VWF, fibrinogen and fibronectin are central to the irreversible platelet activation and thrombus propagation at the site of ruptured plaque (Savage, Saldivar et al. 1996; Vinogradova, Velyvis et al. 2002).
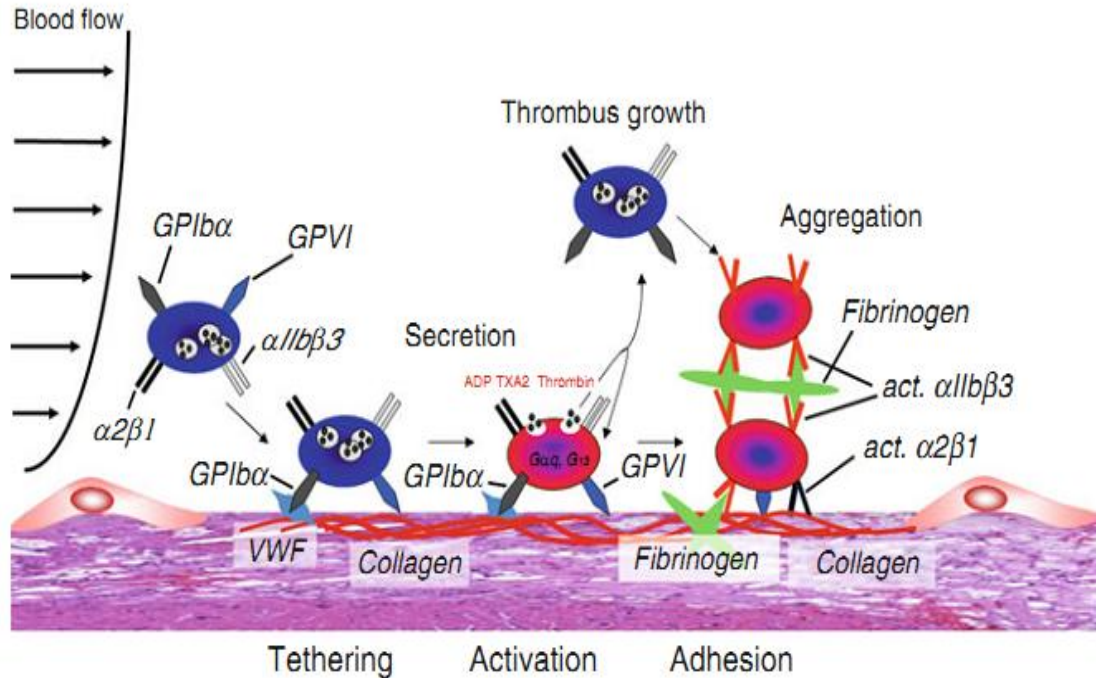
Figure 1.2 Mechanism of platelet recruitment following plaque rupture

Plaque rupture exposes subendothelial proteins VWF and Collagen. Platelets constitutively express receptors for these protein and are recruited to the endothelium from the circulation, initially via GPIbα-VWF interactions (tethering). Once slowed, platelets can bind collagen via its gpVI receptor and downstream signaling initiated from this interaction activate platelets (activation). Platelet activation leads to integrin (αIIbβ3 and α2β1) activation, from inside-out signaling, leading to irreversible platelet activation and firm adhesion (adhesion). Figure adapted from (Schulz and Massberg 2012).

1.1.1.1 Blood coagulation and thrombin generation

An important function of activated platelets is localization of subsequent pro-coagulant events to the site of ruptured plaque. Platelet activation leads to the rapid exposure of the membrane phospholipid phosphatidylserine (PS) from inner membrane leaflet to platelet surface, which provides the anionic surface to support the assembly of coagulation complexes on the platelet plasma membrane. This is critical for localized thrombin generation and fibrin formation (Jackson 2011). Thrombin is one of the most potent endogenous platelet agonists, and plays a seminal role in thrombus formation under all rheological conditions (Kahn, Zheng et al. 1998). Thrombin's effect on human platelets is mediated by surface G protein-coupled protease-activated receptor (PAR) 1 and 4 (Coughlin 1993; Coughlin 1999; Coughlin 2005). Thrombin-induced platelet activation and fibrin generation is crucial for thrombus growth and stability, and hence the drugs that inhibit thrombin generation or thrombin-induced platelet activation, inhibit atherothrombosis (Michelson 2010).

1.1.1.2 Spatial and temporal heterogeneity within growing thrombus *in vivo*- a revised model of thrombus development

Recent studies using high resolution intravital confocal microscopy, genetically engineered mice, and flow chambers demonstrate that the above description of occlusive thrombi formation may be more complicated than previously anticipated. There studies also provide sufficient evidence of spatial and temporal heterogeneity within the growing thrombi (Fig.1.3)

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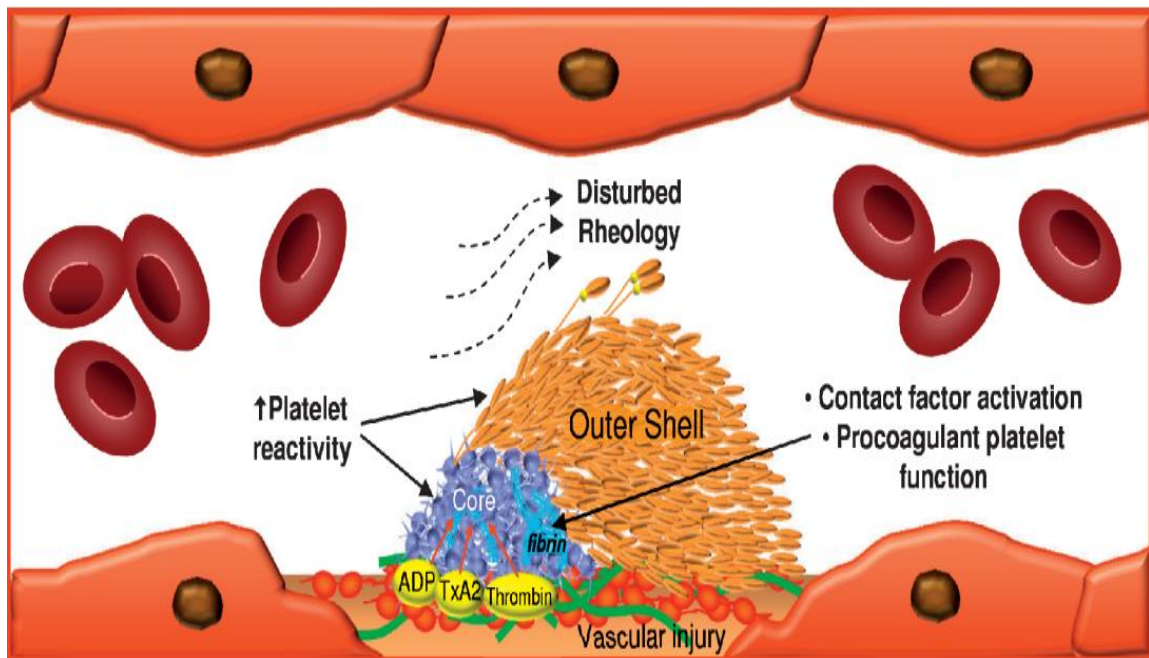
Figure 1.3 Spatial and temporal heterogeniety of thrombus development

Plaque rupture leads to the capture of platelets from circulation on to thrombogenic endothelium matrix proteins and polymers. Subsequent platelet activation via ADP and TxA_2 leads to the formation of highly active platelet core. Shear flow gradient generated at the site, due to narrowing of the lumen, causes platelet tethering and formation of outer shell, composed mainly of discoid and ready to be activated platelets. Adapted from (McFadyen and Jackson 2013).

(Nesbitt, Westein et al. 2009; Stalker, Traxler et al. 2013). Capturing thrombus development in real-time reveals the heterogeneity in the growing thrombus, which appears to have an inner core containing activated platelets and an outer shell comprising of poorly activated discoid platelets. Elegant studies of blood circulation *in vivo* and in flow chambers reveal that the growing platelet aggregates were sensitive to prevailing rheological conditions i.e. to the flow gradient established around the growing thrombus. This flow gradient results in the recruitment of circulating discoid platelets to the growing thrombi through the extension of their membrane, called tethers. These unactivated platelets in thrombi are ready for activation dependent changes leading to thrombus stabilization. (Nesbitt, Westein et al. 2009; Stalker, Traxler et al. 2013).

Importantly, accumulation of discoid platelets on developing thrombi by shear gradients is not impeded by widely used anti-platelet agents aspirin and clopidogrel or thrombin inhibitors (Nesbitt, Westein et al. 2009). Thus, improved understanding of interaction of local conditions at sites of injury or plaque rupture with the platelet signaling network offers a new context and rational into how future anti-thrombotic drugs should be designed.

1.1.2 Platelet morphology and ultrastructure

1.1.2.1 Plasma membrane (PM) and open canalicular system (OCS)

Resting platelets are discoid, roughly 2-3 µm in diameter and their number range from 150,000 to 450,000 cells/µL in human blood. About two-third of the platelets exist in the circulation and the remaining cells are sequestered in the

spleen. Platelets have a life span of ~8-10 days (Hartwig and Italiano 2003; Thon and Italiano 2012).

The platelet plasma membrane is a typical phospholipid bilayer, but also contains an extensive series of complex indentations called the open canalicular system (OCS) (Fig.1.4) (Behnke 1970; Frojmovic, Wong et al. 1992). The OCS is a surface connected tubular system that connects the cytosol with the surrounding medium and facilitates the quick release of secreted substances to the extracellular environment (White and Clawson 1980; White and Krumwiede 1987). The OCS also constitutes an extensive membrane reservoir that upon activation results in increased plasma membrane surface area that facilitates fillopodia formation and platelet spreading (Thon and Italiano 2012). Embedded in the platelet plasma membrane are numerous glycoprotein receptors (GP) and integrins that are involved in the initial adhesion of platelets to the subendothelial matrix and formation of a hemostatic plug (Phillips and Agin 1977; Kunicki 1989). The negatively charged plasma membrane phospholipids (e.g. phosphatidylserine and phosphatidylinositol) present in the inner leaflet of resting platelets, are translocated and exposed on the platelet surface upon activation to provide the surface for binding of proteins involved in coagulation (Heemskerk, Bevers et al. 2002). The phosphatidylserine exposure on the surface of activated platelets is crucial for normal hemostasis and this is evident from a rare bleeding disorder, Scott syndrome, where the patients have reduced

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phosphatidylserine exposure on surface and are unable to make microparticles upon platelet activation (Toti, Satta et al. 1996; Heemskerk, Bevers et al. 2002).

1.1.2.1.1 Platelet derived microparticles (PMPs)

PMPs are small (0.1-1.0 µm in diameter) phospholipid vesicles, which are shed from the cell membrane following platelet activation. PMPs are highly pro-thrombotic because their surface contains exposed phosphatidylserine, which provides the ideal catalytic surface that greatly expedites coagulation (Sandberg, Bode et al. 1985; Sims, Faioni et al. 1988). Elevated numbers of circulating PMPs have been detected during several diverse pathophysiological processes such as acute myocardial infarction, peripheral artery disease, cerebral malaria, rheumatoid arthritis, diabetes, multiple sclerosis, sepsis, and ischemic stroke (Soriano, Jy et al. 2005; Faille, Combes et al. 2009; Boilard, Nigrovic et al. 2010; Lannan, Phipps et al. 2014).
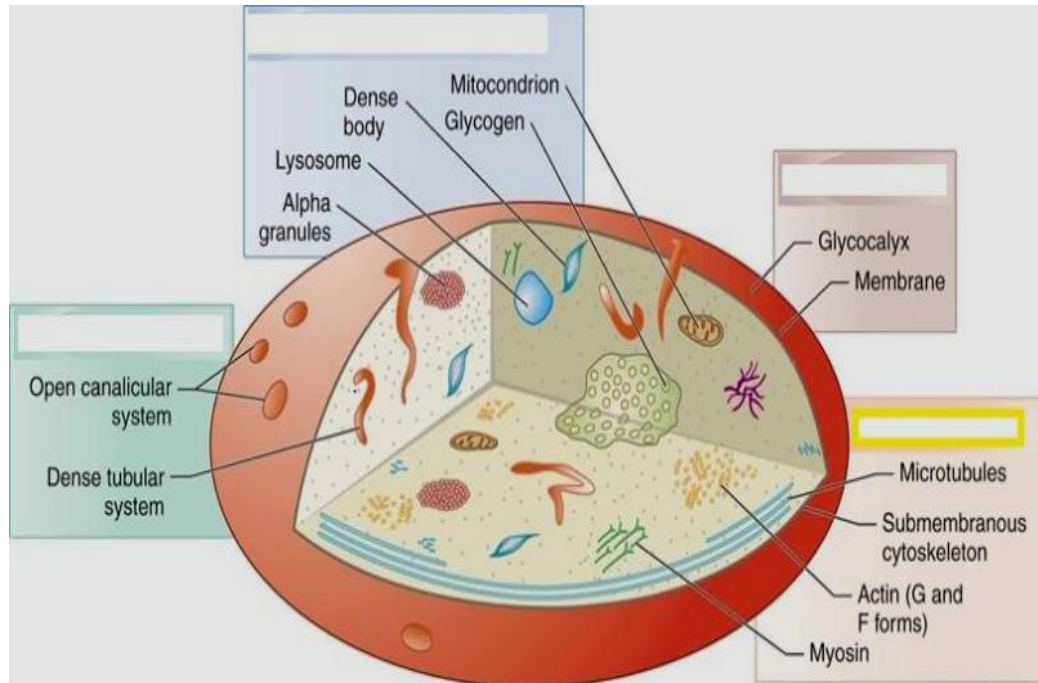
Figure 1.4 Platelet ultrastructure

The role of platelet granules (alpha and dense granules and lysosomes), OCS, dense tubular system and cytoskeleton is explained in the text. Mitochondria are the energy source. Resting platelets fulfill their energy requirements by oxidative phosphorylation to maintain platelet function and membrane asymmetry. Glycogen provides the energy for platelet reactions. Platelet glycocalyx is very dynamic and is covered by glycoprotein receptors. Source (http://www.studyblue.com/notes/note/n/platelets/deck/2298597).

1.1.2.2 Dense Tubular System (DTS)

Another membranous system present in platelets is termed as the DTS that is a closed-channel network of residual smooth endoplasmic reticulum (White 1972). The DTS sequesters ionized calcium and probably also a major site of TxA_2 and prostaglandin synthesis (Gerrard, White et al. 1976; Gerrard, White et al. 1978). The release of intracellular calcium from DTS upon platelet activation contributes to platelet degranulation, cytoskeletal reorganization and redistribution of αIIbβ3.

1.1.2.3 Platelet Cytoskeleton

Platelets undergo rapid shape change, spreading, secretion and/or aggregation upon activation. This transformation in stimulated platelets is largely achieved by rapid cytoskeletal rearrangements within the platelet (Hartwig 2006). The platelet cytoskeleton consists of a marginal band consisting of a microtubule coil, a cytoplasmic actin network, and cytoskeletal rim. Together these structures support the platelet plasma membrane and confer shape to both resting and activated platelets. This inherent capacity of platelets to rapidly reorganize its cytoskeleton allows them to seal the leaks in the vasculature under shear conditions (Hartwig 2006; Thon and Italiano 2012).

The marginal band lies beneath the plasma membrane and confers the discoid shape to the resting platelet. Microtubule disassembly with drugs such as nocodazole or colchicine or depolymerization induced by chilling at 4ºC, cause

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platelets to lose their discoid shape and become round (White and Krivit 1967; White 1968; Hartwig 2006).

The cytoskeletal rim is composed of actin, spectrin, talin, vinculin and actin cross-linking proteins filamin and α-actinin (Fox 2001; Hartwig 2006). Filamins are large cytoplasmic proteins that give mechanical stability to cells by cross-linking actin into dynamic 3-dimensional structures (Pudas, Kiema et al. 2005). There are three filamin isoforms: filamin A (FlnA), filamin B (FlnB) and filamin C (FlnC), but platelets only express FlnA and FlnB. FlnA is an elongated 280-kDa dimeric protein that self associates and is expressed ~10 fold in excess to FlnB (Falet, Pollitt et al. 2010). Structurally, FlnA contains an actin-binding domain (ABD) at the N-terminus, followed by 24 compact immunoglobulin-like repeats of 90–100 amino acids, and a C-terminal domain (Fig. 1.5). Repeat 24 mediates dimerization (Pudas, Kiema et al. 2005) (Nakamura, Pudas et al. 2006). Two unstructured hinge regions separate FlnA repeat domains into rod 1 (repeats 1–15), rod 2 (repeats 16–23), and the self-association domain. Rod 1 that binds actin filaments is elongated and linear, but Rod 2 that contains most binding sites for FlnA partners, has a more compact structure. FlnA has over 70 binding partners and in platelets FlnA links the GPIb-IX-V receptor complex to F-actin. More than 90% of FlnA in platelets is in complex with GPIbα (Nakamura, Pudas et al. 2006; Falet, Pollitt et al. 2010). This interaction has been mapped to FlnA repeat 17 and aa 563–571 (FRSSLFLWV) in the cytoplasmic tail of GPIbα (Nakamura, Pudas et al. 2006).
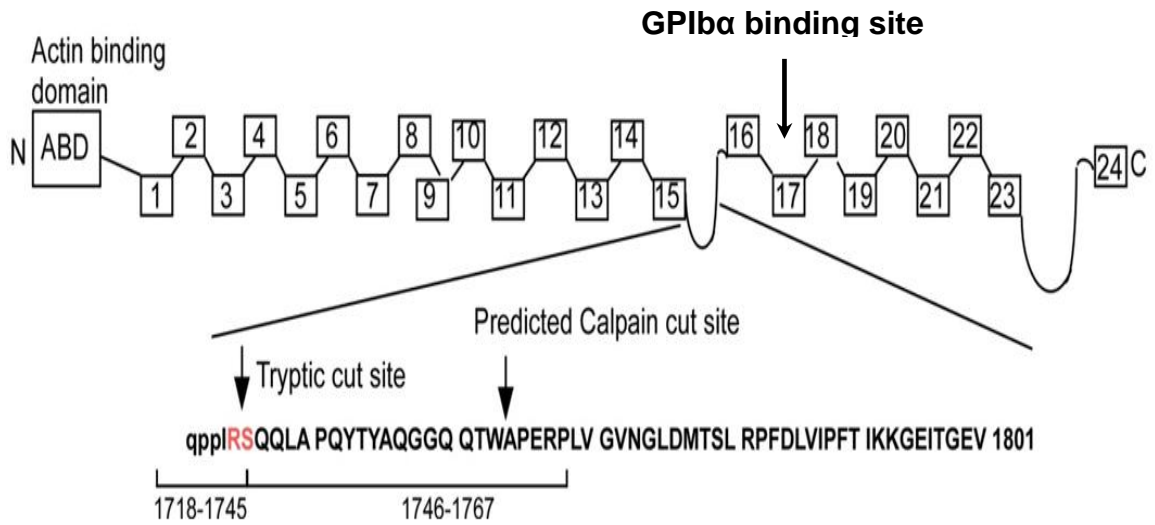
Figure 1.5 Structure of Filamin A (FlnA)

FlnA structure showing N-terminal actin-binding domain (ABD); 24 compact immunoglobulin-like repeats; C-terminal dimerization domain; predicted calpain cut site and proteasome-tryptic cut site; and GPIbα binding site at repeat 17. Adapted from (Gupta, Li et al. 2014).

The FlnA-GPIbα interaction in platelets is critical for anchoring the GPIb-IX-V receptor complex to the membrane skeleton as well as for maintaining cell adhesion under high shear (Cranmer, Ashworth et al. 2011). This FlnA-GPIbα interaction is also vital for formation and release of discoid platelets from megakaryocytes, the platelet precursor cells (Jurak Begonja, Hoffmeister et al. 2011).

The cytoplasmic actin network comprised of actin (which accounts for ~20% of total platelet protein mass) filaments and associated proteins (Oda, Daley et al. 1992). In unstimulated platelets, 40% of the actin is filamentous F-actin, and the rest is globular monomeric G-actin. Upon stimulation, the actin filaments in platelets are severed and resulting smaller fragments are used as the focal point for new, longer actin filaments. This leads to an increase in the F-actin proportion to ~70% to 80% (Kovacsovics and Hartwig 1996). Myosin IIa, the contractile protein, is rapidly phosphorylated by myosin light chain kinase (MLCK) following platelet activation and becomes associated with F-actin (Fox and Phillips 1982) and forms filaments that are anchored to the platelet plasma membrane by attachment (via FlnA) to the GPIb-IX-V complex (Kovacsovics and Hartwig 1996). Myosin IIa phosphorylation following platelet activation is essential for clot retraction and granule centralization (Cohen, Gerrard et al. 1982; Stark, Golla et al. 1991).

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1.1.2.4 Platelet Secretary Granules

Platelets contain secretary granules packed with preformed bioactive molecules, released upon platelet activation, that are essential for normal platelet function. Platelets have three distinct secretary granules; alpha granules, dense granules and lysosomes, each with different morphologies, molecular content, and kinetics of exocytosis (Flaumenhaft 2003; Coppinger, Cagney et al. 2004).

The α-granules are the largest and most abundant platelet granule, outnumbering dense granules by ~10-fold (Koseoglu and Flaumenhaft 2013). These granules contain pro-coagulant proteins (fibrinogen, VWF, platelet factor 4), coagulation factor V, glycoprotein CD62P (P-selectin), CD36, growth-promoting factors and mitogens [platelet-derived growth factor (PDGF), thrombospondin, vascular endothelial growth factor (VEGF), transforming growth factor β (TGFβ) etc. (Coppinger, Cagney et al. 2004).

Dense granules are the smallest platelet granules and humans platelets contain ~3-9 dense granules/platelet (White 1969). They house a variety of hemostatically active molecules such as calcium, magnesium, serotonin and ADP/ATP (ratio 3/2), that are released upon platelet activation to recruit additional platelet at sites of vascular injury (Rendu and Brohard-Bohn 2001; King and Reed 2002). Transport of serotonin in platelet dense granules is essential for liver regeneration (Lesurtel, Graf et al. 2006).

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P-selectin (from α-granules) expression on the surface of stimulated platelets and ATP release from dense granules are frequently used markers to assess the activation state of platelets *in vitro*.

Lysosomes are formed during megakaryocyte maturation earlier than α-granules. Platelets have few primary and secondary lysosomes and their size lies between dense and α-granules (Menard, Meyers et al. 1990). They contain hydrolases (such as cathepsins D and E) elastase and membrane proteins LAMP-1, -2 and -3 (Bentfeld-Barker and Bainton 1982; Israels, McMillan et al. 1996; McNicol and Israels 1999).

1.1.3 Platelet Receptors

Platelets express a great number of receptors (Fig. 1.6), respond to many agonists, and trigger a surfeit of signaling pathways. Importantly, these receptors are not merely redundant as genetic defects or pharmacological inhibition of any of these molecules has a significant effect on platelet function e.g. patients suffering from BSS (with defects in the GP1b-X-V complex) have bleeding disorder or pharmacologic inhibition of αIIbβ3 inhibits platelet aggregation. In the next section, I will focus on the receptors involved in my research work.

1.1.3.1 Adhesive receptors

1.1.3.1.1 Integrins

Integrins are a broadly distributed family of heterodimeric (α and β subunits) cell surface adhesion receptors that occupy ~ 50% of surface area of

an activated platelet. A hallmark of integrins is their ability to cycle from a low affinity ligand binding state (resting platelets) to a high affinity ligand binding state (activated platelets), a property that is of particular importance to blood cells such as platelets (Carman and Springer 2003).

Platelets express two major integrin β subunits, β1 and β3 and five α-subunits. The β1-integrins present in platelets are α2β1, and α6β1(Hynes 2002; Kasirer-Friede, Kahn et al. 2007). Of all the integrins, αIIbβ3 is the dominant integrin on platelet surface and plays a prominent role in platelet aggregation by binding plasma fibrinogen. Approximately 40,000-80,000 copies are present per platelet (Kauskot and Hoylaerts 2012). Upon platelet activation through ADP, thrombin, TxA$_2$ or collagen, αIIbβ3 undergoes conformational change to a high affinity state. This process is termed inside-out signaling (Shattil and Newman 2004). The signaling axis for αIIbβ3 activation involves Rap1, RIAM, talin and kindlin-3 (Banno and Ginsberg 2008; Metcalf, Moore et al. 2010). Fibrinogen binding to the activated αIIbβ3 relays outside-in signaling via the signaling proteins FAK, Pyk2, SFKs (Src, Fyn and Yes), PI3Kβ, or SHIP1 to the actin-myosin cytoskeleton and all of this is important for thrombus stability and clot retraction (Shattil and Newman 2004; Suzuki-Inoue, Hughes et al. 2007; Gratacap, Guillermet-Guibert et al. 2011).
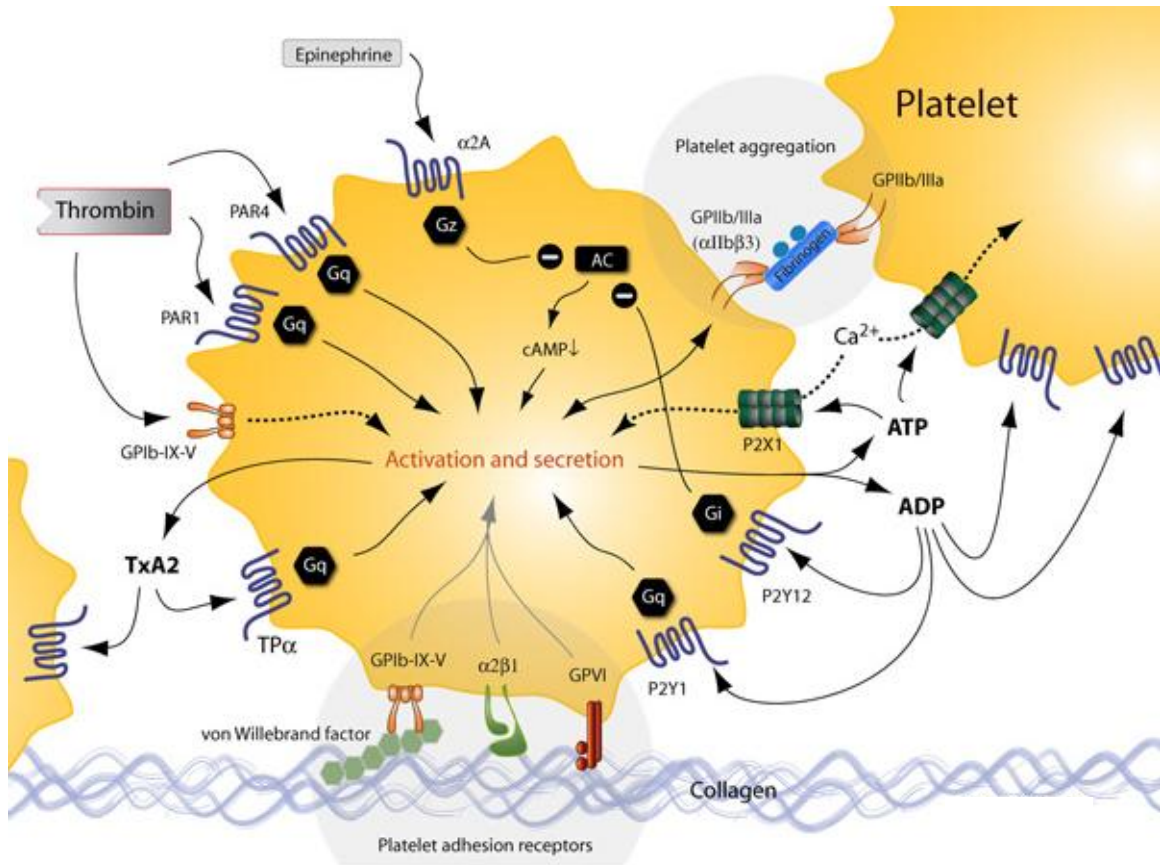
Figure 1.6 Platelet receptors.

The initial recruitment of platelets to injured endothelium is mediated by the adhesion receptors; GPIb-IX-V binds the VWF (green balls), α2β1 and gpVI binding to collagen (white and gray strands), and αIIbβ3 binds fibrinogen (blue bar) to form platelet aggregates. Secondary platelet activation is largely driven by cell surface G- protein coupled receptors, most notably the ADP receptors P_2Y_1 and P_2Y_{12}, ATP receptor P_2X_1, thrombin receptors PAR1 and PAR4, and the TxA_2 receptor, TPα. Adapted from (http://platelets.se/platelets/)

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Since the αIIbβ3 receptor is absolutely essential for irreversible platelet activation and thrombus propagation in vivo (Savage, Saldivar et al. 1996; Vinogradova, Velyvis et al. 2002), pharmacologic inhibitors of this receptor had been developed and are in clinical use. Three inhibitors, abciximab, eptifibatide and tirofiban are FDA approved for percutaneous coronory intervention (PCI, (abciximab and eptifibatide) and acute coronory syndrome (ACS, eptifibatide and tirofiban). The major limitation of these drugs is increased incidence of bleeding, which restricts their use to only high risk patients who have not been pretreated with $P2Y_{12}$ receptor antagonists (Michelson 2010; Muniz-Lozano, Rollini et al. 2013).

1.1.3.1.2 Glycoprotein Ib-IX-V

The GPIb-IX-V receptor complex is crucial for the initial adhesion of circulating platelets to the injured vascular surface via its interaction with its ligand VWF at high shear rates (Savage, Saldivar et al. 1996; Goto, Ikeda et al. 1998; Ruggeri 2001; Ruggeri and Mendolicchio 2007). This receptor complex is constitutively expressed on platelet surface with the density of about 25000 copies per platelet (Modderman, Admiraal et al. 1992) and consists of 4 distinct non-covalently attached subunits namely, glycoprotein Ibα (GPIbα), glycoprotein Ibβ (GPIbβ), glycoprotein IX (GPIX), and glycoprotein V (GPV) in the ratio 2:2:2:1 (Ware 1998; Kauskot and Hoylaerts 2012). The N-terminal extracellular region of GPIbα contains the binding sites for VWF, P-selectin, Mac-1 (integrin α2βM), coagulation factors XI (Baglia, Badellino et al. 2002) and FXII (Bradford,

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Pixley et al. 2000), high-molecular-weight kininogen (Lanza 2006) and thrombin (De Marco, Mazzucato et al. 1994; Adam, Bouton et al. 2003). The intracellular cytoplasmic domain of GPIbα is linked to FlnA and this interaction tethers the GPIb-IX-V receptor complex to the platelet cytoskeleton, maintaining the cytoskeletal architecture of resting platelets and those adhering in vessels at high shear rates (Cranmer, Ashworth et al. 2011).

In the absence of any vascular trauma, plasma VWF does not normally bind to platelets, since the GPIbα binding VWF-A1 domain is inaccessible (Miyata, Goto et al. 1996). However, damage to vessel wall exposes subendothelial collagen, enabling VWF to bind collagen through its A3 domain. This and/or high shear stress induces conformational change in VWF, exposing the otherwise cryptic binding site on VWF-A1 for the GPIb-IX-V complex (Siedlecki, Lestini et al. 1996). The snake venom peptide botrocetin (Read, Smith et al. 1989) or the antibiotic ristocetin (Scott, Montgomery et al. 1991) can also be used to induce vWF-GPIbα interaction in vitro.

GPIbα, which is primarily regarded as the VWF receptor, also contains a high affinity binding site for thrombin that contributes to platelet activation at low thrombin concentrations and plays a significant role in the generation of platelet microparticles and their procoagulant activity (Harmon and Jamieson 1986; Dormann, Clemetson et al. 2000). The importance of thrombin binding to GPIbα became evident after the observation that patients with a rare autosomal recessive condition called BSS (quantitative or qualitative defects in the platelet

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GPIb-IX-V complex) (Lanza 2006; Cox, Price et al. 2011), showed reduced responsiveness to thrombin (Ganguly 1977; Jamieson and Okumura 1978). GPIbα also increases the rate of protease-activated receptor 1 (PAR1, discussed later) hydrolysis by 5 fold, indicating that GPIbα may act as a cofactor that facilitates PAR 1 cleavage (De Candia, Hall et al. 2001). A specific mutation that only blocked thrombin-GPIbα interaction reduced the ability of murine platelets to form thrombi, suggesting this interaction is essential for thrombosis. Importantly, blocking thrombin-GPIbα interaction *in vivo* had no effect on bleeding time, making this interaction a suitable therapeutic target (Guerrero, Shafirstein et al. 2008).

1.1.3.1.3 Collagen Receptors

The subendothelial matrix protein collagen is a key initiator of platelet responses by not only serving as a substrate for platelet adhesion, but also by acting as a potent platelet agonist. Two major direct receptors have been implicated in the platelet responses to collagen: α2β1 and glycoprotein VI (gpVI). α2β1, a member of the integrin family, serves primarily to anchor platelets to subendothelial collagen exposed after vascular injury. Basically, gpVI-collagen interaction is weak, but provides a potent stimulus for intracellular signaling. As a result of this signaling, α2β1 is induced to bind collagen with high affinity thereby contributing to firm interaction between platelets and subendothelial collagen (Jung and Moroi 1998; Polanowska-Grabowska, Simon et al. 1999; Nieswandt, Brakebusch et al. 2001; Chen and Kahn 2003; Horii, Kahn et al. 2006).

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GPVI is predominantly a signaling receptor that belongs to the immunoglobulin (Ig) superfamily whose expression is restricted to platelets and megakaryocytes. Approximately, 4000–6000 copies of gpVI are present/platelet (Clemetson, Polgar et al. 1999; Jandrot-Perrus, Busfield et al. 2000). GPVI exists as a single transmembrane receptor and its ability to generate signals rests on the interaction between its transmembrane domain and immunoreceptor tyrosine-based activation domain (ITAM)-containing Fc receptor γ chain (FcRγ) (Kahn 2004). This gpVI- FcRγ chain interaction is critical for receptor stabilization and downstream signaling as mice deficient in FcRγ are unresponsive to collagen in part due to loss of signaling and in part because of their failure to express the receptor on the surface of their platelets (Poole, Gibbins et al. 1997; Tsuji, Ezumi et al. 1997; Kato, Kanaji et al. 2003). Collagen stimulation induces cross-linking of the gpVI- FcRγ chain complex resulting in sequential activation of Src and Syk family tyrosine kinases which further activates phosphatidylinositol 3-kinase β (PI3Kβ). This leads to phospholipase C γ2 (PLCγ2) activation, where PIP2 hydrolysis increases intracellular calcium and induces diacylglycerol (DAG) production. Both of these events are crucial for αIIbβ3 activation and subsequent platelet aggregation (Watson, Auger et al. 2005).

Platelets from humans with gpVI deficiency are nonresponsive to collagen (Sugiyama, Okuma et al. 1987; Kahn 2004) and gpVI polymorphisms are associated with increased risk of myocardial infarction (Yee and Bray 2004). Interestingly, inhibition or loss of gpVI prevented arterial thrombosis in animal models, but only mildly perturbed the normal hemostasis in both mice and

humans (Kahn 2004), making gpVI a suitable therapeutic target. A soluble gpVI-Fc fusion protein (Revacept) that blocks the gpVI-binding sites on exposed collagen suppresses murine arterial thrombosis without a bleeding phenotype. Revacept is currently being tested in phase II trials (Ungerer, Rosport et al. 2011).

1.1.3.2 G- Protein Coupled Receptors

1.1.3.2.1 Thrombin Receptors

Thrombin's effect on human platelets is mainly mediated by surface GPCRs called protease-activated receptor (PAR) 1 and 4 and GP1bα (described above).

1.1.3.2.1.1 PARs

PAR1 was the first identified protease-activated receptor and out of four known PARs, human platelets express PAR1 and PAR4, whereas, murine platelets express only PAR3 and PAR4. (Coughlin 1993; Kahn, Zheng et al. 1998; Coughlin 1999; Coughlin 2005). PAR1 is a prototype of this family, and accounts for the majority of cellular responses to thrombin. PAR1 is activated through proteolytic cleavage of its extracellular N-terminal, carboxyl to the R^{41} residue, to yield the tethered ligand sequence S^{42}FLLRN. This tethered ligand can bind intra-molecularly to the cleaved PAR1 receptor to initiate signaling (Vu, Hung et al. 1991; Macfarlane, Seatter et al. 2001). Proteolytic cleavage of the extracellular N-terminus of PAR4 yields the tethered ligand sequence GYPGQV. PAR1 or PAR4 activation can also be triggered by short synthetic peptides,

SFLLRN (PAR1) or AYPGKF (PAR4), mimicking the sequence of tethered ligand without any receptor cleavage (Vu, Hung et al. 1991; Scarborough, Naughton et al. 1992; Vassallo, Kieber-Emmons et al. 1992).

Among the PARs of human platelets, PAR1 is the high affinity thrombin receptor, in contrast to PAR4 that is a low affinity thrombin receptor that needs higher concentration of thrombin for activation. Furthermore, both PAR1 and PAR4 exhibit different kinetics of calcium mobilization: PAR1 activation induces a rapid increase in intracellular calcium, on the other hand, PAR4 triggers a more prolonged and sustained increase in intracellular calcium (Covic, Gresser et al. 2000; Jacques and Kuliopulos 2003; Coughlin 2005). Stimulation of PAR1 and PAR4 with its ligand activates a complex array of downstream pathways by coupling directly with G proteins; $G\alpha_q$, $G\alpha_i$ and $G\alpha_{12/13}$. Stimulation of the $G\alpha_q$ pathway activates phospholipase Cβ (PLCβ) that hydrolyzes the membrane-bound phosphatidylinositol 4,5- bisphosphate (PIP2) substrate into DAG and the secondary messenger inositol 1,4,5- triphosphate (IP3). IP3 binds to its receptors on the dense tubular system leading to an increase in cytosolic calcium concentration, while DAG activates protein kinase C (PKC). This signaling pathway initiates calcium-dependent cytoskeletal reorganization, leading to platelet shape change, aggregation and degranulation (Gabbeta, Yang et al. 1997; Offermanns, Toombs et al. 1997). Stimulation of the $G\alpha_{12/13}$ pathway activates Rho, RhoA, and p160ROCK pathways that lead to calcium-independent platelet shape change and dense granule release (Paul, Daniel et al. 1999).

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PAR1 plays a crucial role in platelet activation and thrombus stabilization and in accordance with this PAR1 antagonist, Vorapaxar and Atopaxar, were developed and clinical trials have been performed. Table 1.1 summarizes the clinical trial status as well as limitations of PAR1 antagonists.

1.1.3.2.1.2 ADP Receptors

Adenosine 5'-diphosphate (ADP) is a physiologic, endogenous platelet agonist critical for normal hemostasis and thrombosis. ADP is stored in platelet dense granules and is secreted upon platelet activation. Shear stress can also induce ADP release from erythrocytes. ADP amplifies platelet activation initiated by other agonists, and is vital for maximal platelet aggregation under high shear conditions. ADP acts in an autocrine/paracrine manner and binds two different G-protein coupled purinergic receptors, P_2Y_1 and P_2Y_{12} (Oury, Toth-Zsamboki et al. 2006). P_2Y_1, couples to $G\alpha_q$ and activates phospholipase Cβ (PLCβ) that triggers mobilization of intra-platelet calcium stores. This calcium flux initiates cytoskeletal reorganization leading to platelet shape change and aggregation (Ebbeling, Robertson et al. 1992; Jardin, Lopez et al. 2008). P_2Y_{12} is a $G\alpha_i$-coupled receptor that inhibits adenylate cyclase (AC) leading to reduction in the intracellular levels of the signaling messenger cyclic adenosine monophosphate (cAMP), while $G\beta_\gamma$ dimers activate phosphoinositide 3-kinase (PI3Kβ/γ). This regulates platelet aggregation via actin cytoskeleton-dependent integrin $\alpha IIb\beta 3$ activation (Chen, De et al. 2004). Continuous signaling through P_2Y_{12} is critical for persistent $\alpha IIb\beta 3$ activation and thrombus stability under shear flow conditions (Cosemans, Munnix

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et al. 2006). In accordance with their important role in initial platelet activation and thrombus formation, the ADP receptor (P_2Y_{12}) is the pharmacological target of many anti-platelet drugs in clinical practice. Table 1.1 gives the list of drugs targeting this receptor and their potential limitations.

1.1.4 Current anti-platelet therapies and their limitations

An exaggerated platelet response at sites of atherosclerotic plaque disruption leads to athrothrombosis, the underlying cause of myocardial infarction and ischemic stroke. It is no wonder then, that platelets are the main targets for therapeutic intervention. However, despite improvements in our understanding of the mechanisms contributing to thrombus formation and the presence of several anti-platelet therapies, athrothrombosis is still the leading cause of morbidity and mortality in the developed world (Alwan 2011; Jackson 2011).

All the existing anti-platelet agents work by inhibiting signaling mechanisms that amplify platelet activation (ADP and TxA_2 secretion), platelet aggregation ($\alpha IIb\beta3$), or phosphodiestrases (PDE) that degrade cyclic nucleotides (cAMP and cGMP) (Choi and Kermode 2011). Anti-platelet drugs aspirin (inhibits TxA_2) and clopidogrel (P_2Y_{12} receptor antagonist) alone have limited protection against arterial thrombosis (Awtry and Loscalzo 2000; Savi, Pereillo et al. 2000; Pereillo, Maftouh et al. 2002), but dual therapy with both drugs is the gold-standard anti-platelet therapy (Anderson, Adams et al. 2007; Anderson, Adams et al. 2013).

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While the combined administration of both drugs reduced vascular events (myocardial infarction or ischemic stroke) by ~ 25% in patients with CVD (Choi and Kermode 2011), the relative impact of this reduction is marginal due to the associated increased incidence of severe bleeding (Chen, Jiang et al. 2005; Choi and Kermode 2011). In fact, this is an inherent weakness with most anti-platelet drugs in clinical practice. Table 1 summarizes the available anti-platelet drugs, their target as well as the associated side effects and limitations or the current clinical trial status. It is ironic that the most potent anti-platelet drugs confers the greatest bleeding risk. The list is not exhaustive for all the current clinical trials, but it undoubtedly conveys that all anti-platelet drugs use independent approaches and independent approaches are in fact needed because each therapy has its own problems. *A new mode of affecting platelet reactivity may prove to offer unique advantages in a host of clinical settings.*

The platelet proteome is dynamic, with older denser platelets having decreased protein content. Proteasome inhibition with bortezomib is a key therapy for relapsing multiple myeloma, but what limits this therapy is associated thrombocytopenia (Lonial, Waller et al. 2005). This may be due to direct effect on platelets as bortezomib inhibits platelet aggregation *in vitro* (Avcu, Ural et al. 2008). A second proteasome inhibitor, PSI, prevented thrombosis in renovascular hypertensive rats, (Ostrowska, Wojtukiewicz et al. 2004). Platelets contain a functional 20S proteasome and its regulatory cap (Ostrowska, Ostrowska et al. 2003). Platelets should also contain a functional ubiquitin-proteasome system because collagen activation of gpVI stimulates ubiquitination

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of platelet Syk kinase

(Dangelmaier, Quinter et al. 2005) through the E3 ligase Cbl-b (Daniel, Dangelmaier et al. 2010).

Despite these fragmented observations, the proteasome of platelets is not established as a component of cell signaling or function. Based on this, the studies of this thesis examine the role of ubiquitin-proteasome system in modulating platelet function.

Table 1.1 Antiplatelet agents in the clinic or in clinical trials

Anti-platelet strategy	Drug	Mechanism(s) of action	Side effects and limitations or clinical trial status
Blockade of prostanoid biosyntesis (TxA$_2$)	Acetylsalicylic acid (aspirin)	Irreversible acetylation of cyclooxygenase 1 (COX-1), inhibiting generation of TxA$_2$	**Bleeding,** gastrointestinal toxicity Weak anti-platelet agent, ~25% prevalence of aspirin 'resistance'
GPIIb-IIIa inhibition	Abciximab (ReoPro)	Reversible inhibition of integrin$\alpha_{IIb}\beta_3$ activation, also blocks integrin $\alpha_v\beta_3$	**Bleeding,** thrombocytopenia Restrictions because of intravenous administration
	Eptifibatide (Integrilin)	Rapidly reversible, Arg-Gly-Asp (RGD) mimetic	**Bleeding**, small increase in profound thrombocytopenia Restrictions

Anti-platelet strategy	Drug	Mechanism(s) of action	Side effects and limitations or clinical trial status
			because of intravenous administration
P2Y$_{12}$antagonists	Tirofiban (Aggrastat)	RGD mimetic; rapidly reversible, minimal effects on $\alpha_v\beta_3$	**Bleeding,** severe but reversible thrombocytopenia in small numbers of recipients Restrictions because of intravenous administration
	Ticlopidine (Ticlid)	Active metabolite of parent compound irreversibly inhibits the ADP receptor P2Y$_{12}$	**Bleeding,** gastrointestinal toxicity, rash, neutropenia, thrombotic thrombocytopenic purpura (TTP) (largely replaced by clopidogrel

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Anti-platelet strategy	Drug	Mechanism(s) of action	Side effects and limitations or clinical trial status
			because of increased toxicity)
	Clopidogrel (Plavix)	Active metabolite of parent compound irreversibly inhibits $P2Y_{12}$	Rash, neutropenia, TTP, **major bleeding corresponding with >50% inhibition of $P2Y_{12}$.** Interpatient response variability
	Prasugrel (Effient)	Active metabolite of parent compound irreversibly inhibits $P2Y_{12}$; improved potency and consistency over clopidoogrel. Pro-drug	**Bleeding (more haemorrhagic side-effects than clopidogrel)**

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Anti-platelet strategy	Drug	Mechanism(s) of action	Side effects and limitations or clinical trial status
		metabolism is more efficient.	
	Ticagrelor (Brilinta)	Direct and reversible $P2Y_{12}$ antagonist Recently approved.	Indication is for use with aspirin. **Higher rate of major bleeding (compared to clopidogrel)**; increased dyspnoea and ventricular pauses
Agents that elevate camp and/or cGMP	Dipyridamole (Persantine)	Inhibitor of cyclic nucleotide phosphodiesterase (PDE), thromboxane synthase and adenosine deaminase. Also inhibits reuptake of adenosine; anti-platelet activity and	Headache, dizziness, hypotension, flushing, gastrointestinal toxicity, rash

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Anti-platelet strategy	Drug	Mechanism(s) of action	Side effects and limitations or clinical trial status
		vasodilatory properties	
	Cilostazol (Pletal)	Selective type 3 phosphodiesterase (PDE3) inhibitor, elevates cAMP to inhibit platelet aggregation; causes arterial vasodilation	**Bleeding; headache;** diarrhea; palpitations; dizziness; rash; pancytopaenia (~15% of individuals discontinue use because of side effects)
Thrombin receptor antagonists	SCH530348 (Vorapaxar)	High affinity, reversible PAR1 antagonist	Phase 2 trials, no major bleeding, generally well tolerated; phase 3 trial (**TRACER) recently halted (January 2011), potentially because of**

Anti-platelet strategy	Drug	Mechanism(s) of action	Side effects and limitations or clinical trial status
			unacceptable bleeding; second phase 3 trial (TRA 2P TIMI 50) scaled back for 25% of enrolled participants with a history of stroke. Further drug development is unclear
	E5555 (Atopaxar)	PAR1 antagonist	Currently undergoing phase 2 trials; results show a trend **to increase 'nuisance' bleeding,** safety issues regarding

Anti-platelet strategy	Drug	Mechanism(s) of action	Side effects and limitations or clinical trial status
			liver dysfunction and QT interval prolongation require addressing
Thromboxane antagonism	S-18886 (Terutroban)	Selective prostaglandin endoperoxide (TP) receptor antagonist: shows anti-thrombotic, anti-vasoconstrictive and anti-atherosclerotic properties	TAIPAD study, well tolerated, with a safety profile similar to aspirin; well tolerated when taken in combination with aspirin. Currently under evaluation in a phase 3 trial (PERFORM) for secondary prevention of acute thrombotic complications

Anti-platelet strategy	Drug	Mechanism(s) of action	Side effects and limitations or clinical trial status
Isoform selective inhibitors of PI 3-kinase	AZD6482	Isoform selective inhibitor of PI 3-kinase p110β	Phase 1 trials, 2009, acceptable safety and tolerability profile

Table 1.1 Anti-platelet drugs, their target as well as the associated side effects and limitations or the current clinical trial status. The associated major limitation with the anti-platelet drugs in clinical practice or in clinical trial, bleeding, is highlighted in bold letters. Adapted from (Jackson 2011).

1.2 Overview of ubiquitin-proteasome system

Cellular proteins are subjected to a variety of post-translational modifications that profoundly expand the functional repertoire and dynamics of the eukaryotic proteome. Proteins can be modified by the covalent addition of small molecules such as phosphate groups (phosphorylation), methyl groups (methylation), sugar groups (glycosylation), acetyl groups (acetylation) or entire proteins (Hochstrasser 2000; Pickart 2001; Xu and Peng 2006). The first such protein-based modification to be described was ubiquitin (Ub). Ubiquitin is a small 76- amino acid regulatory protein (~ 8.5 kDa) that is evolutionary conserved throughout eukaryotes (only 3 amino acid difference from yeast to human), but is absent from members of the other two super kingdoms, the eubacteria and the archae bacteria (Hershko and Ciechanover 1998; Pickart and Eddins 2004).

The covalent decoration of cellular proteins with Ub, known as ubiquitination, regulates a diverse array of biological processes, including signaling, protein quality control, organelle biogenesis, cell cycle regulation, DNA repair, transcription, inflammation, stress response, endocytosis and vesicular trafficking (Weissman 2001; Greene, Whitworth et al. 2005; Hurley, Lee et al. 2006; Kerscher, Felberbaum et al. 2006; Ulrich and Walden 2010). Ubiquitination of proteins is mediated through an enzymatic cascade that, in most cases, results in the conjugation of either single Ub on one (mono-ubiquitination) or multiple sites (multi-mono-ubiquitination), or multiple Ub monomers (poly-ubiquitination) to the internal lysine (Lys) of a substrate (Ravid and Hochstrasser

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2008). However, in rare instances Ub is conjugated to the N-terminus or the side chain of the cysteine (Cys) moiety of the substrate (Deshaies and Joazeiro 2009; Komander and Rape 2012). The generation of Ub linkages with distinct topologies confers diversity and versatility in the ways ubiquitination modulates various aspects of eukaryotic biology (Weissman 2001).

Ubiquitination is dynamic and reversible. Removal of Ub moieties, which may modulate Ub signaling, is carried out by a specific class of proteases called deubiquitinases (DUBs). DUBs hydrolyze the isopeptide linkages between Ub and the substrate or between multiple Ub moieties. DUBs therefore play critical roles in regulating the rate of protein turnover and in maintaining pools of free Ub by recycling it from existing conjugates (Amerik and Hochstrasser 2004). In mammalian cells, ~ 100 DUBs have been identified and classified in to five subfamilies (Wilkinson 1997; Reyes-Turcu, Ventii et al. 2009).

Among all the processes regulated by Ub, the best characterized is the role of poly-Ub chains in targeting the proteins for degradation by the 26S proteasome (Elsasser and Finley 2005; Miller and Gordon 2005). The 26S proteasome is a large multimeric, catalytic protease (2.5 MDa) that collaborates with the Ub system and ensures the precise, rapid and irreversible degradation of proteins tagged with poly-Ub chains. Like Ub, the proteasome is evolutionary conserved in eukaryotes, however, simpler forms are found even in archaebacteria and eubacteria. (Dahlmann, Kopp et al. 1989; Lupas, Zwickl et al. 1994; Coux, Tanaka et al. 1996; Demartino and Gillette 2007). The ubiquitin-

proteasome system (UPS) is responsible for much of the regulated protein degradation and maintenance of protein homeostasis. Approximately 80% of misfolded, oxidized, or damaged proteins and short lived regulatory proteins are degraded by UPS (Rock, Gramm et al. 1994; Lee and Goldberg 1998).

Given its essential role in regulating protein turnover, defects in the components of UPS has been implicated in the pathogenesis of many human diseases, including cardiovascular diseases, neurodegenerative disorders, viral diseases and numerous cancers (Herrmann, Ciechanover et al. 2004; Corn 2007; Petroski 2008; Hoeller and Dikic 2009; Lehman 2009).

1.2.1 Ubiquitin Conjugation System

The covalent conjugation of Ub to substrate is achieved by the concerted action of three enzymes: Ub activating enzyme E1, Ub conjugating enzyme E2, and Ub protein ligase E3 (Fig.1.7). Since ubiquitination controls diverse biological processes, not only in the cell, but also during the development of tissues and entire organisms (Mistry, Wilson et al. 2004; Pickart and Fushman 2004; Kerscher, Felberbaum et al. 2006; Yanjiang, Hongjuan et al. 2012), the whole process is tightly regulated in a spatial and temporal manner and displays specificity for the substrates it modulates.

In the first step, Ub activating E1 enzyme activates Ub in an ATP dependent manner by forming a high energy thioester bond between the E1 active site cysteine (Cys) and the carboxy terminus glycine76 (Gly76) residue of Ub substrate (Ciechanover, Finley et al. 1984; Hershko and Ciechanover 1998).

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Ub E1s (*Uba1* and *Uba6*) are multidomain enzymes that activate and transfer Ub to the active site of E2s. This is critical for cellular homeostasis because chemical inhibition of E1 activity in the cell results in the almost immediate shutdown of the entire UPS (Yang, Kitagaki et al. 2007). The E1 enzyme contains two active sites, an adenylation site that binds ATP-Mg^{2+} and activates C-terminal carboxyl group of Ub, and a catalytic cysteine that attacks the Ub adenylate bond to form a E1~Ub thioester.

In the second step, "activated" thioester-bound Ub is transferred from Ub-E1 to the active site Cys of one of a number of E2 Ub conjugating enzymes through a transthiolation reaction. The transfer of Ub from E1 to E2 catalytic cysteine residue involves conformational changes in the ubiquitin fold domain (UFD, E2 binding domain on E1) of E1 that facilitates the correct positioning of the active sites of the enzymes for efficient transfer (Huang, Hunt et al. 2007; Olsen and Lima 2013). The E2s are characterized by the presence of a highly conserved ~140 residue catalytic Ub-conjugating (UBC) fold that

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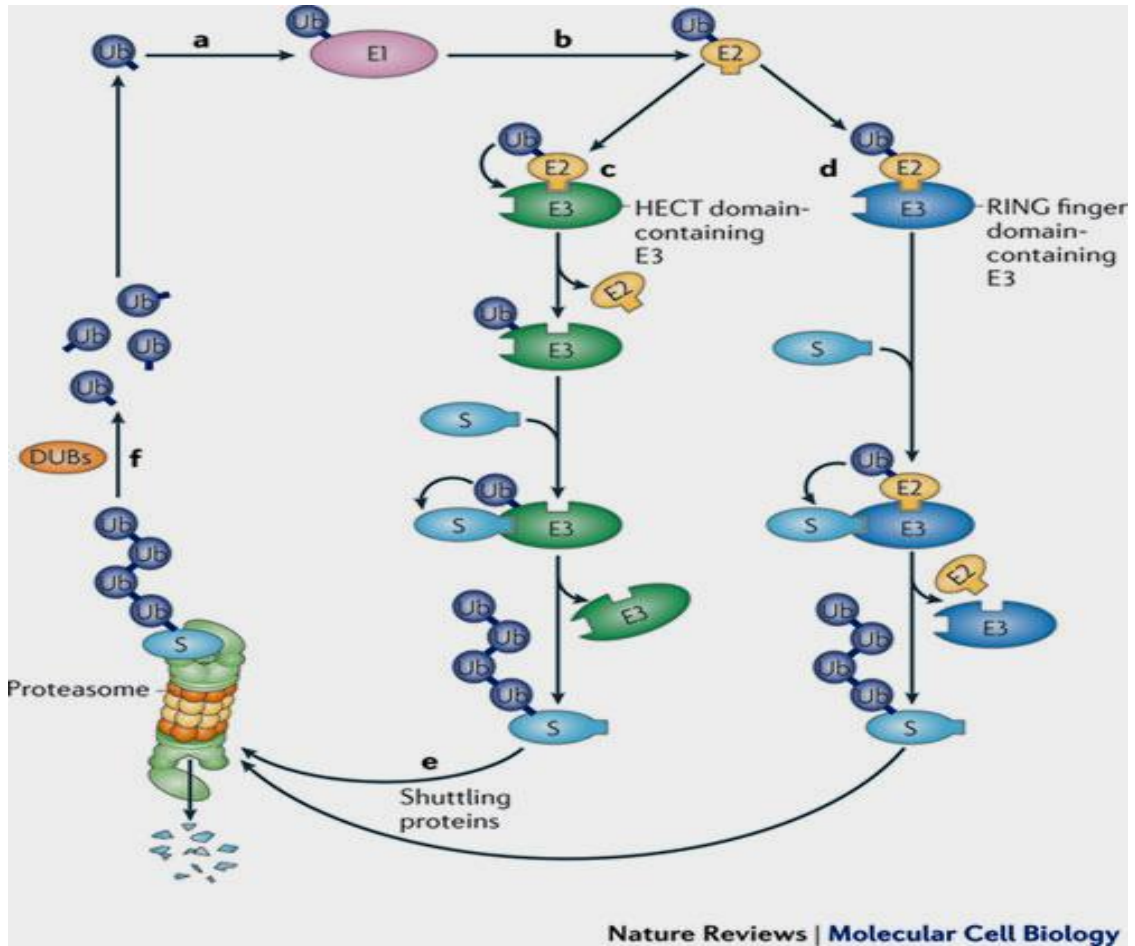
Figure 1.7 Ubiquitin- proteasome system

An overview of ubiquitination pathway. **a)** Ub is activated by an E1 in a ATP dependent manner and **b)** Ub is transferred to an E2 enzyme through a transthiolation reaction. Depending upon the type of E3 ligase involved, the ubiquitin is sequentially transferred from E2-E3- Substrate (S) **c)** HECT E3s or E2-Ub is directly transferred to substrate, where E3 acts as scaffold **d)** RING E3s, **e)** polyubiquitinated proteins are targeted to proteasome for degradation, while **f)** DUBs replete the Ub pool. adapted from (Weissman, Shabek et al. 2011).

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consists of 5 α-helices and 4 anti-parallel β-sheets. In the catalytic groove near the active site Cys is a "HPN" (histidine, proline and asparagine) motif that facilitates both thioester formation between "activated" Ub from E1 and the active site Cys of E2, and substrate ubiquitination. Both histidine and proline may play a role in supporting the protein structure around the active site, where as asparagine is critical for promoting isopeptide bond formation between Ub and a substrate lysine (Wu, Hanlon et al. 2003; Eletr, Huang et al. 2005; Wenzel, Stoll et al. 2011). The E2 Ub conjugating enzyme then catalyzes substrate modification in conjunction with a substrate-specific E3 Ub ligase. E2s play an important role in determining Ub chain length as well the linkage specificity and since both these factors govern the cellular fate of the target protein, E2s are viewed as important regulators of Ub signaling that interact with select E3 proteins rather than just carriers of activated Ub (Olsen and Lima 2013).

The E3 Ub ligases can be classified into three major types based on their catalytic mechanism: the Homologous to E6-associated protein C-terminus (HECT) E3s, really interesting new gene (RING) domain E3s, and structurally related U-box-type E3s. The HECT E3 first loads the ubiquitin from the E2 to their active site Cys and then shuttles it to the substrate (Bernassola, Karin et al. 2008). On the other hand, the RING finger domain and U-box-type E3s act as scaffolds that brings Ub~E2 and substrate in proximity to facilitate Ub transfer from Ub~E2 to substrate (Lipkowitz and Weissman 2011). However, this mechanistic distinction between HECT and RING E3s has become blurred with the identification of RING subfamily of RING-in-between-RING (RBR) E3 ligases

that act as RING/HECT hybrids i.e. they form a thioester~Ub intermediate via a conserved RING domain Cys prior to Ub transfer to substrate (Wenzel, Lissounov et al. 2011).

In mammalian cells, there are two known E1s, approximately 30-40 E2s and more than 600 E3s, making E3s, either alone or in association with its bound E2, the main specificity and selectivity factor of the UPS (Michelle, Vourc'h et al. 2009; Schulman and Harper 2009; Voutsadakis 2010).

1.2.2 Ubiquitin Code

A single round of the E1-E2-E3 enzymatic cascade results in mono-ubiquitination of substrate i.e. a single Ub moiety binds to the substrate. Additional rounds have diverse outcomes: Multi-mono-Ub chains- formed when several single Ub moieties binds to the substrate at multiple sites and/or Poly-Ub chains- formed through the sequential addition of several Ub moities to the previously attached Ub. Poly-Ub chains can be homotypic, using the same lysine residue of incoming Ub moiety or heterotypic, using more than one linkage type. Heterotypic chains are either branched i.e. formed by ubiquitination of one Ub at two or more sites or non-branched. The Ub amino terminal Met1 residue (M1) can also participate in the Ub chain formation (Ikeda and Dikic 2008; Dikic, Wakatsuki et al. 2009; Komander and Rape 2012). All eight Ub-Ub linkages have been found to coexist i*n vivo* (Peng, Schwartz et al. 2003; Xu, Duong et al. 2009).

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Ub attachment to a target protein is more than just a terminal reaction, as both the length (mono-vs. poly-Ub chain) and the linkage type (homotypic or heterotypic) can regulate the fate, stability, activity and subcellular localization of the modified substrates (Pickart and Fushman 2004; Li and Ye 2008).

The canonical view is that poly-ubiquitination at Lys48 (of at least four Ub moieties) tags a protein for proteasome-mediated degradation (Komander and Rape 2012), whereas poly-ubiquitination at Lys63 is non-degradative and plays essential roles in DNA repair, DNA replication and signal transduction (Chen and Sun 2009). Unlike Lys48 and Lys63 chains, not much is known about "atypical chains" that are linked via Lys6, Lys11, Lys27, Lys29, Lys33 and Met1. Current known functions of mono and multi-mono-Ub chains as well as atypical chains are summarized in Fig. 1.8**.**
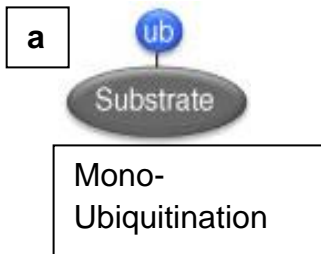
a Mono-Ubiquitination	Histone regulation (Roest, van Klaveren et al. 1996; Robzyk, Recht et al. 2000), Endocytosis (Shih, Sloper-Mould et al. 2000; Hicke 2001), Viral Budding, DNA repair and Nuclear Export (Boyd, Tsai et al. 2000; Geyer, Yu et al. 2000; Brooks and Gu 2006).
b Multi-mono-Ubiquitination	Endosomal Trafficking (Strous and van Kerkhof 2002; Huang, Kirkpatrick et al. 2006), p53 nuclear transport (Li, Brooks et al. 2003).
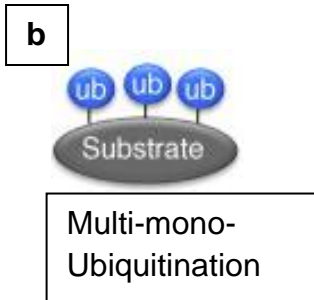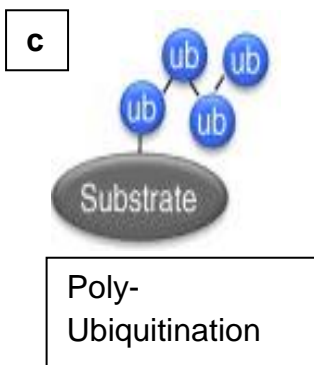
	Lys6	DNA damage response. Reviewed in (Kulathu and Komander 2012).
c Poly-Ubiquitination	Lys11	Cell cycle regulation, Membrane Trafficking, Endoplasmic reticulum associated degradation (ERAD), TNFα signaling. Reviewed in (Kulathu and Komander 2012).
	Lys27	Mitochondrial maintenance or Mitophagy (Geisler, Holmstrom et al. 2010; Glauser, Sonnay et al. 2011), Nuclear translocation (Peng, Zeng et al. 2011) .
	Lys29	Ubiquitin-fusion degradation (Hwang, Shemorry et al. 2010; Metzger and Weissman 2010).
	Lys33	T-Cell receptor signaling (Huang, Jeon et al. 2010).
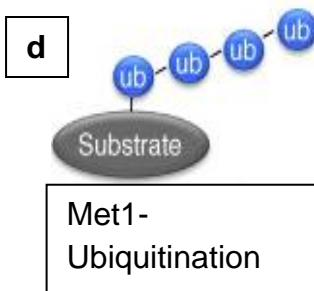
d Met1-Ubiquitination	Nuclear factor-κB (NF-κB) signaling (Iwai and Tokunaga 2009), Cell cycle regulation (Wickliffe, Williamson et al. 2009).

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Figure 1.8 The Ub code

Ub is usually conjugated to the ε-amino group of a Lys residue in a substrate, however, Ub itself possess seven lysines residues each of which, and Met1, can participate in chain formation and generates ubiquitin linkages of different topologies. This figure summarizes the type of ubiquitination and cellular fate of the protein. **a)** Mono-ubiquitination, **b)** Multi-mono- ubiquitination, **c)** Poly-ubiquitination and **d)** Met1 ubiquitination.

1.2.3 The proteasome- a degradation nanomachine

The 26S proteasome is an intricate proteolytic machine that plays an indispensible role in the degradation of misfolded, damaged or regulatory proteins decorated with Ub. The proteasome is a large multimeric complex that is composed of a 20S catalytic core particle (CP) flanked by one or two 19S regulatory particles (RPs, Figure 1.9) (Coux, Tanaka et al. 1996; Baumeister, Walz et al. 1998; Tanaka 2009).

1.2.3.1 20S catalytic core particle

The catalytic 20S core particle is the business center of proteasome. It is assembled into a cylindrical structure formed by axial stacking of 4 hetero-heptamaric rings. Each ring contains 7 different homologous α-subunits and 7 different β-subunits (Unno, Mizushima et al. 2002). The catalytic activities of the 20S core particle are associated with β-subunits with β1-subunit having caspase-like (cleaves after acidic residues), β2-subunit having tryptic-like (cleaves after basic residues) and β5-having chymotrypsin-like activity (cleaves after hydrophobic residues) (Orlowski 1990; Kisselev, Akopian et al. 1999). The combination of three different peptidase activities confers to the 20S core particle the ability to degrade a broad variety of peptide sequences with high efficiency and specificity. The inactive β-subunits are required for proper assembly and maturation of catalytically active β-subunits (Arendt and Hochstrasser 1999). Following degradation, the length of majority of the newly created peptides range

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from 3-15 amino acids that are then hydrolyzed to amino acids by oligopeptidases and/or amino carboxyl peptidases (Glickman and Ciechanover 2002; Tanaka 2009) or the short peptides are presented to class I MHC molecules for antigen presentation to the immune system (Saeki and Tanaka 2012).

Outer α-rings serve as a gated channel to regulate the substrate entry into the catalytic core. The substrate bound for degradation must pass through this channel, lined by α2-, α3- and α4-subunits, to enter the catalytic chamber (Zwickl, Grziwa et al. 1992; Lowe, Stock et al. 1995). The outer α-rings provide binding sites for the 19S regulatory particle and their interaction results in a conformational change in the α-subunits that allow entry of the protein substrate into the central chamber of the 20S core particle (Smith, Chang et al. 2007).

1.2.3.2 19S regulatory particle (19S RP)

The 19S regulatory particle is a ~900 kDa complex that plays an essential role in the recognition, deubiquitination, unfolding of ubiquitinated substrate and in translocation of the unfolded substrate into the 20S core particle for degradation (Coux, Tanaka et al. 1996; Baumeister, Walz et al. 1998; Tanaka 2009). The 19S regulatory particle contains at least 19 subunits that can be subdivided into two groups: regulatory particle of non-ATPase (Rpn) and regulatory particle of triple-ATPase (Rpt). Biochemical experiments have organized the 19S RP into two sub-complexes: the lid and the base (Lander, Estrin et al. 2012).

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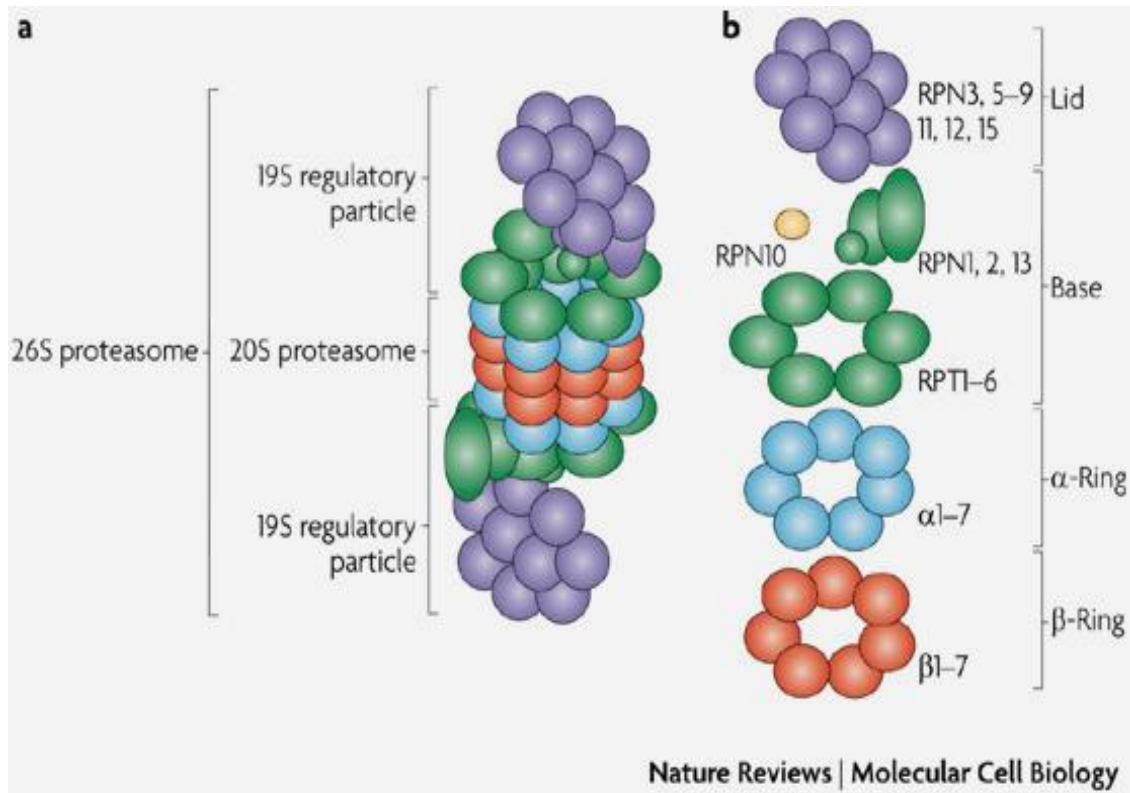
Figure 1.9 The 26S proteasome

a) The 26S proteasome is comprised of 20S core particle flanked by two 19S regulatory particles. **b)** Individual subunit composition of 26S proteasome. The 19S regulatory particle is organized into two sub-complexes, lid and base, and the catalytic 20S core is arranged as a cylindrical barrel made up of 2 outer α- and 2 inner β-rings. Adapted from (Murata, Yashiroda et al. 2009).

The lid is composed of 9 non-ATPase subunits (Rpn3, Rpn5–Rpn9, Rpn11, Rpn12, and Rpn15). The lid subunits, Rpn3, Rpn5, Rpn6, Rpn7, Rpn9 and Rpn12 contain protein–protein interaction motifs called PCI [proteasome–CSN (COP9 signalosome)–eIF3 (eukaryotic translation initiation factor 3)] and function as scaffolding subunits (Finley 2009). The best established function of the lid is deubiquitination. Rpn11, which is a JAB1–MPN–MOV34 (JAMM) domain containing DUB, tightly couples deubiquitination and substrate degradation (Bar-Nun and Glickman 2012). Rpn8 acts as a non-catalytic binding partner of Rpn11 (Finley 2009).

The base contains six ATPases (Rpt1-Rpt6), two large scaffolding subunits (Rpn1 and Rpn2) and two Ub receptors (Rpn10 and Rpn13) that efficiently trap Ub-conjugated substrates (Finley 2009). The 6 triple-ATPases form a hetero-hexameric ring with the specific order of Rpt1-Rpt2-Rpt6-Rpt3-Rpt4-Rpt5 to constitute the molecular motor of proteasome (Tomko, Funakoshi et al. 2010). The ATPase subunits provide the energy needed for deubiquitination, substrate unfolding, and translocation of the substrate through the narrow pore into the degradation chamber (Martin, Baker et al. 2008; Maillard, Chistol et al. 2011). The binding of C-terminus tails of ATPases Rpt2, Rpt3 and Rpt5 on dedicated sites on the 20S core particle (inter-subunit pockets formed by α-subunits of the outer ring) triggers gate opening and facilitating substrate entry (Smith, Chang et al. 2007; Gillette, Kumar et al. 2008).

The major activity of the lid and the base is proposed to be deubiquitination. In mammalian cells, two DUBs, USP14 and UCHL5, are associated with the base complex and they progressively trim poly-Ub chains from the distal end of the chains. USP14 is associated with Rpn1 (Hu, Li et al. 2005) and UCHL5 binds to Rpn2 via Rpn13 (Hamazaki, Iemura et al. 2006; Yao, Song et al. 2006).

1.2.4 Deubiquitination

DUBs are isopeptidases that play pivotal roles in Ub-mediated signaling pathways. They are classified into six subclasses: Ub C-terminal hydrolases (UCHs), Ub specific proteases (USP's), ovarian tumor proteases (OTUs), Josephines, JAB1/MPN/Mov34 metalloenzyme (JAMM) and monocyte chemotactic protein-induced protein (MCPIP). Except for JAMM proteases, which are Zn^{2+} metalloproteases, the rest are cysteine proteases (Fraile, Quesada et al. 2012; Lim and Baek 2013).

DUBs hydrolyze isopeptide bonds in Ub-protein conjugates and are vital for Ub homeostasis. General cellular roles of DUBs are summarized in Fig. 1.10. DUBs regulate ubiquitination in a spatio-temporal manner. DUBs can pair with E3s ligases, which have inherent autoubiquitination activity, in order to prevent their self-ubiquitination and degradation, e.g. USP8 mediated stabilization of neuregulin receptor degradation protein 1 (Nrdp1) ligase induces degradation of its substrate ErbB3 (Cao, Wu et al. 2007). The DUB-E3 pairing can also fine tune editing of one Ub signal for another e.g. cellular inhibitor of apoptosis (cIAP)

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ubiquitin ligases ubiquitinate receptor-interacting serine-threonine kinase 1 (RIPK1) at Lys63, but in order to turn off the signaling, A20 depolymerize Lys63 chains and facilitate the polymerization of Lys48-linked chains on RIPK1 resulting in its degradation (Hymowitz and Wertz 2010).

DUBs display specificity at multiple levels as evident by their ability to distinguish between different Ub chain linkages and chain structure (Komander, Clague et al. 2009). Different subclasses have preference for specific chain linkages e.g. USP14, OTUB1 and A20 process Lys48-linked chains, where as JAMMs and CYLD prefer Lys63- linked chains (Fraile, Quesada et al. 2012). DUBs also modulate protein stability by removing the degradative signal from proteins meant for degradation or even reverse Ub signaling by removing even non-degradative signal.

As mentioned before, three DUBs directly associate with 19S regulatory particles and their activity can prevent degradation of protein even at the face of the proteasome (Guterman and Glickman 2004). The three DUBs associated with 19S regulatory particle are Rpn11, USP14 and UCHL5. Rpn11, a JAMM metalloprotease, is a constituent subunit of 19S RP and is responsible for bulk of proteasome DUB activity. Rpn11 prefers Lys63- linked chains and cleaves at the base of the Ub chain resulting in the release of entire poly-Ub chain (Shen, Schmitt et al. 2013). USP14 is associated with Rpn1 subunit of 19S RP and this association increases its activity by several hundred fold (Hu, Li et al. 2005). USP14 prefers Lys48- linked chains and to cleave at the distal end or within the

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polymeric chain. USP14 is crucial for Ub recycling as inhibition of USP14 leads to increased Ub degradation resulting in the reduction of monomeric Ub. Unlike Rpn11, USP14 is not a constituent subunit of proteasome and may be involved in other cellular processes (Hanna, Hathaway et al. 2006; Koulich, Li et al. 2008; Shen, Schmitt et al. 2013). UCHL5 binds to 19S RP subunits, Rpn2 via Rpn13, and this association is required for its full activation (Hamazaki, Iemura et al. 2006; Yao, Song et al. 2006). An excellent example of temporal regulation of Ub signaling is UCHL5 that catalyzes nucleosome sliding by pairing with the inositol-requiring 80 (INO80) chromatin-remodeling complex (Grabbe, Husnjak et al. 2011).
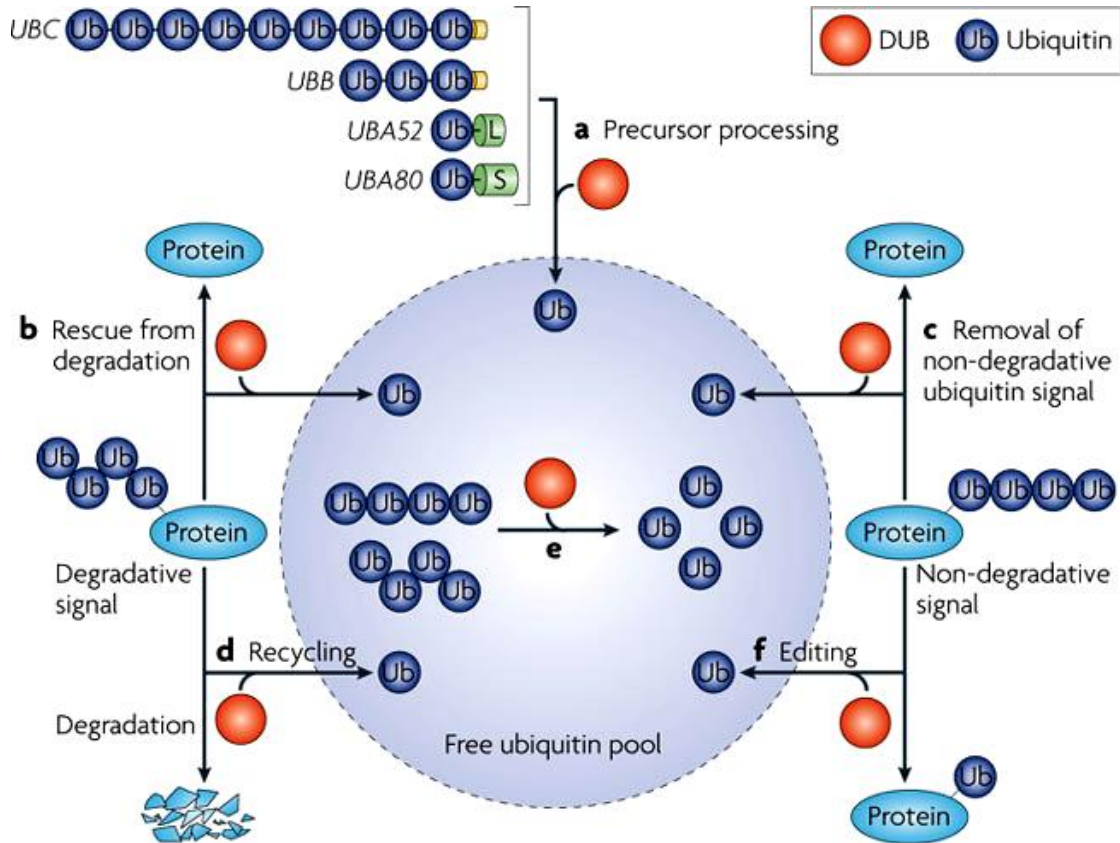
Figure 1.10 General functions of DUBs

DUBs **a)** cleave Ub from its precursor molecules and generates active single Ub molecules b**)** Rescue proteins destined for degradation or **c)** remove non-degradative regulatory signal from the protein **d)** are crucial for Ub homeostasis as they remove Ub from a substrate before its degradation **e)** maintain free Ub pool by removing Ub from unanchored poly-Ub chains **f)** might edit Ub chains by swapping one signal for another and hence change the cellular fate of the target substrate. Adapted from (Komander, Clague et al. 2009).

1.2.5 Targeting UPS

Regulated proteolysis by UPS controls almost all aspects of cellular physiology and it is no wonder that defects in the components of UPS have been implicated in the pathogenesis of several human diseases (Herrmann, Ciechanover et al. 2004; Corn 2007; Petroski 2008; Hoeller and Dikic 2009; Lehman 2009; Schlossarek, Frey et al. 2014). The incentives of targeting this intricately regulated system seems obvious, and the expedited approval of bortezomib (PS-341, Velcade ®; Millennium Pharmaceuticals), to become first FDA approved proteasome inhibitor in clinical practice, for the treatment of multiple myeloma and mantle cell lymphoma has corroborated UPS and its components as legitimate therapeutic targets. (Kane, Bross et al. 2003; Fisher, Bernstein et al. 2006).

Fast track approval of an improved proteasome inhibitor that overcomes bortezomib resistance and has a superior toxicity profile, [Carfilzomib (Kyprolis, Onyx Pharmaceuticals)], for multiple myeloma by the FDA and several orally bioavailable proteasome inhibitors in clinical trials clearly demonstrate the effectiveness of targeting this pathway (Kuhn, Chen et al. 2007; Herndon, Deisseroth et al. 2013; Shen, Schmitt et al. 2013). Given the success of proteasome inhibitors in the treatment of cancer and advances in our understanding of UPS and its components, several small molecule inhibitors have been developed or are in the pipeline that target specific components of this pathway e.g. MLN4924 (NAE, Millenium). This is a small molecule inhibitor for E1 enzyme of a Ub like protein NEDD8, and is in a phase 2 clinical trial

(Soucy, Smith et al. 2009). Several small molecules targeting E3 ligases are either in Phase I clinical trials or are in the preclinical phase (Shen, Schmitt et al. 2013). DUB inhibitors are recent and a USP7/HAUSP inhibitor is already in active preclinical development owing to its role in p53 regulation (Shen, Schmitt et al. 2013). A novel small molecule inhibitor b-AP15 that reversibly targets proteasomal DUBs, USP14, and UCHL5, has anti-cancer properties and it even induced cytotoxicity in bortezomib-resistant cells (D'Arcy, Brnjic et al. 2011).

Taken together, targeting UPS for human therapy seems promising, and small molecule inhibitors targeting specific and smaller portions of UPS pathway have shown encouraging results. Platelets also have components of UPS (Burkhart, Vaudel et al. 2012) system, but the exact role of UPS in modulating platelet function is not clear. However, older platelets have a reduced proteome compared to newly released platelets suggesting a role of proteasome in platelets (Ault, Rinder et al. 1992).

The studies in this thesis were conducted using small molecule inhibitors targeting different components of the Ub-proteasome pathway in order to gain mechanistic understanding of the potential role of this indispensible system in modulating platelet reactivity.

CHAPTER II

Proteasome proteolysis supports stimulated platelet function and thrombosis

2.1 Introduction

Proteasome inhibitors are widely used for the treatment of hematologic cancers, specifically relapsed/refractory multiple myeloma and mantle cell lymphoma, (Kane, Bross et al. 2003; Adams 2004; Kane, Dagher et al. 2007) yet little is known of their impact on platelet function and hemostasis. Potentially, these agents may also target platelets because a cyclic course of the proteasome inhibitor bortezomib (Velcade®) induces significant cyclic thrombocytopenia, (Lonial, Waller et al. 2005) and proteasome inhibition hastens platelet death and turnover (Nayak, Kulkarni et al. 2013). Bortezomib therapy also associates with reduced thrombosis (Zangari, Guerrero et al. 2008; Shen, Zhou et al. 2011). Beyond this, there is little evidence of proteasome action on the platelet proteome, their activation, or on thrombosis.

Platelets are recruited to the vascular wall at high shear through the GPIb-IX-V receptor complex, (Goto, Ikeda et al. 1998) where the GPIbα subunit binds von Willebrand factor (VWF) (Kroll, Harris et al. 1991) while its C-terminal

cytoplasmic domain interacts with the actin binding protein Filamin A with high affinity (Ithychanda, Hsu et al. 2009). This interaction tethers the receptor complex to the platelet cytoskeleton, maintaining the cytoskeletal architecture of resting platelets and those adhering in vessels at high shear rates (Cranmer, Ashworth et al. 2011). The GPIbα subunit also contains a high affinity binding site for thrombin that contributes to platelet activation when thrombin concentrations are low (Greco, Tandon et al. 1996). These interactions are essential for thrombosis (Guerrero, Shafirstein et al. 2008). Thrombotic platelet deposition is modeled in mice by $FeCl_3$ induced injury of carotid arteries (Li, Febbraio et al. 2010; Li, McIntyre et al. 2013) resulting in rapid platelet adhesion and formation of an occlusive platelet-rich thrombus at the site of injury.

Nucleated cells remove proteins from their proteome by proteasome-catalyzed proteolysis. This multimeric complex consists of a 20S catalytic core of non-catalytic α-subunits and three distinct β-subunits that hydrolyze peptide bonds of unfolded protein substrates by β1 caspase-like hydrolysis, β2 tryptic hydrolysis, and β5 chymotryptic cleavage (Chen and Hochstrasser 1996; Arendt and Hochstrasser 1997; Dick, Nussbaum et al. 1998; Nussbaum, Dick et al. 1998). The proteasome is capped by a 19S regulatory subunit that conducts substrate recognition, deubiquitination, unfolding, and protein translocation into the 20S core (Dahlmann, Ruppert et al. 2000; Dahlmann, Ruppert et al. 2001) Substrates for proteasome hydrolysis are recognized by the 19S core through the covalent conjugation of monomeric or polymeric chains of the ~8 kDa ubiquitin to the targeted protein (Nalepa, Rolfe et al. 2006; Jacobson, Zhang et

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al. 2009; Xu, Duong et al. 2009).

Platelets contain several components of the 20S proteasome core (Yukawa, Sakon et al. 1993; Ostrowska, Wojcik et al. 1997; Ostrowska, Ostrowska et al. 2003; Avcu, Ural et al. 2008) and possess at least the chymotryptic activity of the proteasome (Nayak, Kumar et al. 2011). In addition, platelets have components of UPS (Burkhart, Vaudel et al. 2012) system, but the exact role of UPS in modulating platelet function is not clear. Platelets should contain functional E1 ubiquitin activating activity and E2/E3 ligases because collagen activation of gpVI stimulates ubiquitination of platelet Syk kinase (Dangelmaier, Quinter et al. 2005) through the E3 ligase Cbl-b (Daniel, Dangelmaier et al. 2010). Furthermore, clinical inhibitor of proteasome (bortezomib) reduced ADP-induced aggregation (Avcu, Ural et al. 2008) and platelets isolated from patients receiving bortezomib therapy are hyporesponsive to other stimuli (Zangari, Guerrero et al. 2008; Shen, Zhou et al. 2011). Moreover, older platelets have a reduced proteome compared to newly released platelets suggesting a role of proteasome in platelets (Ault, Rinder et al. 1992).

Here we show the expression of a functional ubiquitin/ proteasome system in platelets. By investigating the impact of proteasome inhibition on a well established mouse model of thrombosis and on a range of *ex vivo* activities, we conclusively demonstrate that the platelet proteasome contributes to cellular activation and function. Therapeutic proteasome inhibition in platelets produces a hypothrombotic state, and might also augment anti-platelet therapy.

2.2 Materials and Methods

2.2.1 Chemicals and reagents

Chemicals and reagents were purchased from: sterile filtered hanks balanced salt solution (HBSS) and sterile tissue culture plates (Falcon Labware); endotoxin-free human serum albumin (25% human albumin solution, Baxter Healthcare); endotoxin-free PBS, phenol-extracted LPS (*Escherichia coli* O111:B4) free of lipoprotein contamination (List Biological Laboratories); recombinant soluble CD14, recombinant lipopolysaccharide-binding protein (LBP), Allophycocyanin (APC)-anti-human vWF (R&D Systems); anti-polyubiquitin antibodies FK1 and FK2, anti-UBE1, UbiQapture™-Q kit (Enzo lifesciences); anti-Filamin A, anti-Talin1 (C45f1), anti-β-actin and anti-skp1 (Cell signaling); anti-Cul5 and anti-Asb2 (Abcam); anti-talin 1(YQ 16), anti-GPVI, anti-p53 and SZ2 antibody (SCBT); p53 ubiquitination kit, anti-UBcH 1,5 and 7, anti-RPN2 and anti-11S-α subunit (Boston Biochem); MG132, AYPGKF (PAR4 agonist peptide), SFLLRN (PAR1 agonist peptide) and RWJ56110 (Tocris); Bortezomib (Eurasia); Antichrome TF® (American Diagnostica); Phycoerytherin (PE)-anti-human CD42b (Millipore); fluorescein isothiocyanate (FITC)- CD36 (Cayman Chemicals); Calcein AM and Fura-2 AM (Invitrogen™); Ristocetin and Botrocetin (Sigma-Aldrich). Other chemicals were from Sigma-Aldrich or Biomol Research Laboratories.

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2.2.2 Platelet preparation

Human blood was drawn into acid-citrate-dextrose and centrifuged (200 × *g*, 20 min) without braking to obtain platelet-rich plasma in a protocol approved by the Cleveland Clinic Institutional Review Board. Purified platelets were prepared as stated (Brown and McIntyre 2011). Briefly, platelet-rich plasma was filtered through two layers of 5-µm mesh (BioDesign) to remove nucleated cells and recentrifuged (500 × *g*, 20 min) in the presence of 100 nM PGE_1. The pellet was resuspended in 50 ml PIPES/saline/glucose (5 mM PIPES, 145 mM NaCl, 4 mM KCl, 50 µM Na_2HPO_4, 1 mM $MgCl_2$, and 5.5 mM glucose) containing 100 nM PGE_1. These cells were centrifuged (500 × *g*, 20 min) and recovered platelets were centrifuged again before resuspension in 0.5% human serum albumin in HBSS. For pure platelets, cells were resuspended in AutoMACS sample buffer, 5 µl anti-CD45, anti-CD15, anti-CD14, and anti-glycophorin–coated magnetic beads (Miltenyi Biotec) per 10^9 cells for 25 min with constant rotation before purification in an AutoMACS magnetic separator (Miltenyi Biotec).

2.2.3 Proteasome function

Proteasome-Glo™ 3-Substrate System (Promega) separately assayed hydrolysis of luminogenic substrates for the three proteasome activities [chymotryptic (Suc-LLVY-aminoluciferin), tryptic (Z-LRR-aminoluciferin), and caspase-like or postglutamyl peptide hydrolase (Z-nLPnLD-aminoluciferin)]. Washed platelets (4 X 10^8/ml) were pretreated with or without 30 µM MG132 for 30 min, pelleted and lysed using NP-40 lysis buffer (150 mM NaCl, 25 mM Tris [pH 7.6], 1% nonidet P-40, 2 mM EDTA, 1 mM sodium orthovanadate, 10 mM

sodium fluoride, 100 µM PMSF, 1 µg/mL pepstatin, and 10 µg/mL leupeptin to inhibit non-proteasome proteases) and incubated with luminogenic substrates for 30 min before luminescence was measured. Addition of protease inhibitors make sure that cellular proteases don't cleave the substrate and only

2.2.4 *In vivo* thrombosis

Twelve week old C57BL6 mice were anesthetized with ketamine (90 mg/kg)/xylazine (15 mg/kg) and the right jugular vein and the left carotid artery were exposed via a middle cervical incision. Platelets were labeled by injecting 100 µl of rhodamine 6G (0.5 mg/ml) in saline into the right jugular vein. MG132 (30 µM in DMSO; 6 mg/kg) or DMSO vehicle (10.5 µl) was added to the above solution, and the drug was allowed to circulate for 15 min before $FeCl_3$ injury (Li, Febbraio et al. 2010; Li, McIntyre et al. 2013). Thrombosis was induced in the left carotid artery by stripping the adventitia and placing a piece of black plastic under the vessel to reduce background fluorescence. A 1 X 2 mm piece of filter paper saturated with 7.5% $FeCl_3$ was applied to the carotid artery for 1 minute, the filter paper was removed, and the vessel rinsed with saline. Fluorescent thrombus formation was observed in real-time under a water immersion objective at 10 X magnification. Time to occlusive thrombosis was determined offline using video image capture with a QImaging Retigo Exi 12-bit mono digital camera (Surrey, Canada) and Streampix version 3.17.2 software (Norpix, Montreal, Canada). The end points were set as either cessation of blood flow for >30 seconds or no occlusion after 30 minutes (three times longer than the average

occlusion time), in which case the time was recorded as 30 minutes for statistical comparison.

2.2.5 Platelet transfusion and murine carotid artery thrombosis assay

Eight week old female platelets donor mice were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg) and 700 µl blood was obtained from inferior vena cava puncture into 1 ml syringe containing 100 µl sodium citrate (0.109 M). Blood was transferred into a 2 ml tube containing 800 µl Tyrode's buffer and platelet-rich plasma (PRP) were gathered by centrifugation at 100g for 10 minutes. PRP was further centrifuged at 100g for 10 minutes in the presence of PGE_1 to pellet platelets. 10^9 platelets resuspended in 500 µl saline were treated with MG132 (30 uM) or vehicle for 30 min, centrifuged and re-suspended in 200 µl saline.

Recipient wild type mice were exposed to 11Gy of external beam irradiation from a Cesium 137 source to induce thrombocytopenia with platelet counts <5% of normal after 5~6 days (Chen, Febbraio et al. 2008). At sixth days after irradiation, the thrombocytopenic WT mice (8 weeks old) were anesthetized as before and the right jugular veins and the left carotid artery exposed via a middle cervical incision (Li 2013). The pre-treated donor platelets were then fluorescently stained by adding 100 µl of rhodamine 6G solution (0.5 mg/ml in saline), and injected through the jugular vein of thrombocytopenic mice 10 minutes prior to carotid injury to allow the transfused platelets to reach equilibrium in the circulation. Vehicle DMSO treated platelets were used as

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controls. Details of this thrombosis model have been described previously (Li 2013).

2.2.6 p53 ubiquitination

Washed platelets (10^9/ml) were lysed in NP-40 lysis buffer and incubated with components from a p53 ubiquitination kit (Boston Biochem), but lacking recombinant E1, for 60 min before the reaction was stopped with 2X reducing SDS sample buffer. The products were resolved by SDS-PAGE, and p53 ubiquitination status determined by western blotting with anti- p53 antibody.

2.2.7 Western blotting and liquid chromatography-mass spectrometry

Washed platelets (4 X 10^8/ml) were treated with 30 µM MG132 or buffer for 30 min before treatment with thrombin (0.2 U) or ADP (100 µM). After incubation, platelets were pelleted and lysed using radioimmunoprecipitation assay (RIPA) lysis buffer [150 mM NaCl, 25 mM Tris (pH 7.6), 1% Nonidet P-40, 0.1% sodium dodecyl sulfate, 0.5% deoxycholate, 2 mM EDTA, 1 mM EGTA, 1 mM sodium orthovanadate, 10 mM sodium fluoride, 100 µM phenylmethylsulfonyl fluoride, 1 µg/mL pepstatin, and 10 µg/mL leupeptin]. Samples were kept on ice for 30 min with occasional vortexing to ensure complete lysis before centrifugation (21,000 x g, 10 min). Reducing SDS sample buffer was added before the proteins were resolved by SDS-PAGE.

Three different LC-MS/MS experiments were performed, an identification experiment, truncation analysis with N-terminal tagging, and truncation analysis with chymotryptic digestion with multiple reaction monitoring (MRM). For all of

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these experiments the SDS-PAGE bands were excised from the Coomassie stained gels, washed, reduced and alkylated prior to in-gel digestion with either trypsin (identification and TMT N-terminal tagging experiments) or chymotrypsin. The N-terminal tagging was performed on extracted peptides by adding 100 µl of the tandem mass tags (TMT, Thermo scientific) in 200 mM triethyl ammonium bicarbonate for 1 hr. The reaction was quenched by 5% hydroxylamine for 15 min, combined, dried by speedvac, and dissolved in 1% acetic acid.

Two different LC-MS/MS systems were used in these experiments. Protein identification and MRM analysis was carried out on a LTQ linear ion trap equipped with an Eksigent nano-1D HPLC system. Peptides were resolved on a self-packed Phenomenex Jupiter C18 column (8 cm x 75 µm) with an acetonitrile/0.1% formic acid gradient. The instrument was operated in a either a data dependent mode in which a mass scan was followed by MS/MS scans on the most abundant ions or Multiple Reaction Monitoring (MRM) mode in which specific ions are fragmented and assessed over the entire course of the LC experiment. Proteins were identified by searching the LC-MS/MS spectra against the human reference sequence database with the program Mascot. The TMT-Tagged samples were analyzed on an LTQ-Orbitrap-Velos hybrid mass spectrometer equipped with an Eksigent nano-1D HPLC system. Peptides were resolved on a PicoFrit column packed with ProteoPep 2 (10 cm x 75 µm) C18 with an acetonitrile/0.1% formic acid gradient. The instrument was operated in a data dependent mode in which all high resolution mass scans were obtained in the Orbitrap (resolution at 60,000) to identify the five most abundant ions. These

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ions were then subjected to five MS/MS scans in the LTQ for peptide identification, and five HCD (higher energy collisional dissociation) scans in the Orbitrap for TMT Tag quantitation. The resulting data was then searched against the human reference sequence database (http://www.ncbi.nlm.nih.gov/RefSeq/) using the program SEQUEST. The search parameters include 10 ppm parent ion tolerance, 0.8 Da MS/MS tolerance, full tryptic peptides, oxidized methionine as a variable modification, carbamidomethylated cysteines and TMT tag modifications as static modifications. Positive protein identifications were determined by setting the FDR rate to less than 1% and requiring at least 2 peptides per protein. Peptide TMT quantitation was performed by Proteomics Dynamics Software with an integration window tolerance of 20 ppm and monoisotopic masses for the tabs at 126.127 and 127.131 Da.

2.2.8 Total internal reflection microscopy (TIRF) microscopy

Washed platelets (2×10^8/ml) were treated with 30 µM MG132 or not for 30 min before addition onto a drop of 0.025 U thrombin placed in a glass bottomed microwell (MatTek) dish. Imaging was performed using at 100X with a 1.46 N.A. objective in a Leica AM TIRF MC System (Leica Microsystems, Wetzlar, Germany) equipped with an ImageEM C9100-13 EMCCD camera (Hamamatsu, Bridgewater, N.J). The 488 nm 10-mW diode laser was used for excitation and the penetration depth was set to 70 nm. Within 10 seconds of being placed on the stage, the sample was focused and a time-lapse series was

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initiated to collect images every 3 seconds for 5 minutes. At the end of the series, five static images were collected in both TIRF and differential interference contrast (DIC) modes of five different fields in the sample.

2.2.9 Microparticle isolation and quantitation

Washed platelets (10^9/ml) were pretreated with 30 µM MG132, 40 µM bortezomib or buffer for 30 min before an overnight treatment with thrombin (0.2 U), ADP (100 µM) or 0.1 µg/ml LPS with addition of 0.1 µg/ml each of human recombinant CD14 and LPS binding protein (LBP). Platelets were removed twice by centrifugation (500 x g) before microparticles were collected by centrifugation (100,000 x g, 90 min). The microparticle pellet was resuspended in 0.5 % human serum albumin/HBSS MPs and were counted by flowcytometery using defined numbers of 3-µm polystyrene latex beads (Sigma) as an internal standard for size. Forward scatter (FSC) and side scatter (SSC) were drawn to include 50,000 3-µm events. Microparticle size was determined using 1-µm beads (Sigma). Flowcytometery was performed using settings where the threshold was lowered to 200 and FSC and SSC gates were drawn to include events 1-µm in size or smaller. Microparticles express phosphatidylserine on their surface which can be detected by annexin-V staining. Based on the flowcytometer size gating, only microparticles (1-µm) stained with annexin-V were enumerated.

2.2.10 Peptidyl activity of tissue factor (TF) on microparticles

Tissue factor (TF)-dependent procoagulant activity was assessed with an Actichrome TF assay (American Diagnostica) kit as previously described (Del

Conde, Shrimpton et al. 2005). Washed platelets (10^9/ml) were pretreated with 30 µM MG132 for 30 min before an overnight treatment with thrombin (0.2 U). Platelets were removed twice by centrifugation (500 x g) before microparticles were collected by centrifugation (100,000 x g, 90 min). The microparticle pellet was resuspended in 0.5% human serum albumin/HBSS and were immediately placed in 25 µl of kit assay buffer and TF was calculated. The data are displayed as fold increase in peptidyl activity of TF on microparticles at absorbance 405 nm.

2.2.11 Clot retraction

Washed platelets (5 X 10^8/ml) were incubated with MG132 (30 µM, 30 min) before fibrinogen was added to a final concentration of 500 µg/mL and dispensed (500 µl) into siliconized glass tubes. Clot retraction was initiated by the addition of human α-thrombin (0.2 U/mL) with clot volume imaged over time. The size of the retracted clot was determined at stated times using Imagepro plus software (Media Cybernatics, Bethesda,MD) to calculate retraction.

2.2.12 Aggregation

Washed platelets (2 X 10^8/ml) were pretreated with combinations of 30 µM MG132, 40 µM bortezomib, 10ng SZ2, 35 nM RWJ56110 (PAR1 receptor inhibitor) or buffer for 30 min before stimulation with low (0.025 U) or high concentrations of thrombin (0.1 U) or 50 µM SFLLRN (PAR1 specific agonist). Platelet aggregation was measured by transmittance (Chronolog) with stirring (600 rpm). For ristocetin or botrocetin induced agglutination, washed platelets (2

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X 10^8/ml) were resuspended in a buffer containing 50% HBSS-A and 50% platelet poor plasma (PPP) and were pretreated with 30 µM MG132, 40 µM bortezomib or buffer for 30 min. Agglutination was induced with either 650 µg/ml ristocetin or 300 ng/ml botrocetin and was measured by transmittance (Chronolog) with stirring (1000 rpm).

2.2.13 Flow cytometry

Washed platelets (2 X 10^8/ml) were pretreated with 30 µM MG132 or buffer for 30 min before stimulation with 0.025 U thrombin (30 min) followed by fixation with 2% paraformaldehyde. Anti-CD42b antibody or non-immune isotype control (Millipore) was used to determine the surface expression of GPIbα by flow cytometery.

2.2.14 Expression of data and statistics

All experiments were performed at least three times with cells from different donors, and all assays were performed in triplicate. The standard errors of the mean from all experiments are presented as error bars. Figures and statistical analyses were generated with Prism4 (GraphPad Software). A value of p≤ 0.05 was considered statistically significant.

2.3 Results

2.3.1 The platelet proteasome aids occlusive thrombosis

Human platelets expressed all three primary proteolytic activities of the proteasome, effectively hydrolyzing peptides through chymotryptic, tryptic and

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caspase-like cleavage (Fig. 2.1 A). The luminescent product was the same for each luminogenic proteasome substrate, so the caspase-like activity was approximately four times more efficient than either the tryptic or chymotryptic activity. The trimeric leucinyl proteasome inhibitor MG132 at 30 μM completely blocked platelet chymotryptic and caspase-like activities, and reduced the tryptic activity by half. MG132 at 30 µM is just optimally inhibitory (Fig. 2.1 B). Murine platelets also hydrolyzed these three substrates at equivalent ratios (Fig. 2.1 C) indicating that murine platelets have active proteasome.

We determined whether proteasome inhibition affected thrombosis by systemically injecting MG132 into BL6 mice to achieve an estimated initial circulating concentration of 30 µM along with rhodamine dye to fluorescently label circulating platelets. We initiated occlusive thrombosis 15 min later (after MG132 or DMSO injection) in a surgically exposed external carotid artery by a brief ectopic application of 7.5% FeCl$_3$ (Li, Febbraio et al. 2010; Li, McIntyre et al. 2013). This oxidative insult to the vascular wall resulted in the deposition of fluorescently labeled platelets along the damaged vessel wall that increased over several minutes (Fig. 2.1 D). Typically, complete occlusion of the vessel occurred by 10 min after FeCl$_3$ treatment, but in animals previously injected with MG132 occlusion was significantly delayed to 25 min (Fig. 2.1 E).

The delay in thrombosis after systemically administering MG132 need not solely reflect participation of platelet proteasomes in thrombosis. We therefore isolated and washed platelets from wild-type BL6 mice and treated these isolated cells with 30 µM MG132 or buffer, washed the cells by centrifugation and

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transfused these cells into mice previously rendered thrombocytopenic by gamma irradiation. We induced carotid artery thrombosis with ectopic $FeCl_3$ as before to find control platelets were fully functional and occluded the carotid artery by 10 min (Fig. 2.1 F). We again found occlusive thrombosis was delayed in mice reconstituted with platelets treated with MG132 *ex vivo*. The platelet proteasome thus participates in arterial thrombosis.

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A Chymotryptic activity · Tryptic activity · Caspase-like activity

B Chymotryptic activity · Tryptic activity · Caspase-like activity

C Mouse proteasome activity

D

E

F
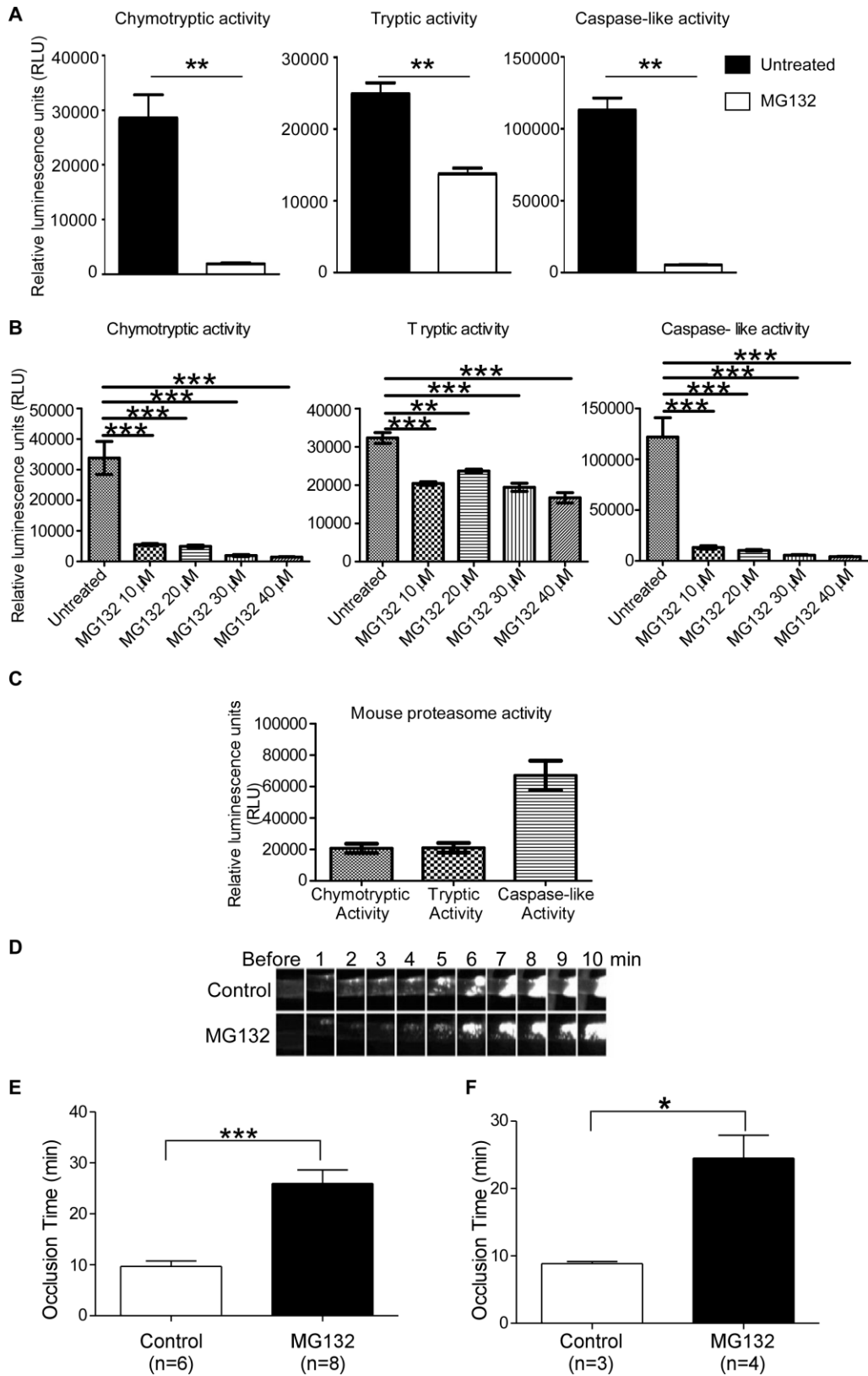
Figure 2.1 Platelets express a functional proteasome that contributes to occlusive thrombosis

(A) Platelets contain a functional proteasome. Proteasome proteolytic activities were individually assayed using luminogenic substrates for indicated catalytic activities in lysates of untreated and MG132-treated platelets (n=3; **p≤ 0.01). **(B)** Proteasome proteolytic activities were individually assayed as mentioned in panel (A) in lysates from untreated platelets and platelets treated with different concentrations of MG132 (10, 20, 30 and 40 µM), n=6; ***p<0.001, **p≤ 0.01). **(C)** Mouse platelets contain a functional proteasome. Proteasome proteolytic activities were individually assayed in mouse platelet lysates using luminogenic substrates for indicated catalytic activities (n=4). **(D)** Fluorescent platelet accretion after $FeCl_3$ damage to carotid arteries is reduced by MG132. Video frames at 1 min intervals of thrombus formation in $FeCl_3$-damaged carotid arteries (n=5). **(E)** MG132 lengthens the time to vascular occlusion. Time to cessation of blood flow in mice treated as in the preceding panel determined by cessation of platelet movement (n=8 experimental, 6 control; ***p≤ 0.001). **(F)** *Ex vivo* MG132 pretreatment of platelets prolongs occlusion time after transfusion into irradiated thrombocytopenic mice. Platelets from donor mice were first pretreated for 30 min with MG132 and were washed before transfusion into normal BL6 mice and $FeCl_3$-induced thrombosis of carotid arteries initiated 15 min later (n=4 experimental, 3 control; *p≤ 0.05).

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2.3.2 Platelets contain a stimulatable ubquitination system

Modification of platelet function by MG132 suggests platelets may designate proteins as substrates for proteolysis by ubiquitination, as in nucleated cells. We determined whether platelets contained E1, E2, E3 enzymes that sequentially conjugate ubiquitin to target proteins. In order to ensure that we assessed only platelet proteins, we also isolated highly purified, negatively selected platelets that were essentially free of nucleated cells (less than 1 monocyte in 10^9 platelets (Shashkin, Brown et al. 2008). Western blotting showed that these platelets contained the E1 activating enzyme UBE1, E2 and E3 ligases, and Rpn2 and the 11S α-subunit of the 19S regulatory complex (Fig. 2.2 A).

We used *in vitro* ubiquitination of exogenous p53, since platelets lack this transcription factor, to determine whether platelet UBE1 was functional. Incubation of recombinant p53 with recombinant E1 and appropriate recombinant E2 and E3 enzymes along with ubiquitin and ATP resulted in robust p53 ubiquitination with formation of numerous slowly migrating adducts (Fig. 2.2 B). Substitution of a platelet lysate for recombinant E1 also promoted *in vitro* ubiquitination of p53, although the higher molecular weight ladder was less prominent than produced by recombinant E1 (Fig. 2.2 B).

We next determined whether endogenous platelet proteins were modified by ubiquitin. Western blotting with FK2 antibody that recognizes both mono- and poly-ubiquitin chains showed that the proteome of quiescent cells contained multiple ubiquitinated proteins (Fig. 2.2 C). The intensity of ubiquitination

increased after thrombin activation, although the pattern and position of the adducted bands was unaltered compared to control cells. ADP stimulation also increased ubiquitination of platelet proteins, but this primarily reflected an increase in modification of very slowly migrating proteins (Fig. 2.2 C).

We investigated whether inhibiting the platelet proteasome altered the amount of ubiquitinated proteins. Pretreatment with MG132 increased both the abundance and the levels of ubiquitinated proteins as detected with the anti-ubiquitin antibody (Fig. 2.2 D). In fact, MG132 was significantly more effective than thrombin or ADP stimulation in enhancing decoration of the proteome with ubiquitin, and the combination of agonist stimulation and MG132 was not different from the effect of MG132 alone (Fig. 2.2 D). We observed a similar increase in ubiquitinated proteins after treatment with bortezomib (Fig. 2.2 E). Probing the platelet proteome with FK1 antibody that recognizes only poly-ubiquitinated proteins revealed less abundant modification in unstimulated cells (Fig. 2.2 F). This was increased by agonist stimulation and was again greatly enhanced by MG132 treatment. Comparison of FK2 (Fig. 2.2 D) and FK1 (Fig. 2.2 F) immunoblots showed distinct patterns of mono-and poly-ubiquitination of the platelet proteome.

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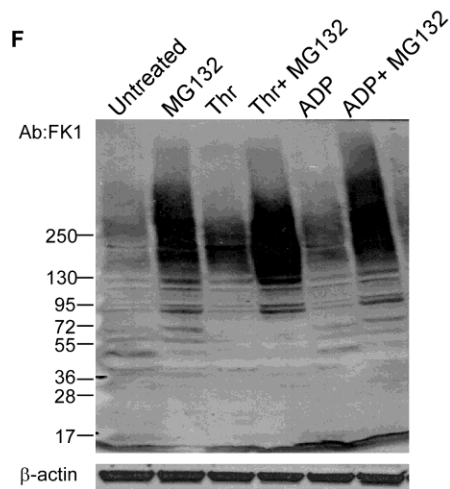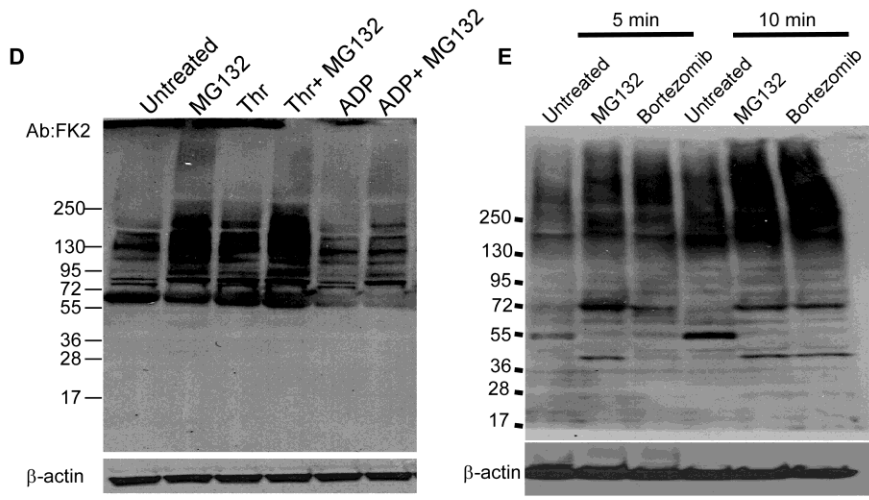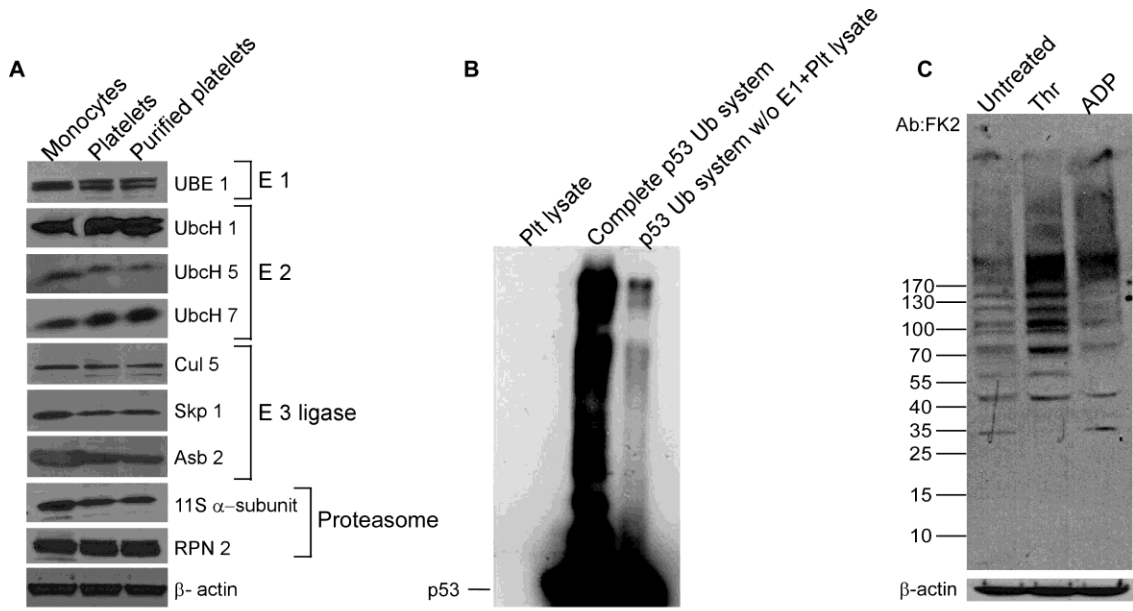
Figure 2.2 Platelets contain a functional ubiquitination system

(A) Platelets contain components of ubiquitin-proteasome system. Immunoblots of platelet E1 activating enzyme, E2 and E3 conjugating enzymes, and proteasome regulatory cap proteins (n=3). **(B)** Platelet E1 is functional. Platelet lysates substituted for recombinant E1 and together with recombinant E2, E3, ubiquitin and ATP ubiquitinated recombinant p53 (n=3). **(C)** Agonist stimulation increases platelet proteome ubiquitination. Lysates of platelets stimulated with buffer, thrombin (thr) or ADP were immunoblotted with FK2 antibody against mono-and polyubiquitinated proteins (n=3). **(D)** MG132 increases proteome ubiquitination. Lysates of platelets, treated or not with MG132 were stimulated, or not, before immunoblotting with FK2 (n=3). **(E)** Both MG132 and bortezomib increases proteome ubiquitination. Western blot of MG132 or bortezomib treated platelet lysate over indicated times as probed with FK1 antibody (n=3). **(F)** MG132 increases poly-ubiquitination of platelet proteins (n=3). Western blotting of platelet lysates with FK1 antibody (n=3).

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2.3.3 Filamin A is ubiquitinated, and truncated by the proteasome

Filamin A links the GPIb-IX-V complex to actin filaments of the cytoskeleton to modify cytoskeletal shape (Cunningham, Meyer et al. 1996). Filamin A was present in the soluble fraction of quiescent platelets as a 225 kD fragment of the 280 kDa native protein (Fig. 2.3 A). MG132 inhibition of the proteasome decreased the amount of this smaller band and increased the amount of intact Filamin A. Capture of ubiquitinated platelet proteins by a sushi domain column followed by immunoblotting using anti-Filamin A antibody showed that the fragment of Filamin A in quiescent cells was constitutively modified with ubiquitin (Fig. 2.3 B). This approach also showed that MG132 increased the amount of Filamin A ubiquitination, and increased the apparent size of the ubiquitinated Filamin A (Fig. 2.3 B). That Filamin A was ubiquitinated and that a larger, more extensively ubiquitinated protein accumulated after MG132 treatment was confirmed by the converse experiment where Filamin A was immunoprecipitated from platelet lysates and then probed for ubiquitin (Fig. 2.3 C) using FK2 antibody. The cytoskeletal protein Talin-1 participates in cell spreading, and similar experimental approaches of western blotting (Fig. 2.3 D), sushi domain capture of ubiquitin adducts (Fig. 2.3 E), and immunoblotting of captured Talin-1 for mono-and poly-ubiquitin chains (Fig. 2.3 F) showed that Talin-1 was present in the cytosol in a rapidly migrating, ubiquitinated form. Again, MG132 treatment increased its apparent size and ubiquitin content.

Coomassie blue staining of soluble proteins showed MG132 treatment did not alter overall platelet protein mass or composition, but there were two

abundant exceptions (Fig. 2.3 G). Two new, slowly migrating bands appeared in the soluble fraction of platelets treated with MG132. Mass spectrometry showed Filamin A to be present in the new band 1, and Talin-1 in bands 1 and 2 of the resolved soluble proteins, corresponding to the altered mobility detected by western blotting.

We used Tandem Mass spectrometry Tags (TMT)—chemically identical tags differentially substituted with heavy atoms that enable quantitative multiplexed analysis—to quantitatively compare the amount of Filamin A peptides in tryptic digests of control and MG132 treated platelets. Untreated (band 3) and MG132 treated (band 1) platelets were separately digested with trypsin, their primary amines exhaustively modified with isobaric TMT tags (m/z 126 and m/z 127, respectively) before combined analysis by mass spectrometry. The peptides confirmed both bands contained Filamin A, and the tags showed the abundance of Filamin A tryptic peptides in band 1 and band 3 uniformly gave a m/z 126/127 ratio of 1.9. This ratio plunged to 0.08 after the tryptic peptide containing residues 1718-1745 (Fig. 2.3 H), demonstrating increased abundance of the full length protein after MG132 treatment. Chymotryptic digestion of band 3 isolated from control cells generated a distinct peptide map and selected reaction monitoring identified a new Filamin A peptide with S_{1746} as its amino terminus. This identifies the R_{1745} - S_{1746} bond as the site of cleavage in control cells, which maps into the unfolded hinge 1 region of Filamin A (Fig. 2.3 I). Notably, this cleavage is tryptic-like, and is distinct from the previously determined calpain cut site (Gorlin, Yamin et al. 1990).

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A Untreated MG132 — Filamin A — 225

B Untreated MG132 — Ubiquitin capture column IB-Filamin A

C Untreated MG132 — IP-Filamin A — IB - FK2

D Untreated MG132 — Talin 1 — 225

E Untreated MG132 — Ubiquitin capture column IB-Talin 1

F Untreated MG132 — IP-Talin 1 — IB - FK2

G Marker 0' min Untreated MG132 — 1 2 3 — 225 150 102 76 52 38 31 24 17

H 126/127 — 1718-1745 — 8_24 428-437 905-916 1440-1450 2194-2207 2568-2576

I Actin binding domain
N ABD 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 C

Predicted Calpain cut site

Tryptic cut site

qpplRSQQLA PQYTYAQGGQ QTWAPERPLV GVNGLDMTSL RPFDLVIPFT IKKGEITGEV 1801

1718-1745   1746-1767

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Figure 2.3 MG132 protects cytoskeletal protein cleavage

 (A) Filamin A is ubiquitinated and its length increased by MG132. Western blot of Filamin A in the soluble fraction of platelets before and after MG132 exposure (n=3). **(B)** Ubiquitination and size of Filamin A are increased by MG132. Western blot of Filamin A in eluates of ubiquitinated platelet proteins captured with sushi columns [agarose beads conjugated to anti-ubiquitin antibody, (n=3)]. **(C)** Filamin A ubiquitination and size are increased by MG132. FK2 western blot of mono- and poly-ubiquitinated proteins immuoprecipitated with anti-Filamin A. **(D)** Talin-1 is ubiquitinated and its size increased by MG132. Western blot of Talin-1 before and after MG132 exposure (n=3). **(E)** Ubiquitination and size of Talin-1 are increased by MG132. Talin-1 in ubiquitinated platelet proteins captured by sushi domain chromatography (n=3). **(F)** Talin-1 ubiquitination and size are increased by MG132. FK2 western blot of mono- and poly-ubiquitinated proteins immuoprecipitated by anti-Talin-1 (n=3). **(G)** MG132 increases high molecular weight proteins. Coomassie stained gel of resolved platelet cytoplasmic proteins. **(H)** Mass spectrometer determination of the *mz* 126/127 ratio of TMT-labeled peptides along the Filamin A sequence (n=2). **(I)** Pictogram of Filamin A structure.

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2.3.4 Proteasome inhibition reduces cytoskeleton-dependent functions

Filamin A is a critical regulator of cytoskeletal structure and function, so we next tested the hypothesis that proteasomal inhibition alters platelet functions regulated by cytoskeletal dynamics, including GPIb function, microparticle generation, and clot retraction.

First, we imaged cytoskeletal dependent spreading by Total Internal Reflection Microscopy (TIRF) that detects only fluorophore closely opposed (≤ 200 nm) to a glass matrix. Calcein-labeled platelets adhered over time after activation by thrombin with extension of filopodia followed by lamellipodia (video supplement) that resulted in adherent, spread cells (Fig. 2.4 A). In contrast, while MG132-treated cells adhered and spread after thrombin stimulation, they did so with less frequency, rapidity, and ultimately were less splayed (Fig. 2.4 B).

Stimulated platelets release pro-thrombotic microparticles from their surface that depends on cytoskeletal rearrangement after stimulation (Morel, Jesel et al. 2011). Thrombin induced a ~6 fold increase in microparticle shedding (Fig. 2.4 C and D), which was significantly reduced by pre-treating the platelets with either MG132 (Fig. 2.4 C) or bortezomib (Fig. 2.4 D). MG132 also blocked microparticle release from LPS-stimulated platelets (Fig. 2.4 E). The majority of these particles express phosphatidylserine on their surface which can be detected by annexin V staining. Both MG132 (Fig. 2.4 F) and bortezomib (Fig. 2.4 G) reduced thrombin induced increase in annexin V positive microparticle shedding. MG132 also suppressed LPS induced shedding of annexin V positive microparticles (Fig. 2.4 H). The surface of these annexin V positive microparticles

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is ~50-100 fold more thrombotic than even the activated platelets (Sinauridze, Kireev et al. 2007) and they promote thrombosis through tissue factor pro-coagulant activity (Fig. 2.4 I), which MG132 significantly inhibited.

Cytoskeletal rearrangement retracts newly formed thrombi, promoting wound repair (Cheresh, Leng et al. 1999; Grinnell 1999). Stimulation of platelets with thrombin induced rapid formation of a thrombus that then consolidated over time (Fig. 2.4 J). MG132 interfered with this process, ultimately decreasing retraction by 60%.
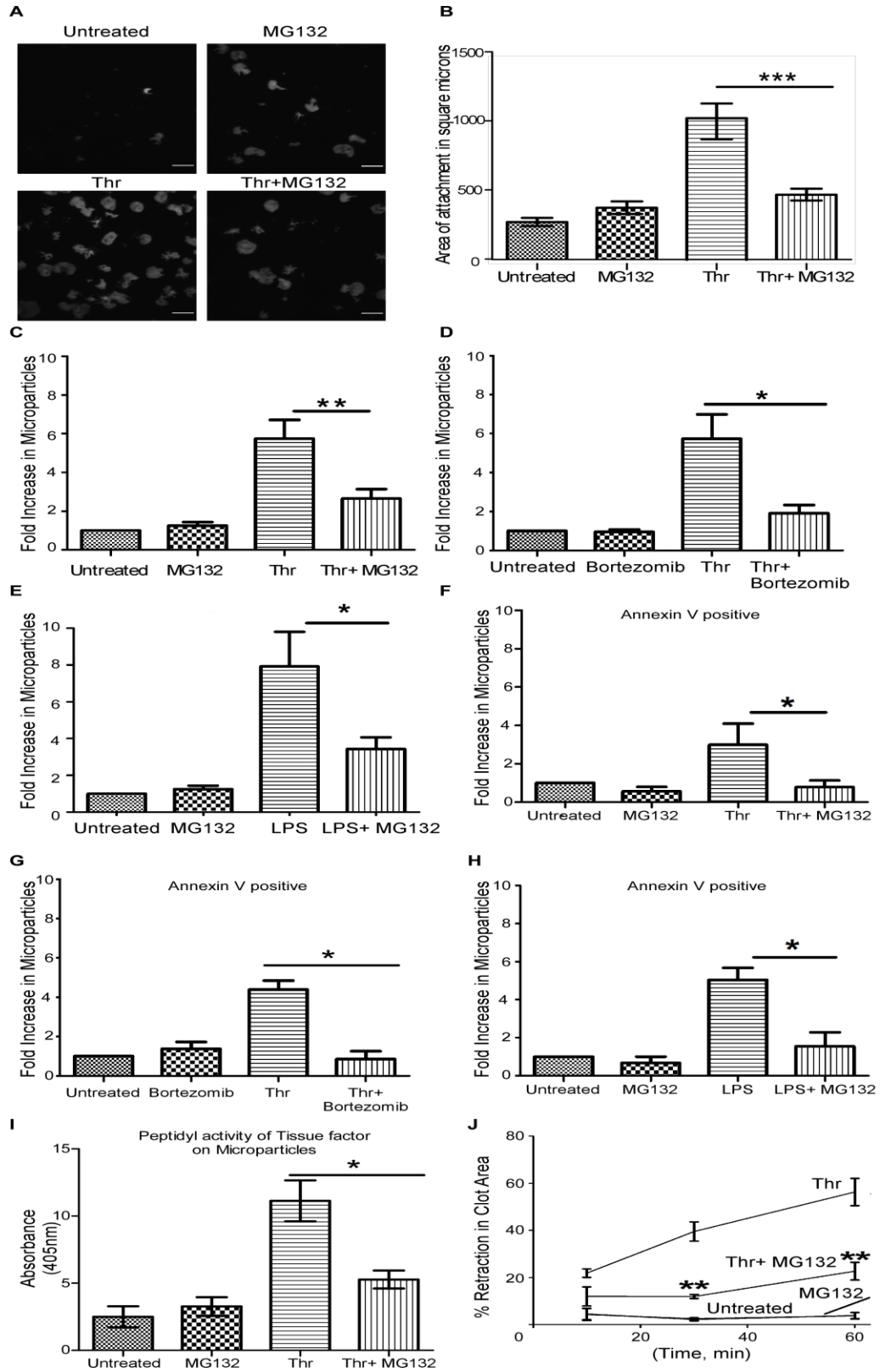
Figure 2.4 MG132 suppresses stimulated spreading, microparticle shedding, and clot retraction

(A) MG132 disturbs thrombin stimulated adhesion and spreading. Interaction of control or thrombin (thr, 0.025 U) stimulated platelets with a glass substrate were imaged by Total Internal Reflection Microscopy after 5 min (n=3) (scale bar = 10 µM). **(B)** Platelet area after MG132 treatment. Platelet area in Panel A was quantified by Imagepro plus software (n=3; $^{***}p \leq 0.001$). **(C)** Microparticle shedding by platelets stimulated with 0.2 U thrombin with or without MG132 (n=3; $^{**}p \leq 0.01$). **(D)** Bortezomib (n=3; $^{*}p \leq 0.05$). **(E)** Microparticle shedding by platelets pretreated with or without MG132 and stimulated with lipopolysaccharide (LPS, n=3; $^{*}p \leq 0.05$). **(F)** Proteasome inhibitors suppress agonist-induced shedding of procoagulant microparticles. Annexin V positive microparticle shedding by platelets stimulated with 0.2 U thrombin with or without MG132 (n=3; $^{*}p \leq 0.05$). **(G)** Bortezomib (n=3; $^{*}p \leq 0.05$). **(H)** Annexin V positive microparticle shedding by platelets stimulated with lipopolysaccharide (LPS, n=3; $^{*}p \leq 0.05$). **(I)** Tissue factor procoagulant activity in platelet microparticles stimulated with 0.2 U thrombin with or without MG132 (n=3; $^{*}p \leq 0.05$). **(J)** MG132 represses clot retraction. Ratio of the image surface area of images of thrombin-induced clots over time (n=3; $^{**}p \leq 0.01$).

2.3.5 Proteasome inhibition selectively reduces aggregation stimulated by low concentrations of thrombin

Thrombin stimulates platelets by cleaving surface PAR1 (protease-activated receptor 1) to create a self stimulatory terminal SFLLRN peptide, (Coughlin and Camerer 2003) but at low concentrations thrombin activates platelets through the high affinity GPIb-IX-V receptor (Greco, Tandon et al. 1996). Both MG132 and bortezomib reduced homotypic platelet aggregation at low concentrations of thrombin (Fig. 2.5 A).

The effect of proteasome inhibitors was on the high affinity GPIb-IX-V complex because the SZ2 antibody against GPIbα (CD42b) fully blocked aggregation at a low concentration of thrombin (Fig. 2.5 A). The PAR1 agonist SFLLRN induced platelet aggregation that was completely blocked by a PAR1 specific antagonist (RWJ56110, Fig. 2.5 B). However, neither MG132 (Fig. 2.5 B) nor bortezomib (not shown), suppressed aggregation induced by SFLLRN. Aggregation induced by a low, submaximal amount of SFLLRN also was unimpeded by MG132 (Fig. 2.5 C). Thus, PAR-1 agonist induced platelet aggregation is proteasome independent. MG132 also failed to affect aggregation induced by AYPGFK stimulation of PAR 4 (Fig. 2.5 D). Furthermore, neither MG132 nor bortezomib inhibited platelet aggregation stimulated by a higher thrombin concentration that acts through PAR1 (Fig. 2.5 E). The modifiers SZ2, MG132, or RWJ56110 by themselves did not induce aggregation (Fig. 2.5 F).

Proteasome protease activity was required by the high affinity thrombin receptor because epoxomicin inhibited platelet aggregation by low thrombin

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concentrations (Fig. 2.5 G) and this inhibition was statistically significant (Fig. 2.5 H). Epoxomicin is a fungal metabolite, structurally unrelated to either MG132 or bortezomib, that specifically inhibits proteasome protolysis without inhibiting calpain, trypsin, chymotrypsin, or cathepsin B at concentrations 50-times higher than we used for blocking platelet aggregation (Meng, Mohan et al. 1999).

The GPIb-IX-V complex is displayed on the surface of quiescent cells, and is down regulated after thrombin stimulation (Michelson, Ellis et al. 1991). I found both MG132 (Fig. 3.5 I) and bortezomib (Fig. 3.5 J) stabilized the GPIb-IX-V complex on the surface of stimulated cells, and actually enhanced surface GPIbα (CD42b) expression. This surface retention of the receptor in platelets treated or not with proteasome inhibitors is significant (Fig. 2.5 K). Ristocetin increases the affinity of GPIbα for VWF, agglutinating unactivated cells, and pre-treatment of quiescent platelets with MG132 (Fig. 2.5 L) or bortezomib (Fig. 2.5 M) reduced ristocetin induced platelet agglutination. Thus despite the increased surface expression of the GPIb-IX-V complex after proteasome inhibition, the complex is less able to interact with VWF than in control platelets.
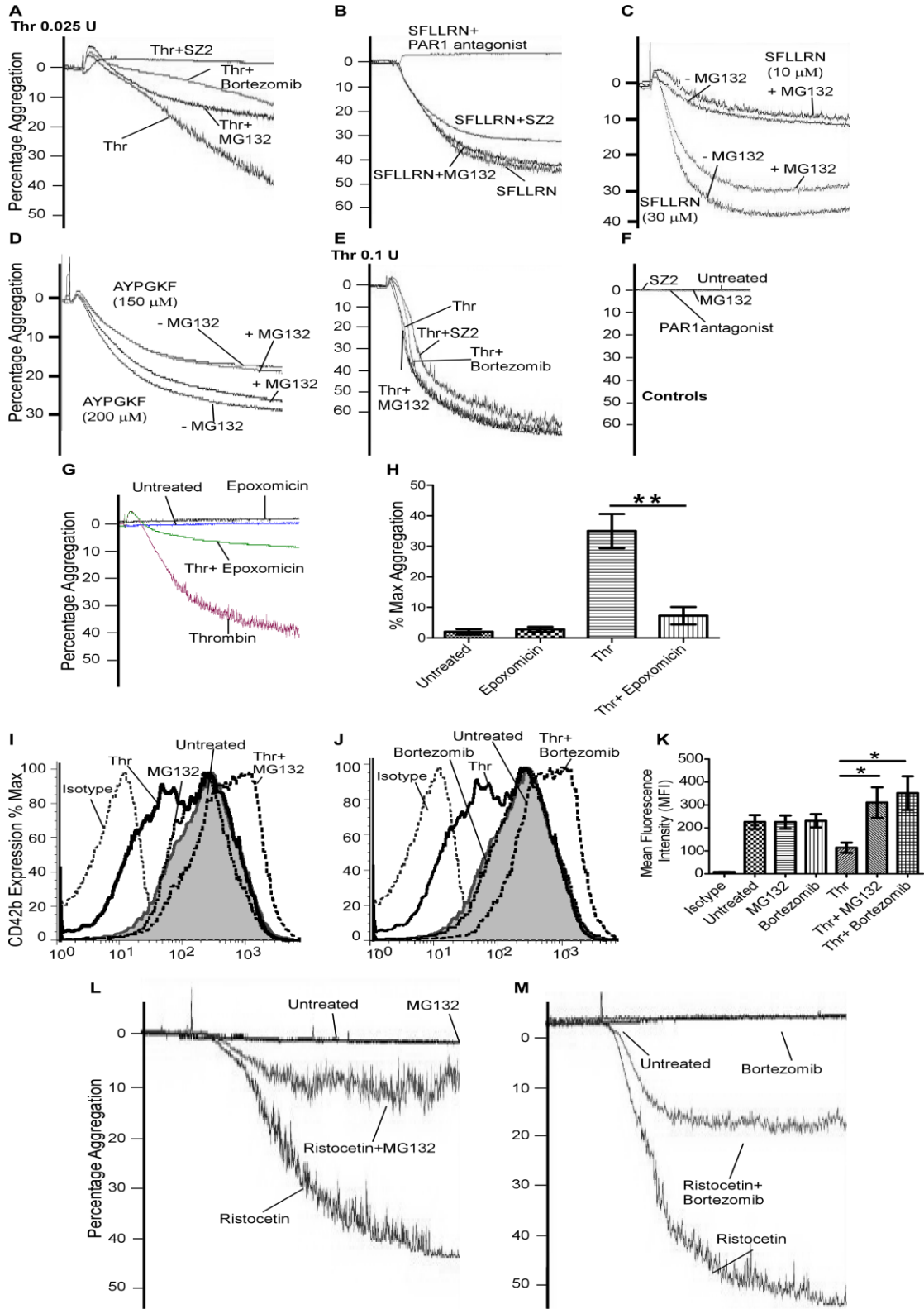
Figure 2.5 MG132 reduces aggregation in response to low dose thrombin, and prevents GP1bα downregulation

(A) MG132 and bortezomib suppress platelet aggregation induced through the high affinity thrombin receptor, GP1b-IX-V. Aggregation induced by 0.025 U thrombin (thr) was reduced by MG132 and bortezomib (n=3). **(B)** MG132 failed to reduce aggregation stimulated by the PAR1 (protease-activated receptor 1) agonist peptide SFLLRN (n=3). **(C)** MG132 (30 μM) pretreatment minimally reduces aggregation stimulated by low concentrations of the PAR1 agonist peptide SFLLRN (n=3). **(D)** MG132 failed to reduce aggregation stimulated by the PAR4 agonist peptide AYPGKF at both 200 μM and 150 μM concentrations (n=3). **(E**) Neither MG132 nor bortezomib block aggregation from thrombin activation of PAR1. Platelet aggregation by 0.1 U thrombin with or without SZ2 antibody (n=3). **(F)** GP1bα or PAR1 inhibition alone does not induce aggregation (n=3). **(G)** Aggregation induced by 0.025 U thrombin (thr) was reduced in platelets pretreated with 1 μM epoxomicin for 30 min (n=3). **(H)** Quantitative representation of percentage maximum (Max) aggregation (n=3; **p≤ 0.01). **(I)** MG132 repressed stimulated GPIbα down regulation. Flow cytometry of surface CD42b (GPIbα) expression on platelets treated or not with MG132 and stimulated or not with 0.025 U thrombin (n=3). **(J)** Bortezomib repressed surface GPIbα down regulation. Flow cytometry of GPIbα expression on control and thrombin stimulated platelets (n=3). **(K)** Mean fluorescence intensity (MFI) of surface CD42b (GPIbα) expression on platelets treated either with MG132 or bortezomib and stimulated or not with 0.025 U thrombin (n=3; *p≤ 0.05). **(L)**

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MG132 suppressed ristocetin induced agglutination. Quiescent platelets pretreated or not with MG132 were agglutinated with ristocetin (n=3). **(M)** Bortezomib suppressed ristocetin induced agglutination. Quiescent platelets pretreated or not with bortezomib were agglutinated with ristocetin (n=3).

2.4 Discussion

Multiple myeloma is associated with an increased incidence of venous (Srkalovic, Cameron et al. 2004; Zangari, Elice et al. 2007; Kristinsson, Fears et al. 2008) and arterial (Libourel, Sonneveld et al. 2010) thrombotic disease, and bortezomib therapy suppresses these thrombotic states (Zangari, Guerrero et al. 2008). Platelets display proteasome chymotryptic activity that is greatly stimulated by soluble agonists (Nayak, Kumar et al. 2011). Additionally, bortezomib represses ADP-induced aggregation (Avcu, Ural et al. 2008) and platelets isolated from patients receiving bortezomib are hyporesponsive to other stimuli (Zangari, Guerrero et al. 2008; Shen, Zhou et al. 2011). A second proteasome inhibitor, PSI, suppresses thrombosis in hypertensive animals (Ostrowska, Wojtukiewicz et al. 2004).

I extend these observations by showing that platelets contain an intact and functional ubiquitin/proteasome system that participates in agonist stimulated responses, especially those aided by cytoskeletal rearrangement. I found that inhibition of the proteasome's proteolytic activity reduced thrombosis at sites of oxidative damaged to murine carotid arterial walls. Since MG132 was introduced into the circulation in this experiment, protection need not have been a direct

effect of MG132 on platelet function, but the target of MG132 in platelets is in fact the proteasome. I treated purified platelets with MG132 *ex vivo*, washed them, and then transfused these cells into mice rendered thrombocytopenic by prior gamma irradiation. This platelet-specific exposure to MG132 interfered with the *in vivo* function of platelets because MG132 treated platelets were deficient in their occlusion of a $FeCl_3$-damaged carotid artery. Remarkably, this *ex vivo* exposure exactly mimicked systemic exposure to MG132. This establishes a role for the platelet proteasome in thrombosis.

Platelets contained a functional ubiquitin system that modified cellular proteins to mark them as proteasome substrates. The proteome of quiescent platelets contained numerous ubiquitin-protein conjugates whose adduction was increased upon stimulation, consistent with the prior observation that collagen activation stimulates ubiquitination of platelet Syk kinase(Dangelmaier, Quinter et al. 2005) through the E3 ligase Cbl-b.(Daniel, Dangelmaier et al. 2010) Two of the proteins decorated with ubiquitin in quiescent platelets were Filamin A and Talin-1. Agonist stimulation modestly increased the amount of ubiquitin esterified in the platelet proteome, but blockade of the proteasome was far more effective than agonist stimulation for this, with the result that heavily ubiquitinated Filamin A, and Talin-1 accumulated in the cytoplasm of cells with diminished proteasome proteolytic activity.

Filamin A is organized into an actin-binding domain (ABD), 24 tightly compacted FERM immunoglobulin-like repeats connected with short linking sequences, and two unstructured hinge regions. Cytoplasmic Filamin A migrated

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more quickly than the intact protein during gel electrophoresis, suggesting it primarily was a fragment. Quantitative comparison of tryptic peptides using tandem mass spectrometry tags showed that MG132 protected Filamin A from cleavage to the smaller 225 kDa fragment. Identification of Filamin A peptides present only after MG132 treatment and identification of the new amino terminal peptide after chymotryptic digestion by multiple reaction monitoring showed cleavage of the Filamin A protein occurred between R1745 and S1746. This is in a hinge region that is not compacted into FERM domains. Cleavage at this site is distinct from the previously determined calpain cut site, (Gorlin, Yamin et al. 1990) and shows the protease was tryptic since it occurred after an arginine residue. The proteasome subunits functionally interact with one another (Kisselev, Callard et al. 2006) to degrade proteins to amino acids and small peptides, but the proteasome also processes large proteins to functional fragments, as shown by NF-κB p105 proteolysis to the p50 transcription factor (Rape and Jentsch 2002). We now identify the longer unstructured Filamin A loop as another site of limited proteasome proteolysis, but our data is associative so we cannot ascribe this proteolysis as causal in MG132 inhibition of platelet adhesion or spreading.

Platelets pretreated with MG132 or bortezomib aggregated less in response to low concentrations of thrombin. Adhesion, spreading, microparticle shedding, and the ability to generate the force to retract formed clots was also reduced by proteasome inhibition. Cytoskeletal interactions aid microparticle shedding from stimulated platelets, and proteolysis by activated calpain (Fox,

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Austin et al. 1991; Basse, Gaffet et al. 1994) and caspase-3 (Shcherbina and Remold-O'Donnell 1999) promote microparticle shedding through cytoskeletal proteolysis (Basse, Gaffet et al. 1994). The reduction of microparticle shedding is additionally revealing because MG132 suppressed shedding after stimulation by LPS activation of TLR4, thrombin activation of PAR1, and ADP activation of P2Y12. So, MG132 interference with stimulated platelet function is not restricted to GPIb-IX-V activation by thrombin.

We establish that platelets, like nucleated cells, express a functional ubiquitin/proteasome system that enables them to ubiquitinate their proteome. This decoration increases upon stimulation, and modulates an array of responses from several receptors that all engage the cytoskeleton. The cytoskeletal proteins Filamin A and Talin-1 are targets of ubiquitination, and proteasome mediated proteolysis. The ubiquitin/proteasome system of platelets affects their response to thrombotic stimuli, and proteasome inhibition effectively delayed arterial thrombosis. Bortezomib is already in clinical practice for multiple myeloma and mantle cell lymphoma so elucidation of its anti-platelet activity defines a new salutary function of these inhibitors.

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2.5 Chapter summary

These data demonstrate that platelets, like nucleated cells, express a functional ubiquitin/proteasome system that enables them to modify their proteome by mono- and poly-ubiquitination. Proteasome inhibition increased ubiquitin decoration of cytoskeletal proteins Filamin A and Talin-1 and increased platelet content of soluble intact filamin A. Proteasome inhibition by MG132 suppressed stimulated platelet spreading as well as clot retraction. Proteasome inhibition reduced platelet aggregation by low thrombin concentrations and ristocetin-stimulated agglutination through the GPIb-IX-V complex. The effects of proteasome inhibitors were not confined to a single receptor as both MG132 and bortezomib suppressed thrombin-, ADP-, and LPS-stimulated microparticle shedding. Systemic MG132 strongly suppressed formation of occlusive, platelet-rich thrombi in $FeCl_3$-damaged carotid arteries. Importantly, this is due to the direct effect of proteasomal inhibition on platelets as transfusion of platelets treated ex vivo with MG132 and washed prior to transfusion into thrombocytopenic mice also reduced carotid artery thrombosis.

Chapter III

Unmasking platelet reactivity through deubiquitination

3.1 Introduction

Platelet activation underlies thrombotic cardiovascular disease that is responsible for significant disability and death in the developed world (Davi and Patrono 2007; Jackson 2011). Platelet activation is transduced from the proteome delivered to platelets as they bud from megakaryocytes during thrombopoiesis, augmented by limited *de novo* protein synthesis (Zimmerman and Weyrich 2008). Platelets additionally contain a functional ubiquitin-proteasome system that targets proteins to the proteasome for degradation by decorating platelet proteins both with single molecules of the 8 kDa ubiquitin protein and by chains of polymerized ubiquitin (Gupta, Li et al. 2014). Polyubiquitin chains assembled by conjugation to any of the seven lysines of ubiquitin alter protein degradation, intracellular trafficking, or protein recognition to participate in a diverse range of cellular processes from morphogenesis to autophagy to intercellular interactions (Kulathu and Komander 2012). Correspondingly, proteasome proteolysis enhances platelet aggregation,

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adhesion, and microparticle shedding (Gupta, Li et al. 2014).

Post-translational modification by covalent ligation with ubiquitin is dynamic with six families of evolutionarily conserved deubiquitinases hydrolyzing these mono- and polymeric ubiquitin adducts (Fraile, Quesada et al. 2012). Deubiquitinase inhibitors alter a range of cellular functions with therapeutic potential (Colland 2010), as anticipated from the range of processes employing ubiquitin adduction. The general deubiquitinase inhibitor PR619 (Altun, Kramer et al. 2011) promotes autophagy, protein aggregation, and the unfolded protein response in nucleated cells (Seiberlich, Goldbaum et al. 2012; Seiberlich, Borchert et al. 2013). A small molecule inhibitor of E1 ubiquitin activating enzyme, PYR41 (Yang, Kitagaki et al. 2007), suppresses arachidonate-stimulated adhesion and migration of tumor cells on a collagen surface (Ray, Rogers et al. 2010), angiotensin II-mediated dendritic cell activation (Chen, Meng et al. 2014), and NF-κB activation in tumor cells (Yang, Kitagaki et al. 2007). However, it also leads to accumulation of ubiquitinated proteins *ex vivo* and *in vitro* by inhibiting deubiquitinases (Kapuria, Peterson et al. 2011), allowing ubiquitin-modified proteins to accumulate. In the context of this work I find PYR41 is a general DUB inhibitor independent of a role as an E1 inhibitor in platelets.

Recently, a small molecule inhibitor, b-AP15, was identified that specifically inhibits proteasome-associated deubiquitinase activity independent of numerous cytoplasmic deubiquitinases (D'Arcy, Brnjic et al. 2011). The proteasome-associated deubiquitinase activity is contributed by three deubiquitinase enzymes: Rpn11, USP14 and UCHL5, associated with the

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regulatory subunit of proteasome (Lam, DeMartino et al. 1997; Borodovsky, Kessler et al. 2001; Verma, Aravind et al. 2002). b-AP15 displays potent anti-tumor activity both *in vivo* and *in vitro* and is highly specific for USP14 and UCHL5, showing no inhibitory activity towards non-proteasomal deubiquitinases (D'Arcy, Brnjic et al. 2011). b-AP15 also reduced viability of multiple myeloma cells and overcomes the resistance to the proteasome inhibitor bortezomib (Tian, D'Arcy et al. 2014).

Platelets express a number of deubiquitinases (Burkhart, Vaudel et al. 2012), but the potential function of these enzymes in platelets is unstudied. I determined whether ubiquitination of the platelet proteome was dynamic and subject to deubiquitination to recover free ubiquitin and remodel existing polymeric ubiquitin chains. I find platelets contain several deubiquitinases that regulate platelet thrombotic aggregation and activation ex vivo, and that deubiquitinase inhibition reduced occlusive thrombosis in vivo. These results suggest modulation of deubiquitinase activity is a potential new approach to anti-platelet therapy.

3.2 Materials and Methods

3.2.1 Chemicals and reagents

Chemicals and reagents were purchased from: sterile filtered HBSS and sterile tissue culture plates (Falcon Labware); endotoxin-free human serum albumin (25% human albumin solution, Baxter Healthcare); PYR41 (Biogenova), PR619 (Life Sensors), b-AP15 (Sigma-Aldrich); polyubiquitin chains (Ub $_{2-7}$)

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K48/K63 linked, HA-Ubiquitin-Vinyl Sulfone (Boston Biochem); anti-ubiquitin antibody (P4D1; Santa Cruz Biotechnology, Inc.); anti-HAUSP antibody (Bethyl Laboratories); anti-Ub K48 antibody (Millipore); Calcein-AM (Molecular Probes$^{®}$, Life Technologies); DUB-GLO^{TM} Protease Assay kit (Promega); anti-Akt, anti-pAkt ser473, anti-pAkt thr308 and anti-pPTEN antibody (Cell Signaling). Other chemicals were from Sigma-Aldrich or Biomol Research Laboratories.

3.2.2 Platelet preparation

Human blood was drawn into acid-citrate-dextrose and centrifuged (200 × g, 20 min) without braking to obtain platelet-rich plasma in a protocol approved by the Cleveland Clinic Institutional Review Board. Purified platelets were prepared as stated (Brown and McIntyre 2011). Briefly, platelet-rich plasma was filtered through two layers of 5-µm mesh (BioDesign) to remove nucleated cells and recentrifuged (500 × g, 20 min) in the presence of 100 nM PGE$_1$. The pellet was resuspended in 50 ml PIPES/saline/glucose (5 mM PIPES, 145 mM NaCl, 4 mM KCl, 50 µM Na$_2$HPO$_4$, 1 mM MgCl$_2$, and 5.5 mM glucose) containing 100 nM PGE$_1$. These cells were centrifuged (500 × g, 20 min) and recovered platelets were centrifuged again before resuspension in 0.5% human serum albumin in HBSS.

3.2.3 Western blotting

Washed platelets (4 X 10^8/ml) were treated 25 µM PYR41, 30 µM PR619, 10 µM b-AP15, or buffer for 30 min before platelets were pelleted (2000 X g, 10 min) and lysed using radioimmunoprecipitation assay (RIPA) lysis buffer [150

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mM NaCl, 25 mM Tris (pH 7.6), 1% Nonidet P-40, 0.1% sodium dodecyl sulfate, 0.5% deoxycholate, 2 mM EDTA, 1 mM EGTA, 1 mM sodium orthovanadate, 10 mM sodium fluoride, 100 µM phenylmethylsulfonyl fluoride (PMSF), 1 µg/mL pepstatin, and 10 µg/mL leupeptin]. Samples were kept on ice for 30 min with occasional vortexing to ensure complete lysis before centrifugation (21,000 x g, 10 min). Reducing SDS sample buffer was added to the resulting supernatants before the proteins were resolved by SDS-PAGE.

3.2.4 Deubiquitination Assay and Ubiquitin chain disassembly

DUB-Glo™ protease assay system (Promega) assayed hydrolysis of luminogenic substrates for the deubiquitinating enzymes (DUBs). Washed platelets (4 X 10^8/ml) were pretreated with or without 25 µM PYR41 or 30 µM PR619 for 30 min, pelleted and lysed in ice cold DUB buffer (50 mM Tris-HCl, pH 7.5, 0.1% NP-40, 5 mM $MgCl_2$, 150 mM NaCl, 10 mM DTT, 1 mM sodium orthovanadate, 10 mM sodium fluoride, 100 µM PMSF, 1 µg/ml pepstatin, and 10 µg/ml leupeptin) and incubated with luminogenic substrate for 30 min before luminescence was measured. Ubiquitin chain disassembly was assessed by incubating recombinant polymerized K48 or K63 linked-ubiquitin chains with lysates (20 µg) from untreated, PYR41-(25 µM) or PR619-(30 µM) treated platelets for 30 min at 30 ºC followed by boiling in reducing sample buffer and resolution by SDS-PAGE. The extent of chain disassembly was assessed by western blotting using P4D1 antibody to recognize both free and polymeric ubiquitin.

Activity-based deubiquitinase labeling used washed platelets (4 X 10^8/ml) treated or not with 25 µM PYR41 or 30 µM PR619 for 30 min before lysis in ice cold DUB buffer. Lysates (20 µg) cleared by centrifugation were incubated with 200 ng HA-Ubiquitin vinyl sulfone (Boston Biochem) for 30 min at 30 ºC, boiled in reducing sample buffer, and resolved by SDS-PAGE. Proteins were transferred to nitrocellulose membranes and immunoblotted with anti-HA antibody to detect covalently labeled proteins.

3.2.5 *In vivo* thrombosis

Twelve week old C57B/6 mice were anesthetized with ketamine (90 mg/kg)/xylazine (15 mg/kg) and the right jugular vein and the left carotid artery were exposed via a middle cervical incision. Platelets were labeled by injecting 100 µl of rhodamine 6G (0.5 mg/ml) in saline into the right jugular vein. PYR41 (25 µM in DMSO; 4.7 mg/kg) or DMSO vehicle (10.5 µl) was added to the above solution, and the drug was allowed to circulate for 15 min before $FeCl_3$ injury (Li, Febbraio et al. 2010; Li, McIntyre et al. 2013). Thrombosis was induced in the left carotid artery by stripping the adventitia and placing a piece of black plastic under the vessel to reduce background fluorescence. A 1 X 2 mm piece of filter paper saturated with 7.5% $FeCl_3$ was applied to the carotid artery for 1 minute, the filter paper was removed, and the vessel rinsed with saline. Fluorescent thrombus formation was observed in real-time under a water immersion objective at 10 X magnification. Time to occlusive thrombosis was determined offline using video images captured with a QImaging Retigo Exi 12-bit mono digital camera

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(Surrey, Canada) and Streampix version 3.17.2 software (Norpix, Montreal, Canada). The end points were set as either cessation of blood flow for >30 seconds or no occlusion after 30 minutes (three times longer than the average occlusion time), in which case the time was recorded as 30 minutes for statistical comparison.

3.2.6 In vitro thrombus formation:

Microfluidics experiments were performed using the Cellix Microfluidics System (Cellix Ltd., Dublin, Ireland). Each micro channel of a Vena8 Fluoro+ biochip was coated with 15 µl of type 1 collagen (150 µg/ml) and the biochip was then placed in a humidified box, and incubated overnight at 4ºC. Each channel of the Vena8Fluoro+ biochip was washed with 1X PBS using the Mirus Nanopump before placing the biochip on the microscope stage. Images were collected using an HC Plan Apo 20X/0.7NA lens was on a Leica DMI6000 inverted microscope equipped with an environmental chamber and a Hamamatsu ImagEM cooled CCD camera. Whole blood collected from healthy volunteers was fluorescently tagged with Calcein-AM and was pretreated with or without 25 µM PYR41 or 30 µM PR619 for 30 min. After the incubation, blood was drawn through the channel at a shear rate of 67.5 dynes for 3 minutes. Images of platelets adhering to the collagen coating were captured every 2 seconds during that time. At the end of the experiment, the tube containing the blood was removed and the 1X PBS in the biochip reservoir was drawn through the channel at 2.5 dynes. Ten images were captured along the length of the channel during that time.

3.2.7 Flow cytometry

Platelet rich plasma (PRPs, 2 X 10^8/ml) was pretreated with either 25 µM PYR41, 30 µM PR619, or 10 µM b-AP15 for 15 min. After pretreatment, PRPs were incubated with 25 µM ADP and were stained simultaneously with PAC1-FITC or CD62P-PE and isotype control antibody (BD Biosciences) for 20 min room temperature. Washed platelets (2 X 10^8/ml) were pretreated with 25 µM PYR41, 30 µM PR619, 10 µM b-AP15, or buffer for 30 min and then were incubated with 0.2 U thrombin or 100 ng convulxin. These cells were then stained simultaneously with PAC1-FITC and CD62P-PE or with isotype control for 20 min room temperature. The samples were then analyzed using flow cytometery.

3.2.8 Total internal reflection fluorescence (TIRF) microscopy

Washed platelets (2 X 10^8/ml) were labeled with calcein-AM for 30 min and were incubated with 25 µM PYR41, 30 µM PR619, or buffer for 30 min before addition onto a drop of 0.2 U thrombin placed in a glass bottomed microwell (MatTek) dish. Imaging was performed at 100X with a 1.46 N.A. objective in a Leica AM TIRF MC System (Leica Microsystems, Wetzlar, Germany) equipped with an ImageEM C9100-13 EMCCD camera (Hamamatsu, Bridgewater, N.J). The 488 nm 10-mW diode laser was used for excitation and the penetration depth was set to 70 nm. Within 10 seconds of being placed on the stage, the sample was focused and a time-lapse series was initiated to collect images every 3 seconds for 5 minutes. At the end of the series, five static images were collected in both TIRF and differential interference contrast (DIC) modes of five different fields in the sample.

3.2.9 Aggregation

PRPs (2×10^8/ml) were pretreated with either 25 µM PYR41, 30 µM PR619, or 10 µM b-AP15 for 15 min. After pretreatment, PRPs were stimulated with 5 µM ADP (Chronolog). Washed platelets (2×10^8/ml) were pretreated with either 25 µM PYR41, 30 µM PR619, or 10 µM b-AP15 for 30 min before stimulation with 0.2 U thrombin, 2 µg/ml Collagen or 100 ng convulxin. Platelet aggregation was measured by transmittance (Chronolog) with stirring (900 rpm).

3.2.10 Expression of data and statistics

All experiments were performed at least three times with cells from different donors, and all assays were performed in triplicate. The standard errors of the mean from all experiments are presented as error bars. Figures and statistical analyses were generated with Prism4 (GraphPad Software). A value of $p \leq 0.05$ was considered statistically significant.

3.3 Results

3.3.1 Deubiquitination of the platelet proteome promotes thrombosis

Arterial thrombosis is modeled *in vivo* by $FeCl_3$-induced injury of the wall of murine carotid arteries that leads to rapid formation of a platelet-rich occlusive barrier at the site of injury (Li, Febbraio et al. 2010; Li, McIntyre et al. 2013). Typically, complete cessation of flow through the artery of DMSO injected mice occurs 12 min after the brief exposure to ectopic $FeCl_3$ (Fig. 3.1 A). Disruption of ubiquitin metabolism by intravenous injection of PYR41 15 min prior to vessel injury significantly lengthened the time to occlusion to 26 min.

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The occlusive thrombus formed in FeCl$_3$-damaged carotid arteries is almost exclusively formed by platelet accretion, so I determined whether platelets contained deubiquitinase activity that was inhibited by PYR41. Deubiquitinase enzymes are specifically covalently adducted during hydrolysis of HA-tagged ubiquitin vinyl sulfone (Borodovsky, Kessler et al. 2001), and this mechanism-based inhibitor modified a number of platelet proteins (Fig. 3.1 B). PYR41 and a structurally unrelated deubiquitinase inhibitor PR619 decreased catalytic labeling of all platelet deubiquitinases. Direct measurement of ubiquitination hydrolytic activity showed platelet lysates contain deubiquitinase enzymatic activity, and that both PYR41 and PR619 showed platelets have active deubiquitinases and these compounds are equally effective in inhibiting them (Fig. 3.1 C).

The soluble proteome of quiescent platelets contains numerous ubiquitin conjugated proteins (Fig. 3.1 D), primarily with slow mobility during electrophoresis. Exposing platelets to either PYR41 or the PR619 deubiquitinase inhibitor for 30 min prior to lysis increased poly-ubiquitination of these soluble platelet proteins. The pattern of ubiquitinated proteins after either PYR41 or PR619 treatment recapitulated that of quiescent platelets, so platelets rapidly and continuously cycle ubiquitin onto select proteins.

Deubiquitinase enzymes are selective for ubiquitin chain linkages (Fraile, Quesada et al. 2012). Lysates from PYR41- or PR619-treated platelets showed limited disassembly of K48- linked (Fig. 3.1 E) and only minor effect on K63-linked (Fig. 3.1 F) poly-ubiquitin chains compared to lysates from untreated

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platelets. This limited chain disassembly may account for the accumulation of

ubiquitin conjugates of high molecular weight observed in Fig. 3.1D.
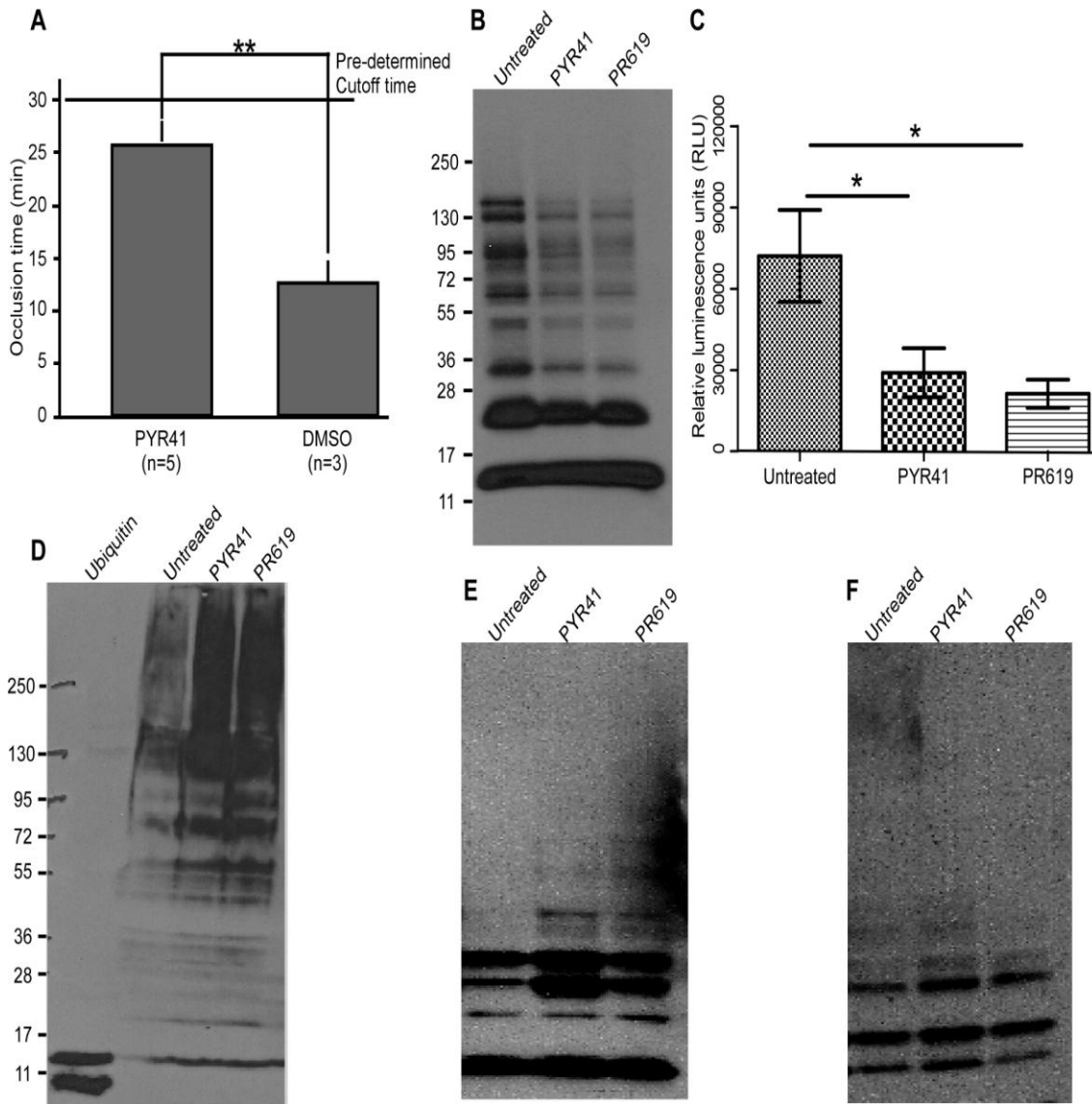
Figure 3.1 Platelets express functional deubiquitinases that promote thrombosis

(A) Deubiquitinase inhibitor, PYR41 prolongs the time to vascular occlusion. Mice were injected with PYR41 or DMSO and thrombosis was induced 15 min later as described in "Methods". Time to complete cessation of blood flow in the murine carotid artery was determined using intravital microscopy (n=5 experimental, 3 control; **p≤ 0.01). **(B)** Platelets contain several deubiquitinases. Western blot of anti-HA antibody after active-site–directed labeling of deubiquitinases with HA-tagged Ub-vinylsulfone in platelet lysates from washed platelets treated with or without PYR41 or PR619 (n=3). **(C)** The isopeptidase activity of platelet deubiquitinases was assayed using a luminogenic substrate in the lysates of untreated and PYR41 or PR619 treated platelets (n=3; *p≤ 0.05). **(D)** PYR41 or PR619 pretreatment increases ubiquitination of platelet proteome. Immunoblot of platelet lysates, treated or not with PYR41 or PR619, probed with anti-ubiquitin antibody (P4D1, n=3). PYR41 or PR619 pretreatment limits hydrolysis of **(E)** Lysine48- linked and **(F)** Lysine63- linked heptameric chain as detected by western blotting with anti-ubiquitin antibody (P4D1, n=2).

3.3.2 Deubiquitinase inhibitors suppress platelet adhesion to collagen under shear flow

Deubiquitinase inhibition in platelets reduced hydrolysis of ubiquitin chains and caused accumulation of ubiquitinated proteins, indicating that the ubiquitin cycling is affected by the inhibitors and that deubiquitinase inhibition directly alters platelet function to reduce adhesion to collagen exposed in injured arteries. To test this, I examined platelet adhesion during the flow of whole blood through a collagen-coated microfluidic channel that generates high shear. Fluorescently labeled platelets in whole blood were immobilized along the chamber, as shown in a typical video frame captured at the distal end of the chamber after 3 min of flow (Fig. 3.2 A). Preincubating cells with either PYR41 or PR619 reduced the number of platelets adhering in the collagen-coated well by 80% (Fig. 3.2 B). This outcome shows the adhesive phenotype of activated platelets depends on rapid deubiquitination of their proteome.

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A
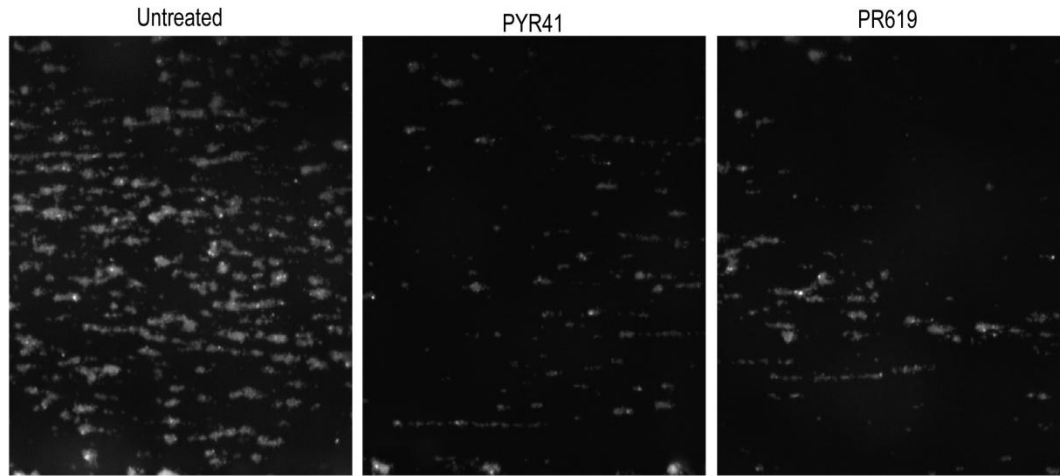
B
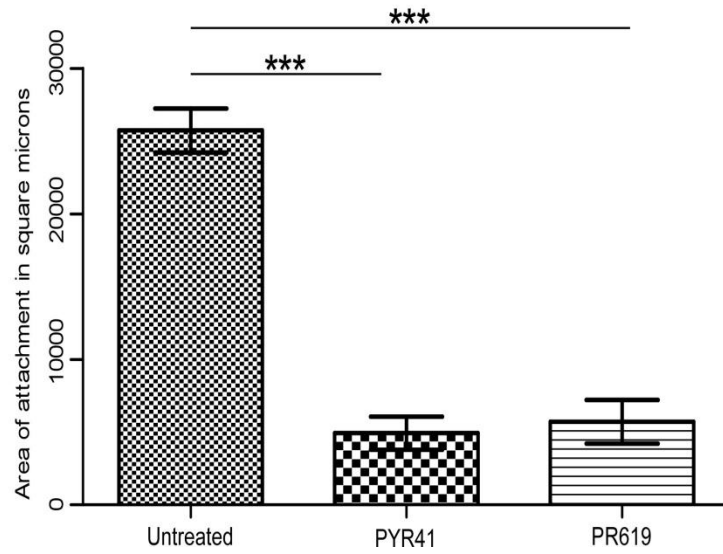
Figure 3.2 Deubiquitinase inhibitors suppressed in vitro thrombosis

(A) PYR41 or PR619 pretreatment blocked platelet adhesion to collagen at high shear. Calcein-AM labeled blood, treated or not with PYR41 or PR616, was perfused over immobilized type 1 collagen fibrils (150 µg/ml) at 67.5 dyne/cm^2 for 3 min. Images are representative fields taken from three independent experiments that yielded similar results (n=3). **(B)** Area of platelet attachment after PYR41 or PR619 treatment. Platelet area in panel A was quantified by ImagePro plus software and results are plotted as area of platelet adhesion in square microns (n=3; ***p≤ 0.001).

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3.3.3 Stimulated homotypic platelet aggregation, adhesion, and spreading are suppressed by deubiquitinase inhibitors

Washed human platelets aggregated in response to thrombin (Fig. 3.3 A), ADP (Fig. 3.3 B), or collagen (Fig. 3.3 C). Pre-incubation with either PYR41 or PR619 suppressed aggregation in response to each of these agonists. Neither compound alone promoted aggregate formation (Fig. 3.3 D), but inhibition by each deubiquitinase inhibitor was statistically significant and the inhibition by the two deubiquitinase inhibitors was equivalent (Figs. 3.3 E-G).

Outside-in signaling as platelets interact with a glass substrate induces cytoskeletal rearrangement, extension of filopodia and lamellapodia. Thrombin stimulation enhances these responses captured by total internal reflection microscopy (Fig. 3.3 H). Both PYR41 and PR619 abolish the adhesion and spreading of unstimulated platelets, while greatly reducing these responses after thrombin stimulation (Fig. 3.3 I).

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A — Percentage Aggregation — Thr+ PR619 / Thr+ PYR41 / Thr

B — Percentage Aggregation — ADP+ PR619 / ADP+ PYR41 / ADP

C — Percentage Aggregation — Collagen+ PR619 / Collagen+PYR41 / Collagen

D — Percentage Aggregation — Untreated / PR619 / PYR41 / Controls

E

Thrombin	–	–	+	+	+
PYR41	+	–	–	+	–
PR619	–	+	–	–	+

F

ADP	–	–	+	+	+
PYR41	+	–	–	+	–
PR619	–	+	–	–	+

G

Collagen	–	–	+	+	+
PYR41	+	–	–	+	–
PR619	–	+	–	–	+

H

Untreated   PYR41   PR619

Thrombin   Thrombin+ PYR41   Thrombin+ PR619

I — Area of attachment in square microns

Untreated   PYR41   PR619   Thr   Thr+ PYR41   Thr+ PR619

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Figure 3.3 Deubiqutinase inhibition reduced platelet response to agonists

(A) PYR41 or PR619 pretreatment attenuated thrombin (thr)- **(B)** ADP- and **(C)** collagen-induced aggregation. **(D)** Neither PYR41 nor PR619 stimulated platelet aggregation in the absence of agonists. **(E)** PYR41 or PR619 imposed a reduction in thrombin (thr)- **(F)** ADP- and **(G)** collagen-induced aggregation is statistically significant (n=3; ***$p \leq 0.001$). **(H)** PYR41 or PR619 reduced thrombin stimulated adhesion and spreading. Interaction of control or thrombin stimulated (thr; 0.2 U) platelets with a glass substrate was imaged by total internal reflection microscopy after 5 min (n=3). **(I)** Platelet area after PYR41 or PR619 treatment. Platelet surface area in panel (A) was quantified by Imagepro plus software (n=3; ***$p \leq 0.001$).

3.3.4 Agonist activation of glycoprotein αIIbβ3 is reduced by deubiquitinase inhibition

Agonist stimulation alters the conformation of the platelet β3 integrin of glycoprotein αIIbβ3 to enhance platelet-platelet and platelet-endothelial cell interactions (Gawaz, Neumann et al. 1999). An epitope present in activated αIIbβ3 is recognized by the antibody PAC-1, and thrombin stimulation greatly increased surface PAC-1 binding compared to quiescent control platelets (Figs. 3.4 A). Pre-treating platelets with the deubiquitinase inhibitor PYR41 for 15 min greatly suppressed PAC-1 binding to the extent that αIIbβ3 activation was not statistically distinct from unstimulated platelets (Fig. 3.4 B). The general deubiquitinase inhibitor PR619 also effectively reduced thrombin-stimulated expression of PAC-1 (Figs.3.4 E, F). Inhibition of deubiquitinase activity by either PYR41 (Figs. 3.4 C, D) or PR619 (Figs. G, H) also fully suppressed thrombin stimulation of alpha granule secretion that was detected by the translocation of P-selectin from alpha granules to the platelet surface.
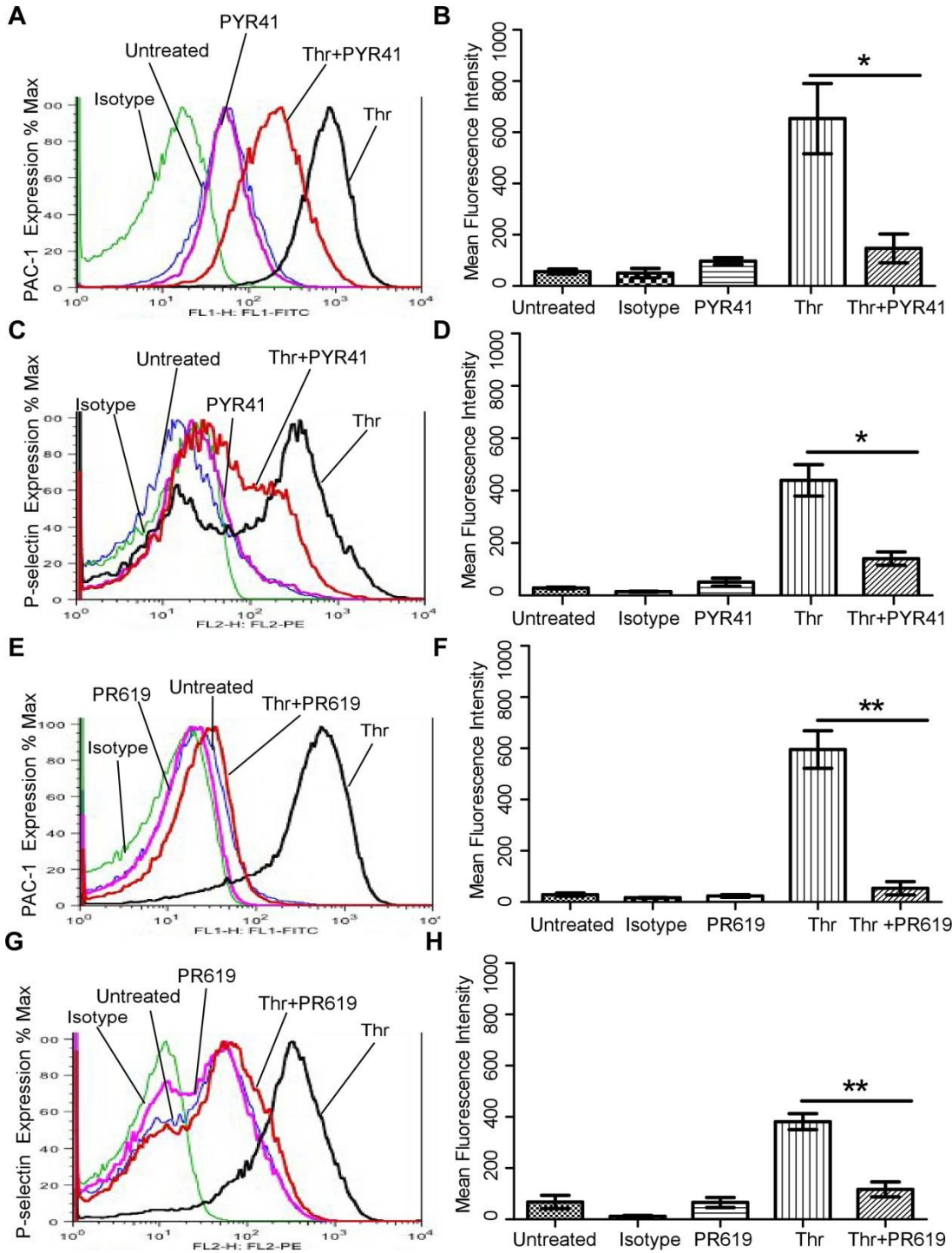
Figure 3.4 Inhibitors of platelet deubiquitinases suppressed protease activated receptor (PAR) mediated αIIbβ3 activation and degranulation

(A) PYR41 pretreatment suppressed stimulated αIIbβ3 activation. Flow cytometry using PAC-1 antibody, that binds active αIIbβ3, on platelets treated or not with PYR41 and with or without 0.2 U thrombin (thr, n=3). **(B)** Mean fluorescence intensity (MFI) of PAC-1 binding on platelets in panel A (n=3; *p≤ 0.05). **(C)** PYR41 pretreatment reduced P-selectin surface expression upon activation. Flow cytometry using PE-conjugated-anti-P-selectin antibody on platelets treated or not with PYR41 and with or without 0.2 U thrombin (thr, n=3). **(D)** Mean fluorescence intensity (MFI) of P-selectin binding on platelets in panel C (n=3; *p≤ 0.05). **(E)** PR619 pretreatment suppressed stimulated αIIbβ3 activation. Flow cytometry using PAC-1 antibody, that binds active αIIbβ3, on platelets treated or not with PR619 and with or without 0.2 U thrombin (thr, n=3). **(F)** Mean fluorescence intensity (MFI) of PAC-1 binding on platelets in panel E (n=3; **p≤ 0.01). **(G)** PR619 pretreatment reduced P-selectin surface expression upon activation. Flow cytometry using PE-conjugated-anti-P-selectin antibody on platelets treated or not with PR619 and with or without 0.2 U thrombin (thr, n=3). **(H)** Mean fluorescence intensity (MFI) of P-selectin binding on platelets in panel G (n=3; **p≤ 0.01).

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3.3.5 Inhibitors of platelet deubiquitinases suppressed ADP mediated αIIbβ3 activation and degranulation

Thrombin at the concentration used above activates platelets primarily through the PAR1 receptor, but activation of platelets through a second receptor, the $P2Y_{12}$ receptor for ADP, was also abolished by deubiquitinase inhibition. Thus, ADP stimulation increased binding of PAC-1 to platelets (Fig. 3.5 A), although not to the extent induced by thrombin, and PYR41 effectively reduced the extent of αIIbβ3 activation after ADP activation to nearly quiescent levels (Fig. 3.5 B). PYR41 pretreatment also suppressed P-selectin exocytosis upon ADP stimulation (Fig. 3.5 C) and this reduction was statistically significant (Fig. 3.5 D). PR619 was equally effective in suppressing platelet integrin activation (Figs. 3.5 E, F).
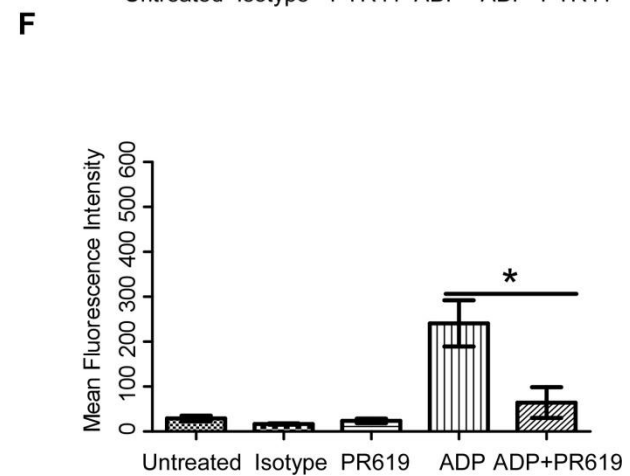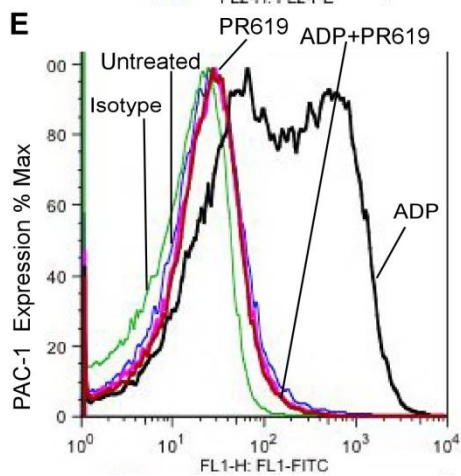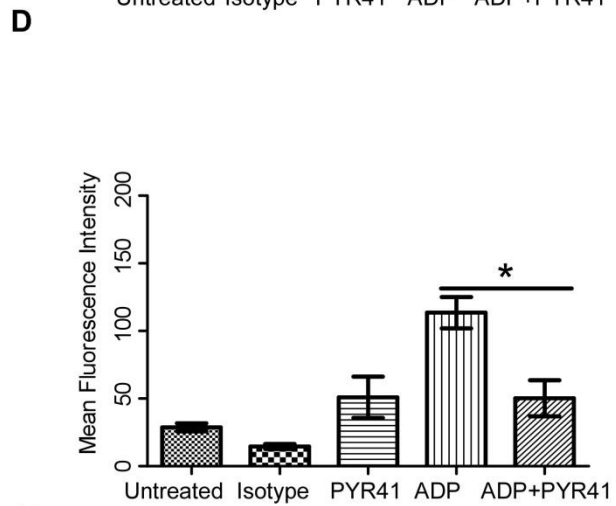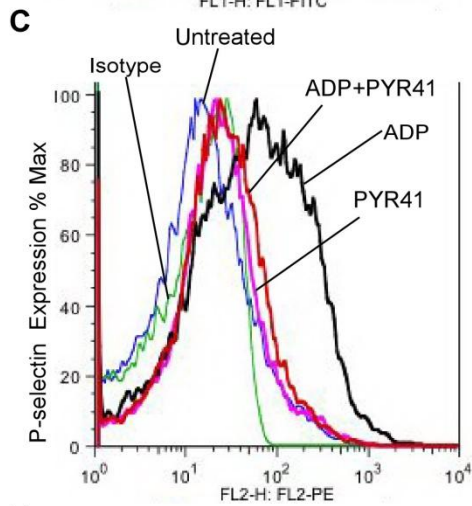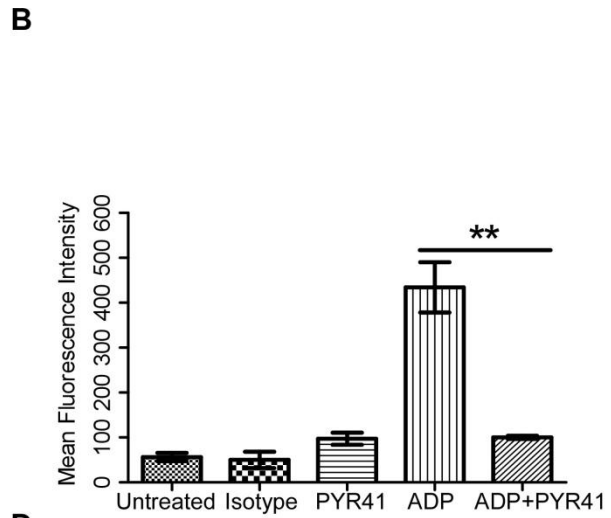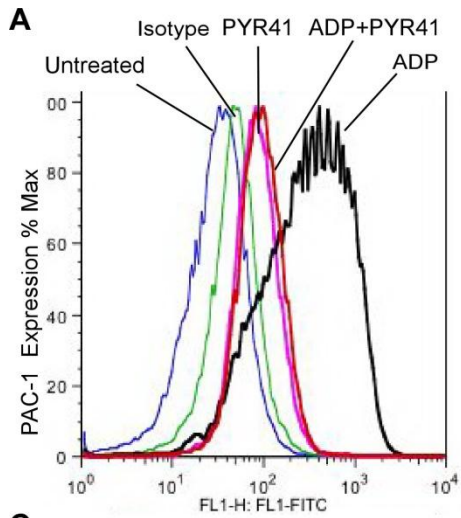
Figure 3.5 Deubiquitinase inhibitors diminished ADP induced platelet αIIbβ3 activation and degranulation

(A) PYR41 pretreatment suppressed stimulated αIIbβ3 activation. Flow cytometry using PAC-1 antibody, that binds active αIIbβ3, on platelets treated or not with PYR41 and with or without 25 µM ADP (n=4). **(B)** Mean fluorescence intensity (MFI) of PAC-1 binding on platelets in panel A (n=4; **p≤ 0.01). **(C)** PYR41 pretreatment reduced P-selectin surface expression upon activation. Flow cytometry using PE-conjugated- anti-P-selectin antibody on platelets treated or not with PYR41 and with or without 25 µM ADP (n=3). **(D)** Mean fluorescence intensity (MFI) of P-selectin binding on platelets in panel C (n=3; *p≤ 0.05). **(E)** PR619 pretreatment suppressed stimulated αIIbβ3 activation. Flow cytometry using PAC-1 antibody, that binds active αIIbβ3, on platelets treated or not with PR619 and with or without 25 µM ADP (n=3). **(F)** Mean fluorescence intensity (MFI) of PAC-1 binding on platelets in panel E (n=3; *p≤ 0.05).

3.3.6 Proteasome associated deubiquitinases regulate platelet activation

A recent report using quantitative mass spectrometry shows platelets contain several cytoplasmic deubiquitinase enzymes including proteasome associated deubiquitinases USP14 and UCHL5 (Burkhart, Vaudel et al. 2012). USP14 and UCHL5 levels are upregulated in many cancers (D'Arcy and Linder 2012) and thus are potential targets for anti-cancer therapies. A small molecule inhibitor, b-AP15, was recently identified that specifically inhibited just these two deubiquitinases without affecting the catalytic activities of proteasome (D'Arcy, Brnjic et al. 2011). Both USP14 and UCHL5 are present in high copy numbers in platelets, 3100 and 1500 copies/platelet, respectively (Burkhart, Vaudel et al. 2012), but whether these enzymes are relevant in platelets is not known. I tested b-AP15 in platelets and observed *ex vivo* treatment of platelets with b-AP15 leads to accumulation of high molecular weight proteins as detected by anti-ubiquitin P4D1 antibody (Fig. 3.6 A).

Next I ascertained the role of proteasome associated deubiquitinases, USP14 and UCHL5, on platelet functions. Inhibition of these deubiquitinases with b-AP15, inhibited thrombin- (Fig. 3.6 B), collagen- (Fig. 3.6 C) and ADP- (Fig. 3.6 D) induced aggregation and, like the general deubiquitinase inhibitors PYR41 and PR619, the inhibitory effect of b-AP15 on agonist-induced platelet aggregation was significant (Fig. 3.6 E). b-AP15 treatment alone had no effect on platelet aggregation. b-AP15 pretreatment also inhibited thrombin- (Fig. 3.6 F) induced αIIbβ3 activation as assessed by PAC-1 binding as well as P-selectin exocytosis from platelet alpha-granules (Fig. 3.6 G). Activation of αIIbβ3 is critical

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for stable thrombus formation and consolidation *in vivo* (Shattil and Newman 2004; Gratacap, Guillermet-Guibert et al. 2011) and pretreatment with b-AP15 also blocked ADP- (Fig. 3.6 H) induced αIIbβ3 activation. USP14 and UCHL5 inhibition had a more profound inhibitory effect on collagen-induced aggregation (Fig. 3.6 C) than the general deubiquitinase inhibitors, so I tested the effect of inhibition of these enzymes on platelet adhesion to collagen under static conditions. b-AP15 pretreatment reduced the number of platelets adhering to a exposed collagen-coated surface (Fig. 3.6 I), suggesting vital role of proteasome associated deubiquitinases in mediating platelet interactions with bound or exposed collagen.
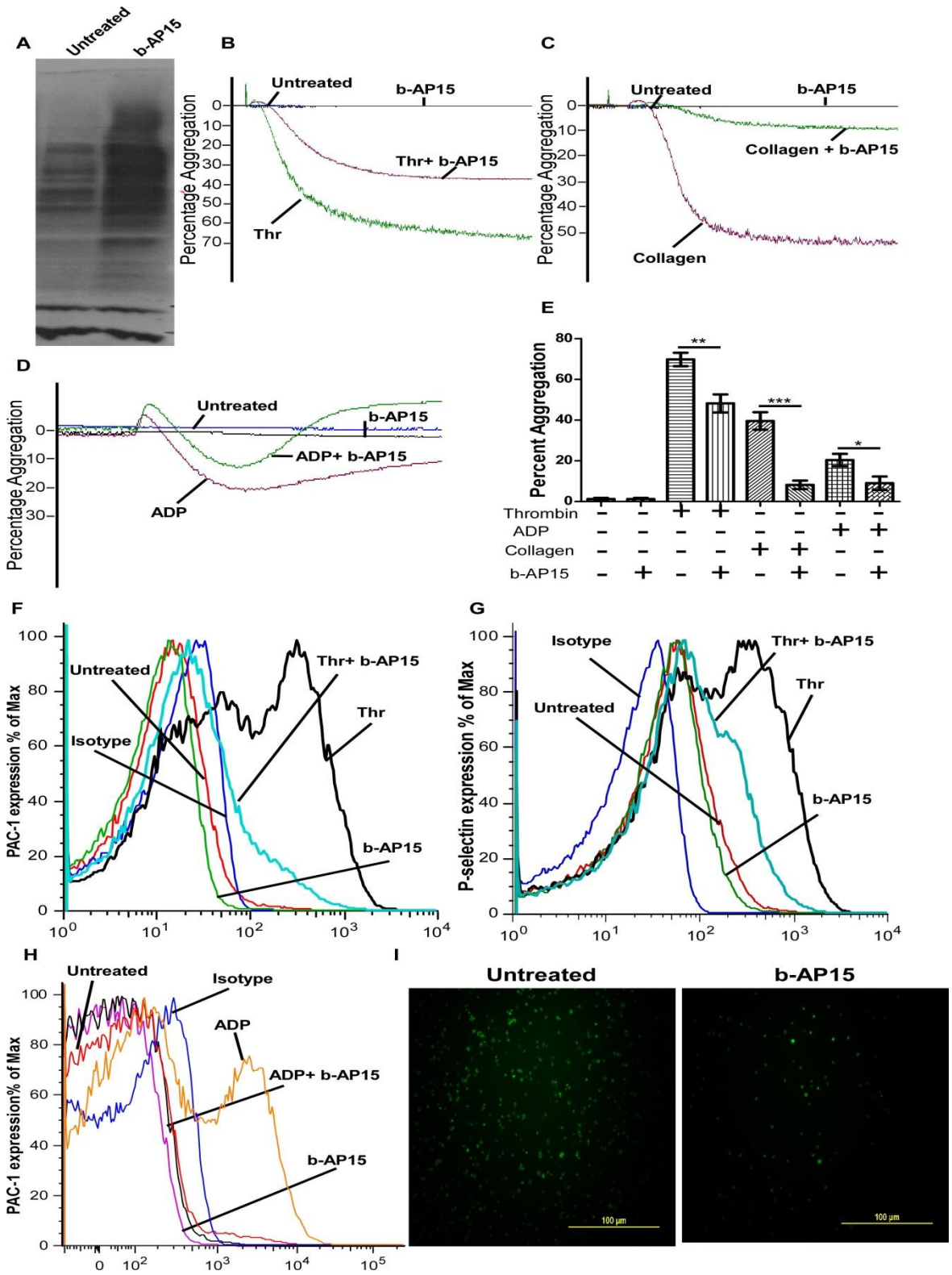
Figure 3.6 Pharmacologic inhibition of proteasome-associated deubiquitinase enzymes, USP14 and UCHL5, reduced platelet responsiveness to agonists

(A) b-AP15 pretreatment increases ubiquitination of the platelet proteome. Immunoblot of platelet lysates, treated or not with b-AP15, probed with anti-ubiquitin antibody (P4D1, n=3). **(B)** Proteasome-associated deubiquitinases promote stimulated platelet aggregation. b-AP15 pretreatment inhibited thrombin- (thr, n=3) **(C)** collagen- (n=3) and **(D)** ADP-induced aggregation (n=3). **(E)** b-AP15 mediated inhibition of stimulated platelet aggregation is statistically significant (n=3; ***p≤ 0.001, **p≤0.01, *p≤ 0.05). **(F)** b-AP15 pretreatment suppressed stimulated αIIbβ3 activation. Flow cytometry using PAC-1 antibody, that binds active αIIbβ3, on platelets treated or not with b-AP15 and with or without 0.2 U thrombin (thr, n=3). **(G)** b-AP15 pretreatment reduced P-selectin surface expression upon activation. Flow cytometry using PE-conjugated- anti-P-selectin antibody on platelets treated or not with b-AP15 and with or without 0.2 U thrombin (thr, n=3). **(H)** Preincubation with b-AP15 suppressed ADP-induced αIIbβ3 activation. Flow cytometry using PAC-1 antibody, that binds active αIIbβ3, of platelets treated or not with b-AP15 and with or without 25 µM ADP (n=3). **(I)** Proteasome-associated deubiquitinases mediate platelet adhesion to collagen coated surface. Microscopic images of platelet adhesion on collagen under static conditions (n=2).

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3.3.7 Platelet deubiquitinases affect collagen mediated responses via the gpVI receptor

Collagen binds and activates platelets via two major platelet surface receptors, α2β1 and gpVI (Nuyttens, Thijs et al. 2011). Since all three deubiquitinase inhibitors had a profound effect on collagen-induced aggregation, I next looked at the effect of these inhibitors specifically on the gpVI receptor, the main signaling receptor for collagen (Nieswandt and Watson 2003). Neither the general deubiquitinase inhibitors, PYR41 and PR619, nor the specific deubiquitinase inhibitor, b-AP15, alone induced platelet aggregation (Fig. 3.7 A), but all three inhibitors suppressed aggregation to convulxin, a snake venom toxin that activates platelets via gpVI receptor (Polgar, Clemetson et al. 1997) (Fig. 3.7 B). Convulxin stimulation increased PAC-1 binding, a marker of αIIbβ3 activation, but pretreatment with PYR41 (Fig. 3.7 C) or PR619 (Fig. 3.7 D) or b-AP15 (Fig. 3.7 E) reduced convulxin induced increase in PAC-1 binding. This suggest that the platelet deubiquitinases are critical for gpVI-mediated transduction of collagen signals.

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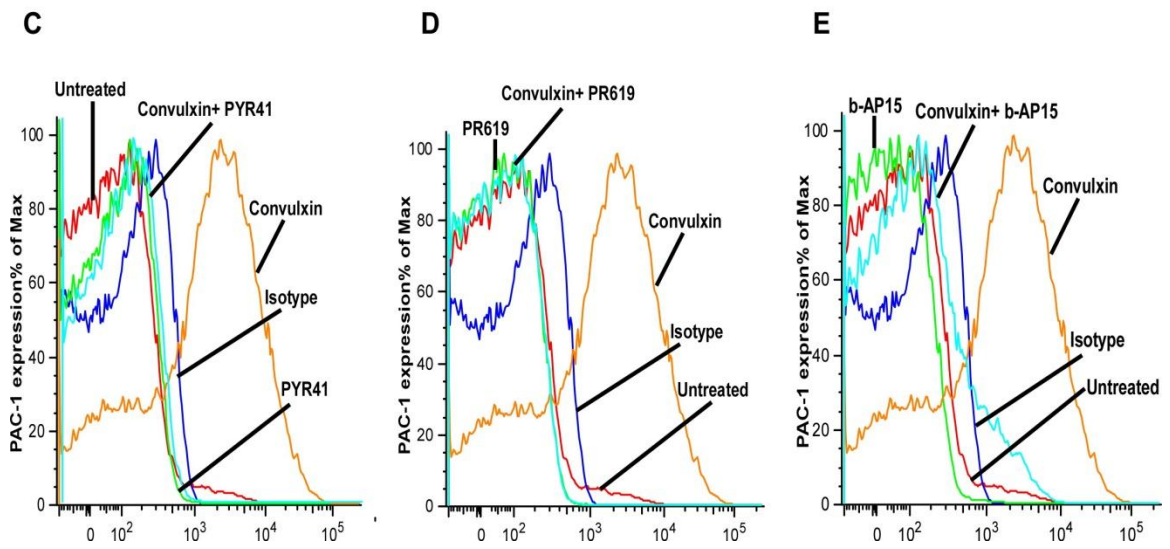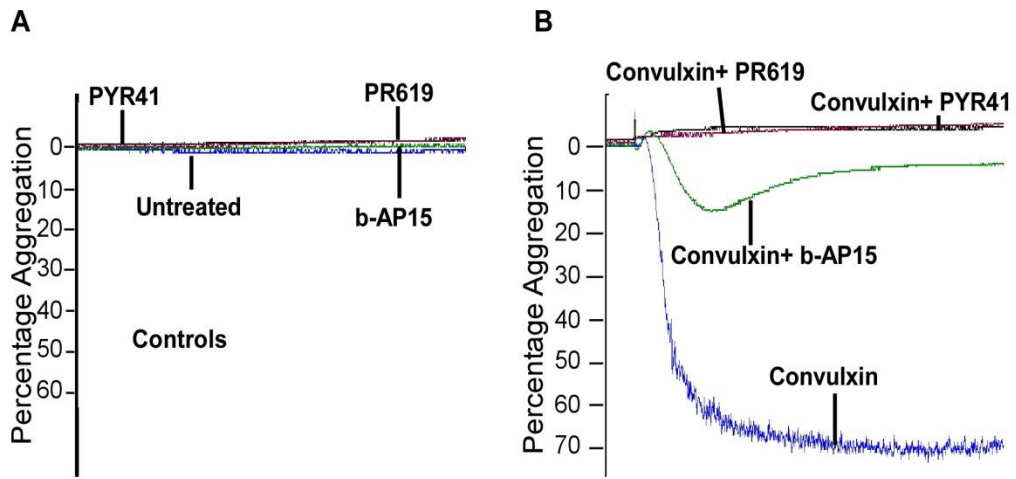
Figure 3.7 Deubiquitinase inhibitors affect collagen responses via the gpVI receptor

 (A) Neither general deubiquitinase inhibitors, PYR41 and PR619, nor b-AP15 stimulated platelet aggregation in the absence of agonists. **(B)** Deubiquitinase inhibitors inhibited gpVI specific platelet responses. Platelets were pretreated with PYR41, PR619, or b-AP15 before initiating platelet aggregation with convulxin (n=2). **(C)** Convulxin-mediated increase in PAC-1 binding was assessed using flow cytometry in platelets pretreated with PYR41 (n=2), **(D)** PR619 (n=2), or **(E)** b-AP15 (n=2).

3.3.8 Inhibition of platelet deubiquitinases modulated platelet signaling responses downstream of thrombin and collagen receptors

Akt, or protein kinase B, is a serine/ threonine kinase that is an established downstream effector of phosphoinositide 3-kinase (PI3K) (Bhaskar and Hay 2007). Platelets express two Akt isoforms, Akt1 and Akt2 (Kroner, Eybrechts et al. 2000; Dittrich, Birschmann et al. 2008) and targeted deletion of each individual isoform affects platelet function. Akt1 deletion in mice resulted in diminished platelet responses to thrombin and collagen (Chen, De et al. 2004; Stojanovic, Marjanovic et al. 2006) and Akt2 deletion suppressed platelet secretion and thrombosis (Woulfe, Jiang et al. 2004).

Platelet stimulation with thrombin resulted in time dependent increase in Akt phosphorylation at both ser473 and thr308 residues (Fig.3.8 A), but pretreatment with either general deubiquitinase inhibitors, PYR41 and PR619, or the specific deubiquitinase inhibitor, b-AP15, reduced thrombin-induced Akt activation as evident from reduced Akt phosphorlylation at both ser and thr residues (Fig. 3.8 A). An equivalent inhibition of collagen-induced Akt phosphorylation, either at ser473 or thr308, (Fig. 3.8 B) was also observed with all three inhibitors of deubiquitinases. The inhibition of Akt phosphorylation at thr308, induced by either agonist, was more profound compared to phosphorylation at ser473. In platelets, Akt activation is both PI3K dependent and independent, but sustained Akt activation is PI3K dependent (Jackson and Schoenwaelder 2010). Class IA PI3K isoform β (PI3K β) is an essential PI3K to promote PLCγ-2 activation downstream of gpVI-FcRγ receptor (Gratacap,

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Guillermet-Guibert et al. 2011). PLCγ-2 activation leads to intracellular calcium release and DAG production and both events are critical for sustained αIIbβ3 activation and fibrin clot retraction (Canobbio, Stefanini et al. 2009; Martin, Guillermet-Guibert et al. 2010). Collagen-induced intracellular calcium release was inhibited when platelets were preincubated with deubiquitinase inhibitors before stimulation by collagen (Fig.3.8 C). Deubiquitinase inhibitors also significantly reduced fibrin clot retraction suggesting impaired outside in signaling from integrin αIIbβ3 (Fig. 3.8 D).

Phosphatase and tensin homologue-deleted on chromosome 10 (PTEN) is a known negative regulator of PI3K/Akt signaling (Carracedo and Pandolfi 2008) that regulates collagen-dependent platelet activation. Tissue specific *pten* deletion in mice resulted in increased Akt phosphorylation and platelet aggregation even at much lower collagen concentrations, indicating that PTEN negatively regulates collagen mediated platelet activation (Weng, Li et al. 2010). PTEN's phosphatase activity is negatively regulated by phosphorylation at ser 380 (Vazquez, Ramaswamy et al. 2000; Yang, Yuan et al. 2013), however this is not studied in platelets so far. I observed both thrombin and collagen increased PTEN phosphorylation at this residue (Fig. 3.8 E) and pretreatment with deubiquitinase inhibitors reduced agonist dependent increase in PTEN phosphorylation and inactivation.

Since deubiquitinases display linkage specificity for ubiquitin chains (Komander, Clague et al. 2009), I used linkage specific antibodies (K63 or K48 specific antibodies) to determine the relative abundance of both type of linkages

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in platelets. I observed accumulation of lysine 48 linkages (Fig. 3.8 F), but interestingly I did not find any accumulation of lysine 63 linkages in untreated, stimulated or deubiquitinase inhibitor treated platelets (not shown). An important role of deubiquitinases is generation of free ubiquitin pool from unanchored polyubiquitin chains. I observed accumulation of high molecular weight polyubiquitin chains upon inhibition of platelet deubiquitinases, suggesting that deubiquitinase enzyme mediated ubiquitin homeostasis is critical for platelet responses.
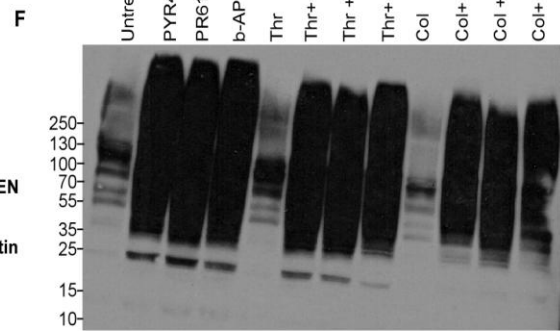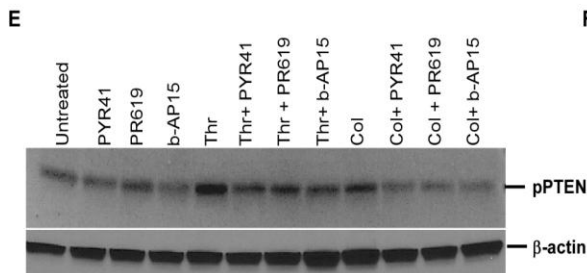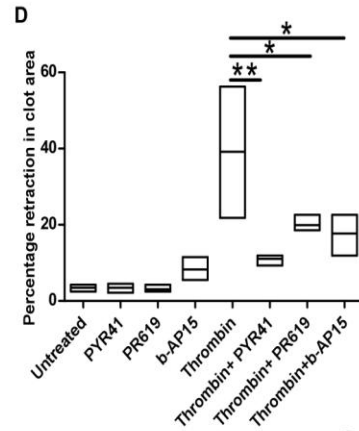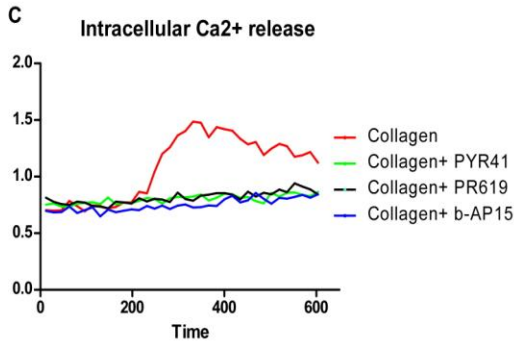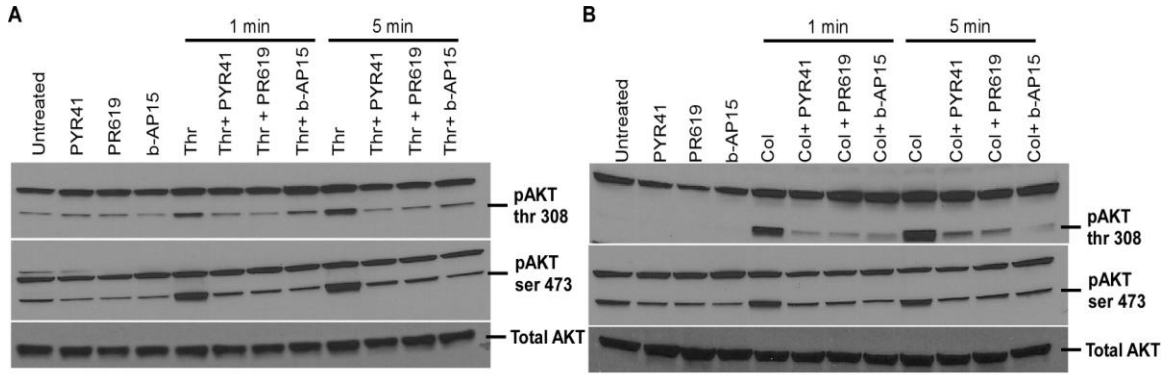
Figure 3.8 Deubiquitinase inhibitors impaired platelet signaling following agonist stimulation

(A) Deubiquitinase inhibitors reduced thrombin induced Akt activation. Platelets treated with or without deubiquitinase inhibitors were stimulated with 0.2 U thrombin (thr) for the stated times and immunoblotted using anti-pAKT ser473, anti-pAKT thr308 or anti-Akt antibodies (n=3). **(B)** Deubiquitinase inhibitors reduced collagen-induced Akt activation. Platelets treated with or without deubiquitinase inhibitors were stimulated with 2 μg collagen for the stated times and immunoblotting was done on platelet lysates using anti-pAKT ser473, anti-pAKT thr308 or anti-Akt antibodies (n=3). **(C)** Deubiquitinase inhibitors blocked collagen-induced intracellular calcium release. Platelets labeled with Fura-2 AM were treated or not with deubiquitinase inhibitors before stimulation with 2 μg collagen. **(D)** Deubiquitinase inhibition suppressed clot retraction. Ratio of the image surface area of images of thrombin-induced clots over time (n=3; $^{**}p < 0.01$, $^*p<0.05$). **(E)** Deubiquitinase inhibition activates PTEN. Immunoblotting of platelets treated or not with deubiquitinase inhibitors before stimulation with 0.2 U thrombin or 2 μg collagen was done using anti-phospho PTEN-ser380 antibody (n=2). **(F)** Deubiquitinase inhibition caused accumulation of lys-48 linked Ub conjugates. Immunoblotting of platelets treated or not with deubiquitinase inhibitors before stimulation with 0.2 U thrombin (thr) or 2 μg collagen was done using anti-K48 linked ubiquitin antibody (n=2).

3.5 Discussion

The proteome of platelets is constitutively decorated by conjugated single ubiquitin molecules and by ubiquitin polymeric chains, whose abundance increases with agonist stimulation (Gupta, Li et al. 2014). Here I present evidence showing that ubiquitin decoration of platelet proteins is dynamic. Turnover of ubiquitin decoration was elucidated by suppressing deubiquitinase activity by any of the general deubiquitinase inhibitors or the specific inhibitor of proteasome-associated deubiquitinases. This greatly increased ubiquitin protein modification within minutes. These changes were apparent in the absence of agonist stimulation, so the protein complement of quiescent platelets undergoes a continuous, and rapid, cycle of ubiquitination and deubiquitination.

Protein deubiquitination is, in fact, a critical step in unlocking platelet reactivity. The general deubiquitinase inhibitors PYR41 or PR619 blocked activation of αIIbβ3 integrin, blocked alpha granule release and hence blocked the agonist-stimulated increase of surface P-selectin. These inhibitors also effectively blocked *ex vivo* homotypic platelet aggregation. The general inhibitor PYR41 additionally blocked formation of platelet-rich thrombi that completely occluded blood flow in damaged carotid arteries. I conclude the complete suppression of stimulated platelet function by deubiquitinase inhibition means that ubiquitin modification of the proteome likely maintains platelets in an inactive state and that deubiquitinase action may be required to release tonic ubiquitin inhibition.

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Deubiquitinase inhibitors affect platelet signaling downstream of platelet receptors. Thrombin and collagen induced Akt phosphorylation was reduced by both the pan deubiquitinase inhibitors, PYR41 and PR619, and the specific inhibitor of proteasome associated deubiquitinase activity, b-Ap15. Akt activation in platelets is both PI3K-dependent and PI3K-independent (Laurent, Severin et al. 2014), however, class IA PI3K isoform β (PI3K β) is mandatory for gpVI-FcRγ receptor-mediated Akt activation, platelet aggregation, intracellular calcium release and degranulation (Kim, Mangin et al. 2009). I attribute these effects to the upregulation of PTEN activity upon pretreatment with deubiquitinase inhibitors. PTEN negatively regulates Akt activation by converting phosphatidyinositol 3,4,5-trisphosphate (PIP$_3$) to phosphatidylinositol 4,5-bisphosphate (PIP$_2$) and its been reported to negatively regulate collagen-induced platelet activation (Weng, Li et al. 2010). Deubiquitinase inhibitors also significantly reduced clot retraction over time, indicating impaired outside-in integrin signaling. Phosphoinositide-dependent protein kinase 1 (PDK1) is activated upon an increase in PIP$_3$ following PI3K activation and activated PDK1 phosphorylates Akt at residue thr308 (Moore, Hunter et al. 2011). Platelet specific knockdown of PDK1 and subsequent loss in Akt phosphorylation reduced thrombin-induced platelet aggregation, clot retraction and thrombosis (Chen, Zhang et al. 2013).

Activation of PI3K and its effector, Akt, is essential for most aspects of collagen-induced platelet activation, and thrombin- and collagen-induced αIIbβ3 integrin outside-in signaling. The effect of PI3K/Akt axis on thrombin-induced

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aggregation is only seen at low thrombin concentrations and is overcome at high agonist concentrations (Martin, Guillermet-Guibert et al. 2010; Chen, Zhang et al. 2013). Additionally, the effect of thrombin-induced aggregation can be reversed using P_2Y_{12} inhibitors, suggesting it's not a direct effect but is due to thrombin-induced platelet ADP secretion (Kim, Mangin et al. 2009). However, it is controversial because P_2Y_{12} knockout mice showed Akt phosphorylation in response to thrombin, indicating a direct effect. In the same study it was shown that the Src family of kinases (SFKs) act upstream of PI3K/Akt axis. The discrepancy was attributed to nonspecific effects of P_2Y_{12} inhibitors used in the first study (Xiang, Zhang et al. 2010). I recognize the fact that the strong effect of pan deubiquitinase inhibitors, PYR41 and PR619, on thrombin-induced aggregation is independent of Akt activation, but I have seen a reduction in thrombin induced intracellular calcium release after pretreatment with pan deubiquitinase inhibitors (not shown) as I found when collagen was the agonist. This did not occur with b-AP15, which also explains the strong effect of pan deubiquitinase inhibitors and mild effect of b-AP15 on thrombin induced aggregation.

Furthermore, I have not identified a direct target that is modulated by deubiquitinase inhibition and that needs further experimentation. However, there could be another possibility for the switch in platelet responsiveness since it is notable that quiescent platelets lacked free ubiquitin monomer. Also the anti-ubiquitin-active band with the most rapid mobility during SDS-PAGE is ~ 17 kDa (Fig. 3.1 D), which is consistent with the anticipated mobility of di-ubiquitin. I have

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not lost monomeric 8 kDa ubiquitin from my gels of platelet lysates since I detect recombinant monomeric ubiquitin. Moreover, I observed accumulation of only K48-linked substrates upon pretreatment with deubiquitinase inhibitors (Fig. 3.8 F), but this is not due to proteasome inhibition as none of the three inhibitors inhibited the proteasome catalytic activity (Altun, Kramer et al. 2011; D'Arcy, Brnjic et al. 2011; Kapuria, Peterson et al. 2011) that also results in K48-polyubiquitination of the platelet proteome (Gupta, Li et al. 2014). So, it is possible that all platelet ubiquitin is either fully adducted with protein or exists as unanchored polymeric ubiquitin chains (Strachan, Roach et al. 2012). Quantitative mass spectrometry reports 3600 copies of polyubiquitin B per platelet, the trimeric ubiquitin precursor, but no free monomer (Burkhart, Vaudel et al. 2012). Instead, I propose monomeric platelet ubiquitin would not be present or available to conjugate to new protein targets until ubiquitin sequestered in either protein or unanchored ubiquitin chains is first released by deubiquitinase action. This could mean the essential role of deubiqutination in platelet activation is either to release ubiquitin from existing depots that would allow its conjugation to a new target protein, or the relevant change could be a required remodeling of existing polyubiquitin chains.

The deubiquitinase inhibitors b-AP15 and PR619 strongly suppressed stimulated platelet function *ex vivo*, and I suggest the effect of PYR41 similarly was through inhibition of deubiquitinase activity. This compound was identified as an inhibitor of ubiquitin E1 activity (Yang, Kitagaki et al. 2007), although it was effective in suppressing E1 activity *in vitro* (not shown). However, it did not

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interfere with ubiquitin activation in platelets and, like b-Ap15 and PR619, PYR41 caused a rapid and massive increase in ubiquitin decoration of soluble platelet proteins. This is incompatible with E1 inhibition, which could indicate ubiquitin activation through the second E1 enzyme except that this protein was not discovered in the platelet proteome (Burkhart, Vaudel et al. 2012). However, PYR41 is routinely used as an E1 inhibitor as evident from several publications and it is recently shown that PYR41 at 25 µM concentration blocked cyclophilin A dependent ubiquitination of CXCR7. This is induced by SDF-1α and its a prerequisite for the surface externalization of CXCR7 in platelets (Chatterjee, Seizer et al. 2014). Although I observed a strong inhibitory effect of PYR41 on platelet deubiquitinases, I have not discounted the possibility that PYR41 might act as a dual inhibitor of both E1 activity and deubiquitinase activity. I therefore performed parallel to the PYR41, experiments with general deubiquitinase inhibitor, PR619, and specific deubiquitinase inhibitor, b-AP15, and I show that compounds inhibiting a range of deubiquitase family members suppressed platelet function.

Taken together, I have identified a new pathway that regulates platelet function. I have identified that platelets contain several deubiquitinases and both the general deubiquitinase inhibitors as well as specific inhibitor of proteasome-associated deubiquitinases modulate platelet reactivity to various agonists. Inhibition of platelet deubiquitinases suppressed platelet aggregation, secretion and adhesion of isolated platelets in response to all agonists. Additionally, systemic blockade of deubiquitination also reduced arterial thrombosis in FeCl$_3$-

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damaged carotid arteries. I also show that the deubiquitinase inhibitors, PYR41, PR619, and b-AP15, affect downstream signaling from the collagen receptor, as evident from reduced Akt activation, likely via upregulation of PTEN activity. This is the first report that establishes agonist-dependent regulation of PTEN activity in platelets. Platelet deubiquitinases are critical for platelet responsiveness and the specific inhibitor of proteasome associate deubiquitinases, b-AP15, is already in active preclinical trials. This strongly makes the case that platelet deubiquitinases can be a new class of anti-thrombotic drug targets.

3.6 Chapter summary

These data further show that platelet ubiquitin-proteasome pathway is crucial in modulating platelet reactivity. I show that ubiquitination of platelet proteins is dynamic, platelets express several deubiquitinases, and platelet deubiquitinases are functional. Specific inhibition of the proteasome-associated deubiquitinases (b-AP15) as well as pan deubiquitinase inhibitors (PYR41 and PR619) increased mono- and poly-ubiquitination of platelet proteins. These agents also blocked platelet degranulation upon stimulation. Deubiquitinase inhibition suppressed $\alpha IIb\beta 3$ activation, platelet aggregation, and adhesion/ spreading in response to diverse platelet agonists. Inhibition of platelet deubiquitinase activity strongly suppressed formation of platelet-rich occlusive thrombi in $FeCl_3$-damaged murine carotid arteries and inhibition of platelet deubiquitinase activity prevented *in vitro* thrombus formation on collagen-coated surfaces at high shear rates. These inhibitors also blocked downstream signaling

from platelet receptors by inhibiting agonist-induced Akt phosphorylation associated with upregulation of PTEN activity. This is the first report that established agonist-dependent regulation of PTEN activity in platelets. These data uncovers a new mechanism that maintains circulating platelets in a quiescent state.

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Chapter IV

Ubiquitin-specific protease 7 (USP7) activity promotes platelet activation

4.1 Introduction and Rationale

Arterial thrombotic occlusion causes myocardial infarction and ischemic stroke and is the single most common cause of death and disability in the developed world (Sanz, Moreno et al. 2012). Platelets play central roles in the development of arterial thrombosis and are therefore the main target of anti-thrombotic therapies. Current anti-platelet therapies, most commonly aspirin and/or clopidogrel, are not fully effective in the prevention of arterial thrombosis, and novel anti-platelet agents are therefore sought (Meadows and Bhatt 2007; Michelson 2011). We have previously demonstrated that platelets contain a functional ubiquitin-proteasome system, that proteasome mediated proteolysis is required both for *ex vivo* platelet activation and for *in vivo* development of arterial thrombosis (Gupta, Li et al. 2014). We have also recently established that, like nucleated cells, the ubiquitination of platelet proteins is dynamic, so platelet deubiquitinases that catalyze this turn-over are functional and modulate platelet reactivity (unpublished).

Targeting ubiquitin-proteasome system in platelets seems promising, and

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small molecule inhibitors targeting specific and smaller portions of ubiquitin-proteasome pathway have shown encouraging results in other settings targeting USP7 in nucleated cells. A recent quantitative mass spectrometry shows platelets express deubiquitinase USP7 (ubiquitin-specific protease 7 or Herpesvirus-associated ubiquitin-specific protease, HAUSP). This is interesting as USP7 participates in the control of the cell cycle, which is irrelevant to platelets, but quantitative mass spectrometry shows platelets contain 1300 copies of USP7 peptides per platelet (Burkhart, Vaudel et al. 2012). USP7/HAUSP modulates proteins involved in cell cycle regulation, as well as oncogenes and tumor suppressors and is therefore a strategic target for cancer therapy (Reverdy, Conrath et al. 2012). USP7/HAUSP inhibitors are already in active preclinical development owing to their role in p53 regulation (Shen, Schmitt et al. 2013). These inhibitors induce apoptosis of tumor cells (Reverdy, Conrath et al. 2012) and apoptosis of multiple myeloma cells that have overcome resistance to the proteasome inhibitor bortezomib (Chauhan, Tian et al. 2012).

USP7/HAUSP is abundant in platelets and we have observed (chapter III, Fig. 3.1 B) reduction in the intensity of the protein band at ~ 130 kD, which corresponds to the molecular weight of USP7/HAUSP, after treatment with generic deubiquitinase inhibitors. Whether USP7/HAUSP modulates platelet reactivity has not been studied. Here I show that specific inhibition of USP7/HAUSP suppresses platelet aggregation in response to collagen and adhesion under flow. This study indentifies USP7/HAUSP inhibition as a potential new approach to anti-platelet therapy.

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4.2 Results

4.2.1 USP7/HAUSP activity promotes platelet activation

Active deubiquitinase enzymes are specifically covalently adducted during hydrolysis of the suicide substrate, HA-tagged ubiquitin-vinyl sulfone (Borodovsky, Kessler et al. 2001), and we used this approach to determine whether platelets have active USP7/HAUSP. Platelets treated with either pan DUB inhibitors (PYR41 and PR619) or structurally unrelated USP7/HAUSP inhibitors (HBX 41108 and P22077) were incubated with HA-ubiquitin-vinyl sulfone. Immunoprecipitation with anti-HA antibody was used to enrich active deubiquitinases. Immunoblotting with anti-HAUSP antibody confirmed the presence of active USP7/HAUSP in platelets as a full length 130 kDa protein, and I found that its activity was suppressed by the general inhibitors PYR41 and PR619 (Fig. 4.1 A).Selective small molecule inhibitors of USP7/HAUSP, HBX 41108 and P22077, were equally effective or better in blocking deubiquitination by this enzyme (Fig. 4.1 B).

Selective USP7/HAUSP inhibition by HBX 41108 abolished platelet deposition from whole blood onto the collagen-coated surface of a microfluidic channel during flow at high shear (Fig. 4.1 C) and this inhibition was significant (Fig. 4.1 D). Neither HBX 41108 nor P22077 alone induced platelet aggregation (Fig. 4.1 E), but each inhibitor suppressed collagen induced platelet aggregation in purified platelet preparations (Fig. 4.1 F). This inhibition was significant, and

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equivalent (Fig. 4.1 G).
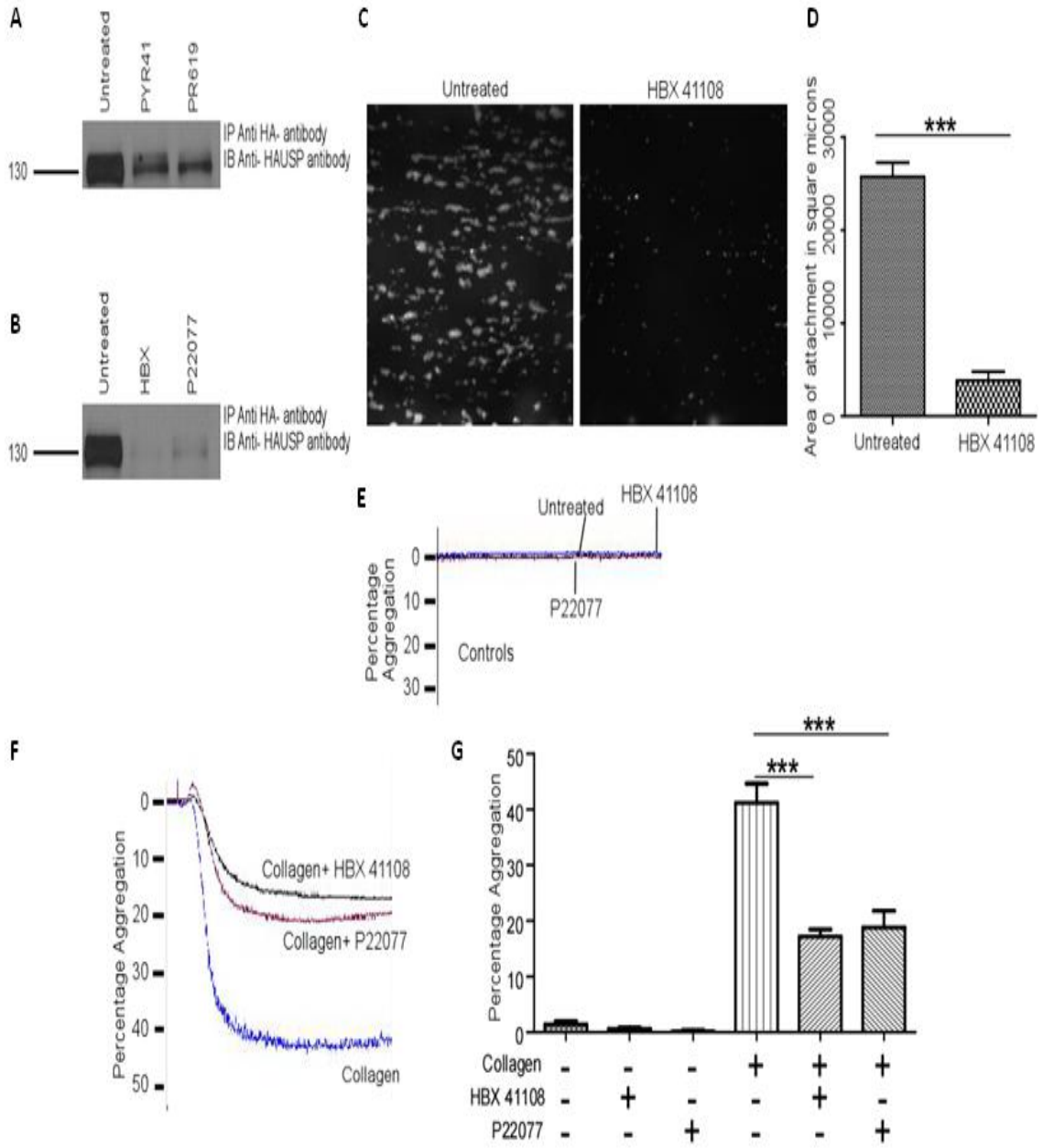
Figure 4.1 USP7/HAUSP inhibition blocked collagen-stimulated aggregation and adhesion under flow

(A) Platelets contain active USP7/HAUSP. Western blot of anti-HA immunoprecipitates with anti-HAUSP antibody after active-site–directed labeling of deubiquitinases with HA- tagged Ubiquitin-Vinyl sulfone (HA-UbVS) in platelet lysates from washed platelets pre-treated with or without PYR41 or PR619 for 30 min (n=3). **(B)** Western blot of anti-HA immunoprecipitates with anti-HAUSP antibody after active-site–directed labeling of deubiquitinases with HA- tagged UbVS in platelet lysates from washed platelets pre-treated with or without HBX 41108 (30 µM) or P22077 (30 µM) for 30 min (n=3). **(C)** USP7/HAUSP inhibition by HBX 41108 blocked platelet adhesion to collagen at high shear. Calcein-AM labeled blood, treated or not with HBX 41108 (30 µM) for 30 min, was perfused over immobilized type 1 collagen fibrils (150 µg/ml) at 67.5 dyne/cm^2 for 3 min. Images are representative fields taken after 3 min from three independent experiments that yielded similar results (n=3). **(D)** Area of platelet attachment after HBX 41108 treatment. Platelet area in panel C was quantified by ImagePro plus software and results are plotted as area of platelet adhesion in square microns (n=3; ***p≤ 0.001). **(E)** Neither HBX 41108 (30 µM) or P22077 (30 µM) stimulated platelet aggregation in the absence of agonists. **(F)** HBX 41108 (30 µM) or P22077 (30 µM) pretreatment for 30 min blocked collagen-induced aggregation (3 µg/ml). **(E)** HBX 41108 or P22077 imposed reduction in collagen-induced aggregation was statistically significant (n=3; ***p≤ 0.001).

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4.3 Chapter summary

These data confirm that components of the platelet ubiquitin-proteasome system are potential anti-thrombotic targets. I show inhibition of a specific deubiquitinase, USP7/HAUSP, strongly suppressed formation of platelet-rich thrombi that formed on collagen-coated surfaces at high shear rates and suppressed platelet aggregation in response to collagen. The deubiquitinase family contains nearly 100 members that accept a range of monomeric and polymeric ubiquitin-conjugated protein substrates, and the polymeric ubiquitin chains themselves are polydisperse from varied polymer length and branching from the seven lysine residues of ubiquitin that are sites of polymerization (Nijman, Luna-Vargas et al. 2005). Deubiquitinases, thus, generally show broadly overlapping substrate specificities.

USP7/HAUSP, member of the DUB family, in contrast, is selective and recognizes ubiquitinated p53 and MDM2, with a critical role in cell cycle regulation (Hu, Gu et al. 2006; Faesen, Dirac et al. 2011). Participation of USP7/HAUSP in deubiquitinating both p53 and its MDM2 E3 ligase, and its participation in the cell cycle, suggests therapeutic opportunities in inhibiting USP7/HAUSP in cancer biology (Colland, Formstecher et al. 2009; Nicholson and Suresh Kumar 2011). This has led to the development of potent and selective USP7/HAUSP inhibitors (Reverdy, Conrath et al. 2012).

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In this study, I find two specific inhibitors of USP7/HAUSP that blocked platelet aggregation by collagen and platelet adhesion under flow. Inhibition or loss of collagen receptor, gpVI, prevented arterial thrombosis in animal models without affecting normal hemostasis in both mice and humans (Kahn 2004), making gpVI a suitable therapeutic target. These data define a focused role for this deubiquitinase in response to just collagen. This shows USP7/HAUSP is required for successful platelet interaction with collagen fibrils exposed in damaged vessels, but does not then participate in platelet activation by soluble agonists. This strongly makes the case that platelet USP7/HAUSP can be a new class of anti-thrombotic drug target that specifically dampens platelet responsiveness to collagen.

Chapter V

Conclusion

5.1 Summary and Conclusion

Overall, the studies presented in this thesis provide evidence that the ubiquitin-proteasome system is an important regulator of platelet responsiveness to diverse stimuli. Pharmacologic inhibitors against specific components of UPS (Fig. 5.1) were used to determine the individual effect of selective inhibition of these components on platelet function. Proteasome inhibition modulated platelet aggregation, spreading, microparticle shedding, clot retraction, thrombus formation and thrombus stability. The requirement for platelet deubiquitinase activity in platelet responses varied among platelet agonists. Specific deubiquitinase inhibitors, as well as general deubiquitinase inhibitors, suppressed stimulated platelet aggregation, adhesion/spreading, platelet degranulation, αIIbβ3 activation and outside-in signaling from αIIbβ3. Systemic blockade of deubiquitination also reduced arterial thrombosis in FeCl$_3$-damaged carotid arteries. The inhibition of proteasome-associated deubiquitinases or soluble deubiquitinase USP7/HAUSP, showed that the platelet responses

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downstream of the collagen receptor were dampened. However, since pan deubiquitinase inhibitors block stimulation by all major agonists, there are receptor-specific as well as receptor-non-specific processes that depend on deubiquitination.

To conclude, this study has identified a novel pathway that regulates platelet function and reactivity. Further understanding of the mechanisms that regulate this pathway in platelets and characterization of specific proteins that are being modulated by this pathway seems vital for the development of new and improved anti-platelet therapies.
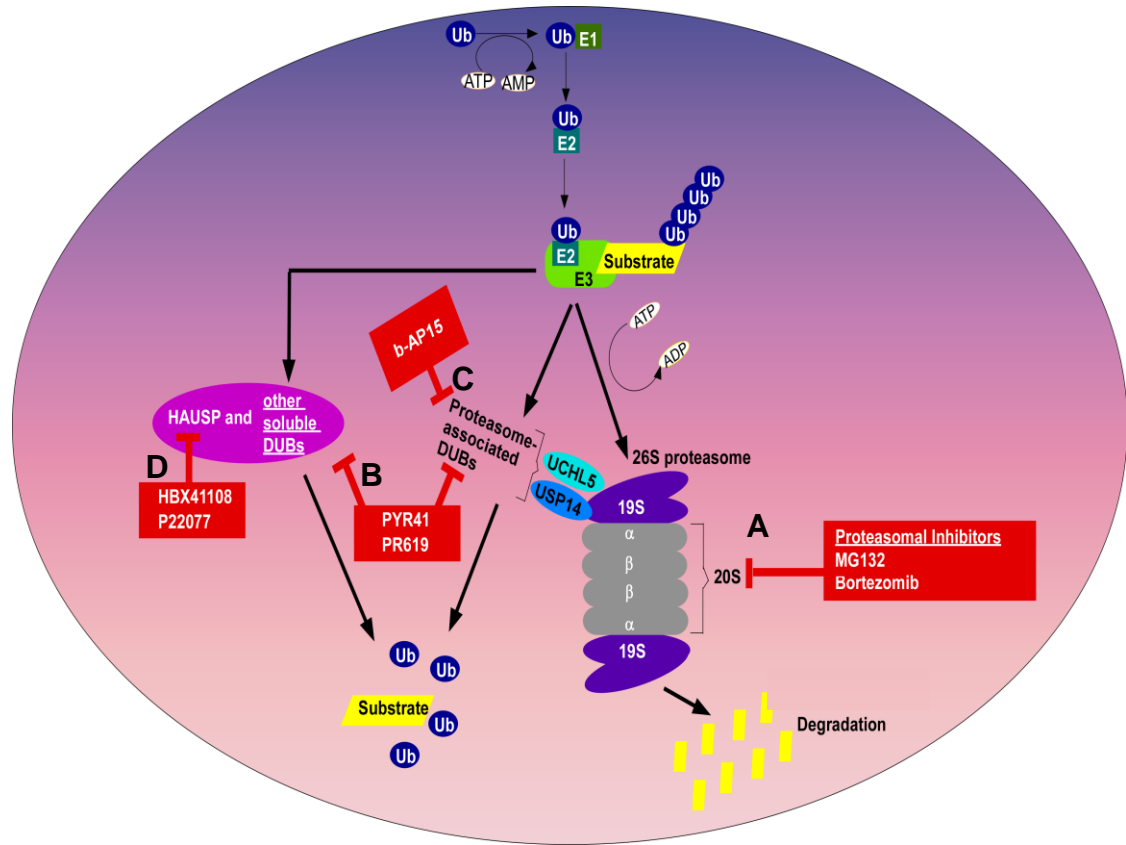
Figure 5.1 Pharmacologic inhibition of different components of UPS modulates platelet reactivity

(A) Proteasome inhibitors MG132 and bortezomib inhibit the catalytic activities of the proteasome. **(B)** PYR41 and PR619 are two structurally unrelated pan DUB inhibitors that inhibit both soluble DUBs as well as proteasome associated DUBs i.e USP14 and UCHL5. **(C)** b-AP15 is a specific and selective inhibitor of proteasome associated DUBs i.e USP14 and UCHL5. **(D)** HBX 41108 and P22077 are irreversible inhibitors of USP7/HAUSP.

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