DUAL ROLES FOR POLO-LIKE KINASE 3 IN THE REGULATION OF PLATELET FUNCTION

by

Brendan Bachman

A thesis submitted to the Faculty of the University of Delaware in partial fulfillment of the requirements for the degree of Master of Science in Biological Sciences

Spring 2013

DUAL ROLES FOR POLO-LIKE KINASE 3 IN THE REGULATION OF PLATELET FUNCTION

by

Brendan Bachman

Approved: _____
Ulhas P. Naik, Ph.D.
Professor in charge of thesis on behalf of the Advisory Committee

Approved: _____
Randall L. Duncan, Ph.D.
Chair of the Department of Biological Sciences

Approved: _____
George H. Watson, Ph.D.
Dean of the College of Arts and Sciences

Approved: _____
James G. Richards, Ph.D.
Vice Provost for Graduate and Professional Education

ACKNOWLEDGMENTS

I would first and foremost like to thank Dr. Ulhas Naik, not only for giving me this project, but also for the guidance he gave me during my time at the University. The time spent in his lab and discussing my project with him was instrumental in teaching me how (and how not) to think like a researcher, and these lessons will shape my thinking for the rest of my life. I know that having me as his student required perhaps an extra degree of patience, and for that also I have to thank him. Without this patience and his timely encouragement just when it was needed, I may not have completed this degree at all.

Anyone who works in or closely with the Naik Lab knows that while Dr. Naik resides at the head of the lab, Meghna Naik sits firmly at its center. Her years of experience, her confidence, and her competence at her work are quite literally an inspiration to the students who work around her. Many of the skills I learned during my time in the lab came directly from Meghna, and she was often the first person I would consult when things just wouldn't work. A better bench scientist you would be hard-pressed to find.

JC Kostyak was for many years another integral part of the Naik Lab, offering his guidance, humor, and of course, his fishing pictures. I had the privilege of working closely with him before he graduated, and during my early time in the lab he too was an inspiration for the scientist I hoped to be.

Arjit Nigam and I have been working in the Naik Lab since we both entered the University of Delaware in the same year. He and I over the years have

consulted each other countless times on our experiments and techniques as we both learned them, usually, if not always, at the same time. He was always around to bounce ideas off of, commiserate on failed experiments, and above all to be a friend.

My committee members, Dr. Dave Edwards, Dr. Erica Selva, and Dr. Donna Woulfe have also provided invaluable guidance over the years on various aspects of my project. They have also put up with my frequently clumsy and often overdue attempts to schedule meetings and consult with them, which deserves recognition of its own.

I would like to thank my family for all their support and encouragement. I must especially thank my mother Maggie, for whom my graduation with an advanced degree is a day long dreamed of and hard fought for, and my sister Ceili, who having recently entered a graduate program of her own, now understands all too well the source of my troubles over these years.

A special thanks goes to all the other members of the Naik lab I did not mention but who I have worked with over the years. You have all contributed to this project in some way and I am grateful to all of you.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

ACD: Acid citrate dextrose
ADP: Adenosine diphosphate
ATP: Adenosine triphosphate
BSA: Bovine serum albumin
CVD: Cardiovascular disease
DAG: Di-acyl glycerol
DIC: Differential interfering contrast
DMS: Demarcation membrane system
FITC: Fluorescein isothiocyanate
GDP: Guanine diphosphate
GEF: Guanine nucleotide exchange factor
GPCR: G-protein coupled receptor
GPIb-IX-V: Glycoprotein Ib-IX-V complex
GPVI: Glycoprotein VI
GTP: Guanine triphosphate
IP_3: Inositol triphosphate
ITAM: Immunoreceptor tyrosine-based activation motif
KO: Knock out
LSC: Liquid scintillation chromatography
MK: Megakaryocyte
PAR: Protease activated receptor
PBS: Phosphate buffered saline
PCR: Polymerase chain reaction
PECAM-1: Platelet endothelial cell adhesion molecule 1
PI3-K: Phosphoinositide 3- kinase
PIP_3: Phosphoinositol triphosphate
PKC: Protein kinase C
PLC: Phospholipase C
PPP: Platelet poor plasma
PRP: Platelet rich plasma
PTB: Phospho/Tyr binding
PTEN: Phosphatase and tensin homolog
RCF: Relative centrifugal force
RIAM: Rap1-GTP-interacting adapter molecule

SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SERT: Serotonin transporter
TBST: Tris-buffered saline w/ Tween
TPO: Thrombopoietin
TxA2: Thromboxane A2
VRK1: Vaccinia related kinase 1
vWF: von Willebrand factor
WASP: Wiskot-Aldrich syndrome protein
WT: Wild type

ABSTRACT

A consequence of the increase in life expectancy of developed nations is that their citizens become increasingly subject to diseases that may manifest only in the later years of life. Cardiovascular disease (CVD) is one such disease, and it often ranks as the number one killer of the denizens of the Western world. Indeed, the increasing potential for us to live lives that are ever more sedentary means that even the middle-aged have become susceptible to it, under the right conditions. Perhaps it is no surprise then, that much time and effort has been spent in the past several decades to find the causes and potential treatments for this disease. Despite this, our understanding of the inner workings of the platelet, a cell of the blood and prime participant in the pathology of CVD, is still surprisingly limited.

Polo-like kinase 3 (Plk3) is a protein that may be key to these inner workings. My research has identified a novel role for Polo-like kinase 3 in controlling platelet function. This protein appears to not only affect the activity of the integrin $\alpha_{IIb}\beta_3$, but also to assist in the process of granular secretion. Using a mouse model in which the *Plk3* gene was ablated, I established that *Plk3*$^{-/-}$ platelets exhibit hyperaggregation compared to WT platelets. They also bind fibrinogen more effectively and will adhere to a fibrinogen coated surface to a greater extent than WT platelets. Conversely, granular secretion is reduced in *Plk3*$^{-/-}$ platelets when stimulated with thrombin. Taken together, these results indicate that the Plk3 protein plays a dual role in regulating platelet function.

Chapter 1

INTRODUCTION

Platelets are the primary agent responsible for haemostasis, or cessation of blood flow, after an injury to a blood vessel wall in humans. However, they also play a key role in pathological thrombosis, where unnecessary or unwanted clot formation can lead to heart attack or stroke and the death of the person. Currently, cardiovascular disease is the leading cause of death in the developed world, resulting in more deaths each year than all forms of cancer combined. It is also responsible for billions in health care costs each year in the United States alone. Roughly 66% of all cardiovascular disease is caused by platelets[1], so elucidating the ways in which platelets are regulated and activated is an endeavor which would be beneficial to a great many people.

Platelets circulating in the blood stand poised to activate and begin the process of coagulation at the first recognition of the appropriate stimuli, and frequently it is the context in which activation occurs that determines whether or not the result is beneficial haemostasis or pathological thrombosis. It has long been a goal of this field of study to understand how platelet activation begins and how it is controlled so that we might prevent pathological activation. In this study, I identify a previously unknown role for the protein known as Polo-like Kinase 3 (Plk3). Genetic ablation in mice results in altered platelet function. Previous work performed in our lab found that genetic knockout of Plk3 resulted in mice with an impaired hemostatic phenotype. My

work also found that *Plk3*$^{-/-}$ mouse platelets are deficient in their ability to secrete their granular contents, a key step in stable thrombus formation. However, I have found that *Plk3*$^{-/-}$ mouse platelets show increased aggregation in response to several key agonists, as well as an increased ability to bind to fibrinogen. Both of these facts point to a more active integrin $\alpha_{IIb}\beta_3$, a key platelet surface protein whose proper function is crucial to hemostasis. Taken together, my data illustrates a potential dual-regulation role that is performed by Plk3 in platelets to control platelet activation and thrombus formation.

1.1 Platelets, structure and origin

Human platelets are small (2-5uM in diameter) and discoid in shape[2]. They circulate in the blood in a concentration of anywhere from 150,000-300,000/μL.[3] They are cell like, but are not considered true cells as they lack some features crucial to other cell types, most notably a nucleus. Therefore, they cannot divide to produce more platelets on their own, but are instead produced in large quantities by cells known as megakaryocytes (MKs). Megakaryocytes, like other cells of the hematopoietic lineage, are produced primarily in the bone marrow.[4] To produce platelets, they undergo a process similar to normal mitosis known as endomitosis, in which they copy their DNA and organelles many times but do not actually divide. A furrow actually forms in the membrane but shortly after reverses.[5] In this way, megakaryocytes can reach a DNA content of up to 64N, although just three cycles resulting in 16N is more common.[6] This DNA replication is accompanied by increased synthesis of the proteins and organelles that will eventually go into platelets, and the volume of the cytoplasm increases greatly.[7] Even the plasma membrane of the megakaryocytes is greatly increased, forming a unique structure known as the

demarcation membrane system, or DMS. The DMS contains many invaginations and extensive channels, extending far into the cytoplasm and yet continuous with the extracellular space. This greatly increases the surface area of the membrane, in preparation for the large quantity of membrane that will be lost when platelets are formed.[8,9] Granules are also formed during this time, before eventual packaging into platelets.[10,11]

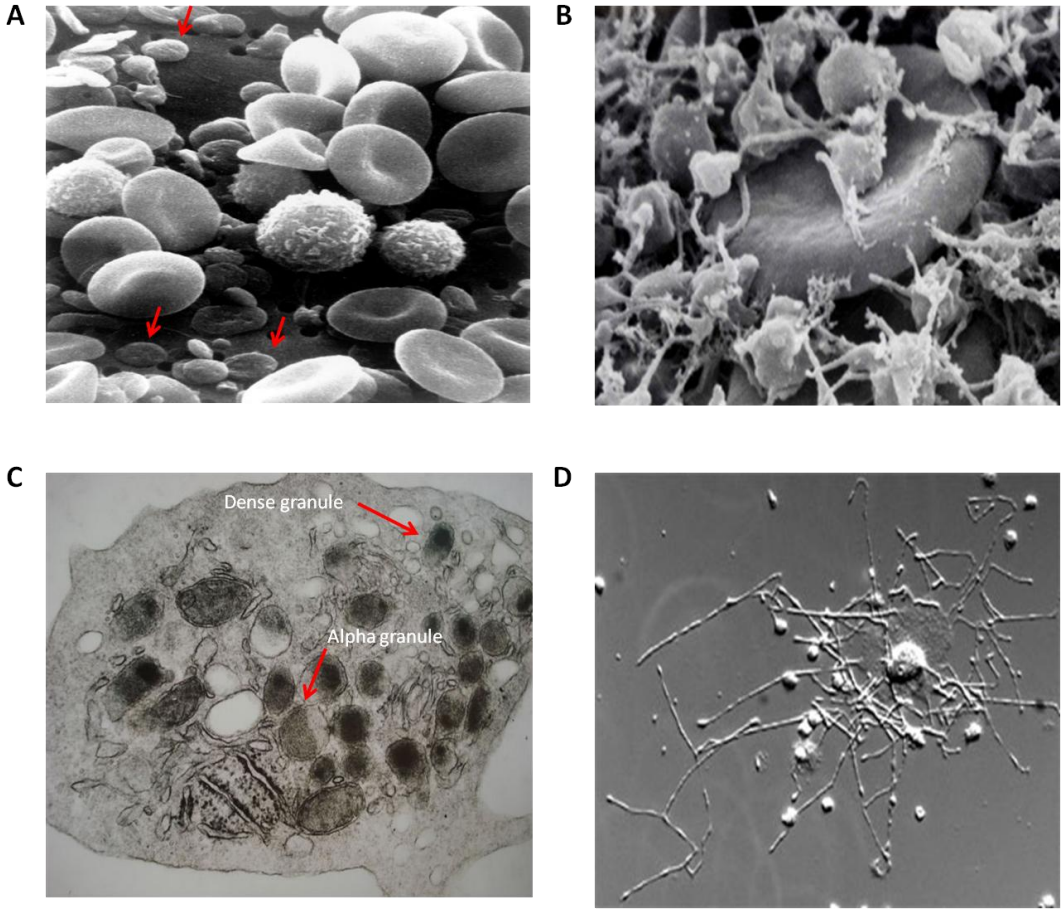


Figure 1.1 Platelet morphology and structure.

Panel A shows the cells of the blood. Red blood cells and white blood cells are the largest, but numerous smaller platelets can also be seen. Three example platelets are indicated by the red arrows. Panel B shows activated platelets surrounding a RBC. Note the numerous filopodial protrusions on each platelet. Panel C shows an electron micrograph of a platelet with the two types of platelet granues clearly visible. Panel D shows proplatelets processes that have disengaged from the megakaryocyte, and are beginning to bud off into platelets.

When a megakaryocyte is ready to produce platelets, it begins to undergo a series of dramatic changes. The membrane forms long ribbon like structures called pro-platelet processes[12]. These are long, thin extensions of the plasma membrane supported by microtubules and actin filaments, and they may be covered in what appear to be partially formed platelets. However, mature platelets are only formed from the very ends of these processes[13]. These processes extend through sinuses in the bone marrow into the bloodstream where they begin to bud off into platelets[14]. A single megakaryocyte can produce approximately 5000 platelets in this manner.[15]

The production of platelets by megakaryocytes is largely regulated by thromobopoietin (TPO). TPO is a small protein produced in the liver[16], that stimulates megakaryocytes to increase in size, number, and DNA content (ploidy) in preparation for platelet production.[17] which is in turn regulated by the number of platelets currently in circulation. Platelets circulating in the blood bind to and sequester circulating TPO, thereby establishing a feedback system that can easily control the number of platelets in the blood. As the level of platelets falls, circulating TPO rises, and more platelets are produced. If platelet levels rise, TPO levels begin to fall and platelet production drops off.[18] A number of other chemokines, including IL-3 which is crucial in the early stages of megakaryocyte development, are also important at different stages of platelet formation.[19]

1.2 Platelets are the primary mediators of hemostasis

After an injury to a blood vessel wall occurs, blood loss must be stopped quickly lest even a small injury become a serious threat to the organism. This occurs through a process known as hemostasis, and it is largely carried out by platelets[20]. The

layer of endothelial cells lining the inside of the vessels is for the most part inhibitory to platelet activation. However, when the vessel is injured, the underlying subendothelial matrix is exposed, and many of the proteins contained there are highly activating to platelets[21]. Collagen exposure is generally the first event to begin the activation of platelets and the start of haemostasis.

Circulating vWF immediately begins to bind to the newly exposed collagen in the matrix layer. The shear stress exerted upon these fibers once they are immobilized causes them to stretch, exposing previously inaccessible binding sites for platelet receptors.[22] The interactions between these immobilized vWF chains and the GPIbα receptor in the platelet GPIb-IX-V complex begins to slow the platelets circulating in the blood down to the point where other receptors can begin to form stronger, longer lasting bonds with their respective ligands.[23] Collagen in particular is key to truly beginning platelet activation in flowing blood. As subendothelial collagen binds to its primary platelet receptor (GPVI), it starts a series of signaling events. A kinase that is constitutively bound to the cytosolic portion of GPVI phosphorylates a tyrosine in the ITAM motif of an FcR γ-chain, with which it is also associated. This leads to activation of the phospholipase C isoforms PLCγ2. PLCγ2 hydrolyzes PIP_2 into IP_3 and diacyl-glycerol (DAG). IP_3 leads to a dramatic rise in intracellular calcium, while DAG leads to activation of protein kinase C (PKC).[24]

Fibrinogen, circulating in the blood and released from platelet alpha granules, is converted into fibrin by the protease activity of thrombin.[25] Fibrin and fibrinogen bind to the activated $\alpha_{IIb}\beta_3$[26] and this binding is the primary mediator of platelet to platelet linkage in the forming clot.[27] The integrin becomes associated with the platelet cytoskeleton, and as the platelets begin to contract they exert the tension on the fibrin

linkages that serves as the mechanical force behind clot retraction. Clot retraction in turn serves to reduce the profile of the clot, minimizing both the occlusion of the vessel in which the clot has formed and the sheer stress being exerted on the clot.

1.3 Platelet GPCRs

Activation is also achieved through the stimulation of other receptors on the platelet surface. As will be discussed in detail later, the activation of a few platelets causes them to secrete their granular contents. These granules contain, among other substances, agonists capable of activating more platelets and recruiting them to the injury site. Several types of G-protein coupled receptors (GPCRs) are found on the platelet surface and participate in this response.

1.3.1 The protease activated receptors (PARS)

The Protease Activated Receptors (PARs) are, as the name suggests, activated by the action of serine proteases. In this case the protease is not actually the ligand, but protease "binding" to the receptor results in cleavage of the N-terminal sequence, producing a tethered ligand that then activates the receptor.[28] Humans express three PARs that respond to the protease known as thrombin (PAR1[28], PAR3[29], and PAR4[30]). PAR1 appears to be the most sensitive of these three, and responds the most strongly to thrombin stimulation.[28] It is in part due to the tethered ligand mechanism of action that thrombin is one of the strongest agonists that can be used to activate platelets, because a single molecule of thrombin can quickly activate a great many PAR receptors. However, as with many aspects of platelet activation, this activation of the receptor is terminal. After being cleaved by thrombin and becoming active, the receptor is quickly internalized or shed into membrane microparticles during

aggregation.[31] As platelets have no mechanism by which to replenish their surface receptors, platelets stimulated in this way are desensitized to thrombin.[31]

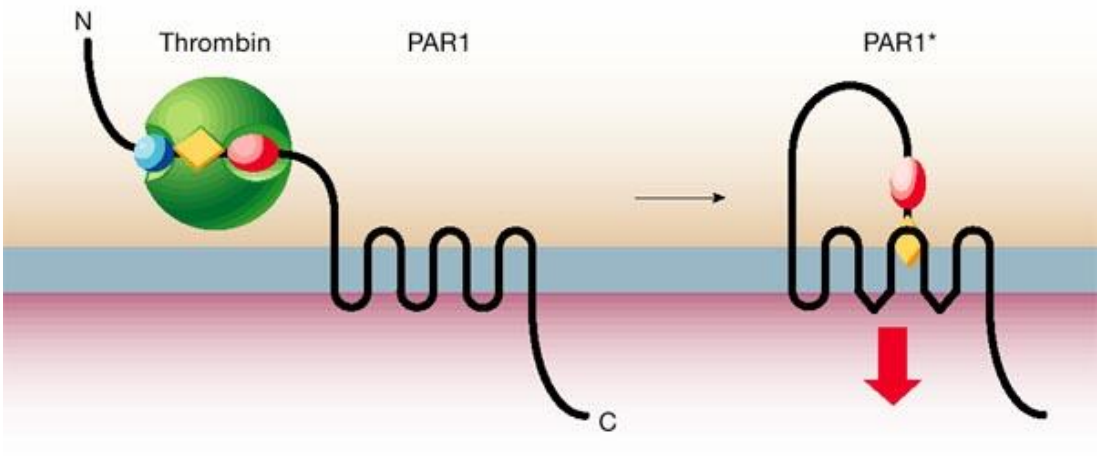


Figure 1.2 Mechanism of PAR activation.

The serine protease thrombin cleaves the N-terminal end of the receptor, forming a tethered ligand that activates the receptor. PARs can be activated by proteases other than thrombin, but this is the primary route of activation for the PARS active on platelets (PAR1 and PAR4). Adapted by permission from MacMillan Publishers Ltd: Nature. *Thrombin signalling and protease-activated receptors.* Vol. 407, 258-264 (14 September 2000).

1.3.2 The platelet purinergic receptors

Also present on the platelet surface are the purinergic receptors, $P2Y_1$[32] and $P2Y_{12}$[33]. These receptors are also GPCRs, and they recognize the nucleotide signaling molecule ADP[32,33]. The activation provided by these receptors is generally dependant

on the activation of both receptors, and activation of $P2Y_1$ alone leads to weak or transient activation[34]. A major function of $P2Y_{12}$ is to inactivate adenylyl cyclase[35], but $P2Y_{12}$ is also believed to lead to activation of one or more PI3-K isoforms.[36] This leads to activation of Akt, a crucial step for platelet activation.[37]

1.4 GPCR signaling

GPCRs mediate intracellular signaling events in platelets by acting as guanine-nucleotide exchange factors (GEFs) for heterotrimeric G-proteins. These G-proteins consist of an α, a β, and a γ subunit. When inactive, all three are bound together and associated with the cytoplasmic side of the receptor, and the α subunit is bound to GDP. When receptor-ligand binding occurs, a conformational shift in the cytoplasmic region of the receptor causes the α subunit to exchange GDP for GTP, and the G-protein becomes active. The G-protein is no longer bound to the receptor, and the α subunit dissociates from the β and γ subunits. Both the α subunit and the βγ heterodimer are now free to effect signaling events downstream of the receptor.[38]

There are multiple forms of G protein alpha subunits (usually termed G_αs), and the signals that propagate from certain GPCRs are often a consequence of what G_α proteins are associated with them. G_i family proteins are often responsible for inhibition of adenylyl cyclase[39], and are associated with both PARs and P2 receptors.[33,40] G_q family proteins are almost exclusively tied to activation of PLC isoforms.[41]

1.5 Platelet Cytoskeleton

With all the mechanical changes that platelets undergo during activation, it is easy to understand that the platelet cytoskeleton is an extremely important component

of the platelet. The majority of the cytoskeleton is, logically, composed of actin. The actin network of platelets is so extensive that actin is the single most abundant protein to be found in platelets.[42] Many of the actin filaments are linked in a rather rigid network that helps to maintain the shape of the platetet.[43] A microtubule coil, imparted when the platelet first buds off from the proplatelet process[13], also helps gives the platelet its discoid shape. This coil consists of a single tubule wrapped 8-12 times around the outer edge of the platelet,[44] composed primarily of β1 tubulin.[45] As platelet activation begins, the platelet cytoskeleton immediately begins to re-shape itself. As PLC activity rises, so does the presence of membrane phosphoinositides[46], which bind to and inactivate the capping proteins that inhibit extension of actin filaments.[47] Rises in intracellular calcium activate gelsolin, a protein that severs actin filaments allowing them to reorganize in different configurations.[48] WASp, which may be activated by ITAM motifs following collagen binding to the GPVI receptor, activates the Arp2/3 complex. Arp2/3 stimulates new actin filaments to form, leading to an interconnected network of growing filaments crucial for lamelipodia formation.[49,50]

The spreading that occurs during activation allows not only for wound coverage, but also the formation of direct platelet-platelet connections. These platelet-platelet interactions are mediated primarily through the integrin $\alpha_{IIb}\beta_3$, an important platelet surface molecule that will be discussed in more detail below. This integrin becomes attached to the platelet cytoskeleton during platelet activation.[51] After spreading and the establishment of platelet-platelet linkage through $\alpha_{IIb}\beta_3$, the platelets begin to exert contractile forces through the action of cytoplasmic myosin, resulting in the process of clot retraction that is required to stabilize the forming thrombus.[52]

1.6 The integrin $\alpha_{IIb}\beta_3$

One of the most crucial effects that platelet activation has is the activation of one of the major platelet surface molecules, the integrin $\alpha_{IIb}\beta_3$. Like other integrins, it is a heterodimeric transmembrane protein. The majority of the dimer is found in the extracellular space, but there is a significant C-terminal intracellular portion of both subunits. The β_3 subunit has the larger cytoplasmic domain.[53] Like other integrins, it is believed that this receptor is normally held in an inactive, folded conformation when the platelet is in the resting state. Upon platelet activation, signals transduced to the cytoplasmic tails of the integrins cause the receptor to adopt an upright, active conformation. This process, termed inside out signaling, is a key aspect of integrin function.[54]

In resting platelets, the cytoplasmic tails of the integrin are closely associated through ionic interactions. In order for the integrin to adopt its upright, active conformation, separation of the tails is required, and this process is likely to be mediated by the interaction of multiple proteins with the tails. Studies have shown that the cytoplasmic protein known as talin is the most likely culprit to be ultimately responsible for the separation of the tails.[55] A PTB like domain in talin recognizes an NPXY motif in the β_3 tail, and interacts with several residues that appear to be crucial for holding the cytoplasmic tails together.[56] Talin is induced to effect this change in the integrin mainly by the action of phospholipase C. DAG and intracellular calcium rise, both a product of PLC activity, result in the activation of CalDAG-GEF1.[57] CalDAG-GEF1 is a guanine nucleotide exchange factor that activates the GTPase Rap1.[58] When active, Rap1 binds to RIAM, which mediates the connection to talin.[59] Through the formation of this complex, talin is recruited to the plasma membrane and brought into close proximity to the integrin tails. This pathway, which represents the

connection from platelet activation to integrin activation, has only recently been elucidated.

When in this active conformation, the binding site of the integrin is exposed, and it is able to bind its primary ligand, fibrinogen.[60] When the integrin is bound to fibrinogen, a further conformational change occurs that causes the cytoplasmic tails, particularly the β_3 tail, to bind proteins that facilitate such necessary functions as shape change and also allow the integrin to associate with the actin cytoskeleton.[61] This change is known as outside in signaling, and is also facilitated by clustering of the integrins after ligand binding.[62] Interestingly, the protein that is responsible for inside-out signaling seems to also be important for linking the actin cytoskeleton to the cytoplasmic tail of the β_3 integrin. Studies have shown that a talin mutant that is incapable of activating the integrin but still binds to the cytoplasmic domain is sufficient to mediate clot retraction if the integrin is activated by an exogenous source, such as addition of $MnCl_2$.[63]

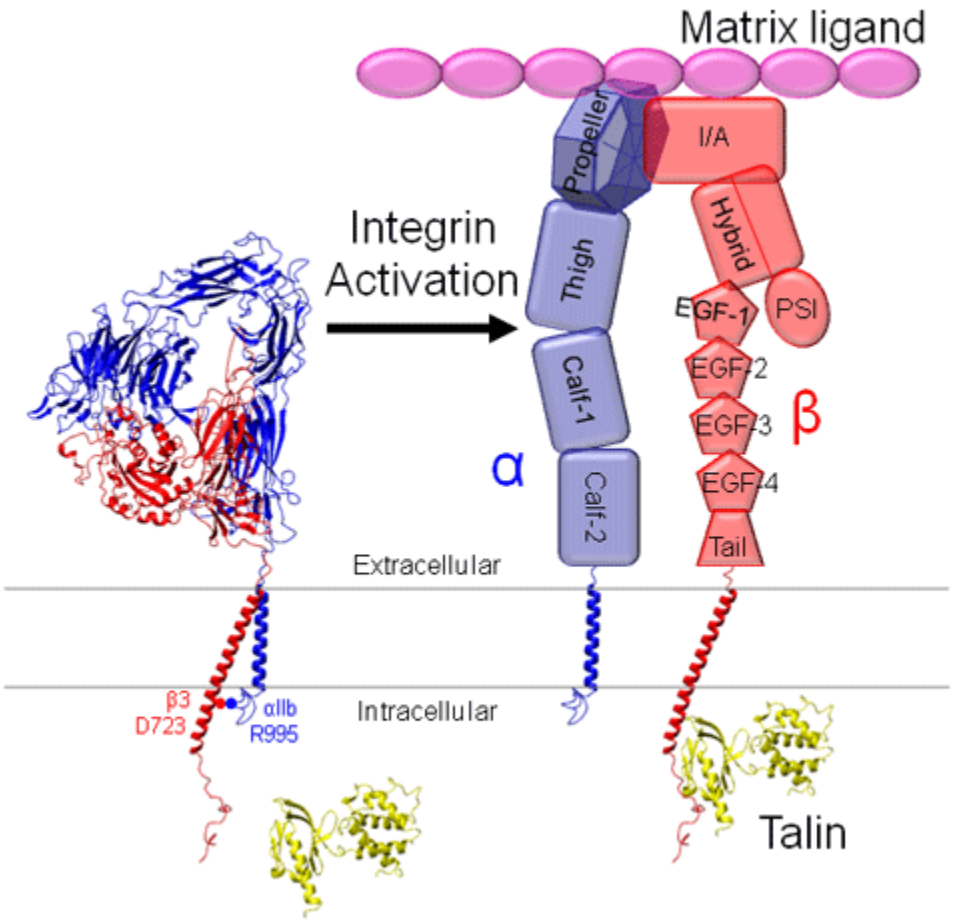


Figure 1.3 Inside out signaling of the $\alpha_{IIb}\beta_3$ integrin.

This diagram shows the integrin structure as well as the activation of the integrin by talin. Once the integrin becomes activated, it is able to bind its ligand.

The proper function and activity of the integrin may be linked to phosphorylation of a threonine residue on the β_3 cytoplasmic tail.[64] This threonine, Thr-753, has been shown to have an inhibitory effect on the activity of the integrin. Thr-753 is proximal to amino acid sequences known to be important for the binding of

proteins (such as talin) to the cytoplasmic tail, and phosphorylation at this site could inhibit or alter the binding of important signaling factors.[65] Disruption or interruption of these protein interaction sites may play a crucial role in regulation of both inside-out and outside-in signaling.

1.7 Polo-like kinase 3

Plk3 stands for Polo like kinase 3. It belongs to a family of proteins with five currently known members, all serine/threonine kinases.[66] All five members also contain a Polo-box domain from which the name of the family is derived. This domain is highly conserved among multiple organisms from yeast to humans[67], and numerous studies have shown that it plays a crucial role in regulation of the cell cycle.[68] Plk1, the first identified family member, is known to be important in mitosis and the cell cycle.[69] The most likely function of the Polo-box is the proper intra-cellular localization of Plk proteins, as the Polo-box itself is non-catalytic, but it is also important for protein to protein interactions and will bind to phosphorylated serine or threonine residues.[70]

1.7.1 Function of Plk3 in platelets

However, as platelets are not truly cells and do not undergo mitosis, this is obviously not the role that Plk3 has in platelet function. Other studies have shown that, in addition to playing a role in the cell cycle, Plk3 also plays a role in regulating microtubule integrity. In one study, mutants of Plk3 were sufficient to cause serious distortions in the morphology of cells in which they were expressed, indicating that Plk3 plays a direct role in this process.[71] Plk3 and Plk2 have also both been shown to have functions in neurons unrelated to mitosis, as neurons are cells that rarely undergo

mitosis over the course of their existence. In this same study, Plk3 and Plk2 were also shown to be regulated in neurons by a protein known as Calcium and integrin binding protein, or CIB-1.[72] This is significant because platelets express both Plk3 and CIB1.

1.8 Calcium and Integrin binding protein 1

CIB-1, first identified by Dr. Ulhas Naik in 1997, has a large amount of sequence similarity to calmodulin and calcineurin B. As would be expected from this similarity, it contains EF-hand motifs that allow it to bind certain metal ions.[73] In a resting platelet, where the concentration of Mg^{2+} is higher than Ca^{2+}, the EF hands are bound to Mg^{2+} and CIB1 is inactive. However, when the concentration of Ca^{2+} in the cell rises dramatically (as happens in platelets when they become activated), Ca^{2+} displaces Mg^{2+} and CIB1 assumes an active conformation in which it can bind to other proteins.[74] CIB1 was established as a binding partner of $\alpha_{IIb}\beta_3$ by performing yeast two-hybrid assay.[73] CIB1 appears to be crucial for proper integrin functioning in outside-in signaling, and when CIB1 binding to the integrin is inhibited, mouse platelets fail to spread properly on immobilized fibrinogen.[75]

CIB1 has been shown by our lab to be inhibitory to Plk3 after binding to it. Studies done in our lab indicate that the kinase ability of Plk3 is greatly reduced upon CIB1 binding.[76] Furthermore, it has been shown that CIB1 associates with the integrin and can be immune-precipitated along with it.[75] While Plk3 has no known role in platelets, the fact that it is regulated by CIB1 and that CIB1 is integral to $\alpha_{IIb}\beta_3$ function and signaling is, at the very least, intriguing. It is possible that CIB1 plays a role in sequestering Plk3, thereby preventing it from reaching potential phosphorylation targets in platelets.

1.9 Phenotype of *Plk3*$^{-/-}$ mice

Plk3 KO mice do not show any outward signs of illness or abnormality. However, the Dai group did determine that KO mice were more susceptible to developing malignant tumors than WT mice. This phenotype seemed only to present itself when the mice reached more advanced ages, however. When the mice were 18 months of age or older, their incidence of tumors would exceed 60%, whereas their WT counterparts had only ~10% chance.[77] This data seems to indicate a role for Plk3 that is more akin to the known roles of its other family members, possibly regulating key aspects of the cell cycle, cell division, and oncogenesis.

Our lab also determined that these mice have a mild haemostatic deficiency. They took longer to stop bleeding in a tail bleeding assay, and also had a higher incidence of re-bleeding. In a carotid artery injury assay, *Plk3*$^{-/-}$ mice also took longer to reach full occlusion of the vessel, or did not reach full occlusion at all.[unpublished data]

1.10 Granular Secretion

Granular secretion plays a vitally important role in hemostasis. As a platelet monolayer begins to cover an injury in a vessel wall, the platelets also inherently begin to cover the exposed collagen which was originally responsible for activating them. There is still a need for platelets to be recruited to the injury site to form a stable clot and adequately seal the injury, but without additional agonist from another source this is unlikely to happen. Granular secretion provides this source. The granules travel from the cytoplasm to merge with the platelet membrane and release their contents, some of which are agonists that are capable of activating other platelets. Like most forms of vesicle exocytosis in mammalian cells, granule fusion with the membrane is mediated by SNARE proteins.[78] Granules likely fuse with the plasma membrane that

composes the open canalicular system. Because this system connects with the exterior of the platelet through channels in the membrane, the contents of the granule are released into the extracellular space.[79]

Alpha granules contain a wide variety of proteins and other molecules. Some of these are not involved in clot formation or stabilization, but rather in other processes related to vessel injury, like immune response and angiogenesis. However, they contain many adhesion molecules and receptors, such as integrins and P-selectin. They also contain two other proteins that are crucial for coagulation: fibrinogen and Factor V.[80] Factor V is a cofactor of the thrombinase complex, and therefore contributes to the coagulation cascade.[81] Fibrinogen is the unprocessed form of fibrin, which is a primary mediator of physical platelet-platelet connections through $\alpha_{IIb}\beta_3$, as has been discussed. Each platelet contains about 80 α-granules, so they are the far more numerous type of granule.[82]

Dense granules contain a large quantity of small molecules as well as metal ions such as calcium. In addition to these, platelet dense granules also contain ADP, ATP, and serotonin.[83] ADP and serotonin in particular are key players in the activation and recruitment of platelets to the injury site.[84]

Chapter 2

MATERIALS AND METHODS

2.1 Animal model

To study the effects of in-vivo genetic ablation of *Plk3*, our lab obtained mice from the lab of Wei Dai at the New York University School of Medicine. This lab had disrupted the *Plk3* gene in these mice via homologous recombination, as previously described in Yang et al, 2008. Chimeras were generated in a C57BL6 background, and individuals heterozygous for the disrupted *Plk3* gene were bred and confirmed by PCR and Southern blot. Individuals homozygous for the disrupted gene were then obtained by backcrossing these heterozygotes.[77]

Plk3$^{-/-}$ mice appear completely normal and have no obvious genetic defect. Litter sizes are normal and the offspring have no developmental abnormalities. They do not suffer from health problems in any greater frequency than wild type C57BL6 mice.

For our experiments, selected animals were at least 7 weeks old and typically between 20-25 grams in weight. Animals that appeared sick or distressed, either outwardly or only upon internal examination, were not used for experiments.

Animals were kept at the University of Delaware in a facility where temperature, feeding, and light cycles were all carefully controlled. All experiments were approved by the University of Delaware's Institutional Animal Care and Use Committee.

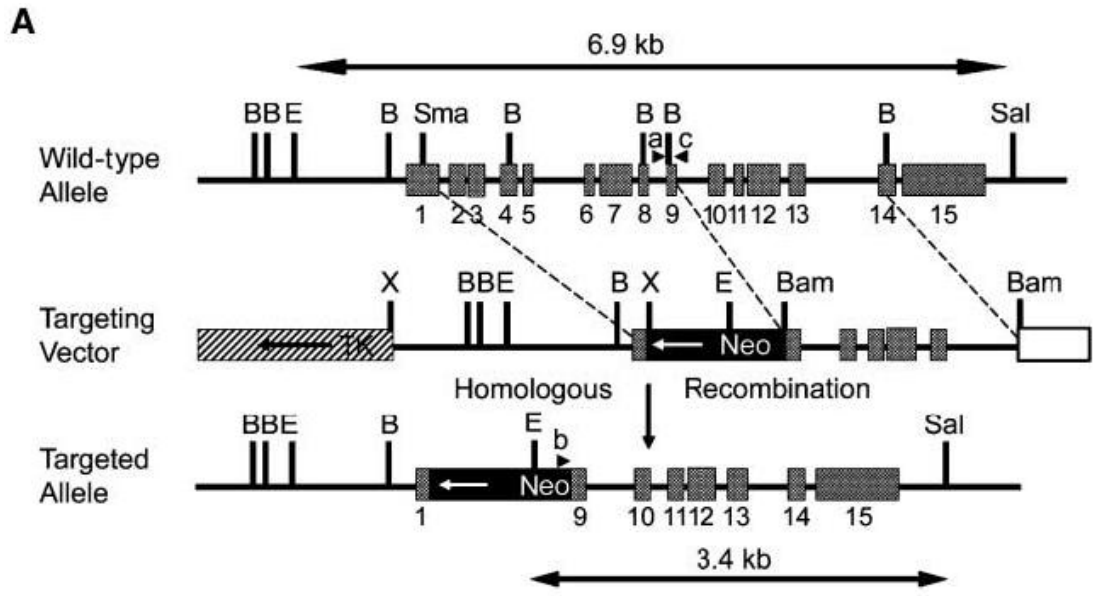


Figure 2.1 Disruption of mouse *Plk3* locus.

This figure illustrates the method by which the Plk3 gene was disrupted. A number of exons were replaced with a neomycin resistance gene by means of homologous recombination. Reprinted by permission from the American Association for Cancer Research: Yang, et al, *Polo-like Kinase 3 Functions as a Tumor Suppressor and Is a Negative Regulator of Hypoxia-Inducible Factor-1A under Hypoxic Conditions*, Cancer Research, 1 June 2008, 68 (11), 4077-85, doi: **10.1158/0008-5472.CAN-07-6182**

2.2 Drawing of blood from mice

Blood was drawn and platelet suspensions prepared using protocols previously established in our lab.[85] These protocols were in turn developed from accepted and established protocols in the field, with minor modifications.[86]

Mice were anesthetized using a mixture of ketamine and xylazine in a dosage of approximately 1uL per gram bodyweight of the mouse. Anesthesia was injected

directly into the abdominal cavity using an insulin syringe with a 29 gauge needle (Lo-Dose, BD Biosciences). Mice were placed back into their enclosures while the anesthesia was allowed to take effect over a period of 10-15 minutes. After this period, mice were removed and immobilized belly-up in the surgical area, and their abdomens were brushed briefly with a 70% ethanol solution. An incision was then made in the subcutaneous layer from the groin area to the bottom of the ribcage, and the skin was peeled back exposing the peritoneal wall. A similar incision was made in the peritoneal wall taking reasonable care to avoid any visible blood vessels. The exposed viscera were then pushed gently aside to reveal the inferior vena cava. Using a 27 gauge needle attached to a 1cc syringe (BD Biosciences), approximately 1mL of blood was drawn directly from the vena cava. Syringe and needle were briefly treated with a heparin solution (10,000 U/mL) just prior to blood drawing. After the draw, the needle was removed from the syringe and the blood was transferred to a 5mL polyethylene tube containing a specific volume of one of two anticoagulant substances. For preparation of PRP, 1/9 of the drawn blood volume in a tri-sodium citrate solution was used. For preparation of washed platelets, 1/6 of the drawn blood volume in ACD (acid-citrate dextrose) solution was used.

For procedures requiring the use of PRP, whole blood mixed with sodium citrate was diluted in a 1:1 ratio with Tyrode's buffer (137mM NaCl, 2.7mM KCl, 12mM $NaHCO_3$, 0.4mM NaH_2PO_4, 1mM MgCl, 2mM $CaCl_2$, 5.55mM glucose) with Ca^{2+} and BSA added, and then centrifuged at 150 rcf for 7 minutes. After centrifugation, PRP was removed using a 1mL pipette and transferred to a new 5mL polyethylene tube. Platelet concentration of PRP was determined using a Beckman-

Coulter Z2 Coulter Counter or hemocytometer, and then diluted to the appropriate concentration for the experiment to be performed.

For procedures requiring the use of washed platelets, whole blood mixed with ACD was diluted in a 1:1 ratio with Tyrode's buffer containing BSA (but not Ca^{2+}) and then centrifuged at 150 rcf for 7 minutes. After centrifugation, PRP was removed using a 1mL pipette and transferred to a new 5mL polyethylene tube. Platelets were then pelleted by centrifugation at 400 rcf for 10 minutes. Plasma was aspirated away from the pellet using a 1mL micropipettor. Platelets were re-suspended in a small volume of Tyrode's containing Ca^{2+} (but not BSA). This suspension was counted on a Beckman-Coulter Z2 Coulter Counter or hemocytometer to determine the concentration of platelets and diluted with Tyrodes ($+Ca^{2+}$, -BSA) to the appropriate concentration for the experiment to be performed.

Blood for an experiment was always drawn fresh each time an experiment was to be performed.

2.3 Counting of platelets for dilution

Counting was performed on Beckman-Coulter Z2 Coulter Counter, or on a hemocytometer. For counting on the Coulter counter, 10uL of the suspension to be counted was placed into 990uL of Isoton II diluent in the appropriate cuvettes. The cuvette was raised onto the aperture and the counting program was started. Before washing, the outside of the aperture was cleaned extensively with water, and between each count the aperture was flushed with Isoton and the outside was again rinsed with water. Water was removed from the outside of the aperture before counting to avoid diluting the sample to be counted. The machine took three 500uL samplings of the

suspension, and averaged these values together to get the approximate counts/mL value.

For counting on a hemocytometer, 2uL of the sample to be counted was diluted in 198uL of Tyrode's buffer. 10uL of this diluted suspension was then placed on a pre-cleaned hemocytometer and the platelets were allowed to settle for at least 10 minutes. Platelets were then counted on a light microscope under 200X magnification. Only the centermost field was counted, and of that field only 5 squares were counted: the upper left, upper right, lower left, lower right, and center squares. The surface area of the 5 grids in which platelets were counted totals $0.2mm^2$, and the volume of the counted area is 0.02uL. The value obtained was then multiplied by 5 to estimate the total number of platelets in the central field, multiplied by 10,000 to estimate the number of platelets in 1mL of the diluted suspension, and then multiplied by 100 to estimate the number of platelets in the original suspension.

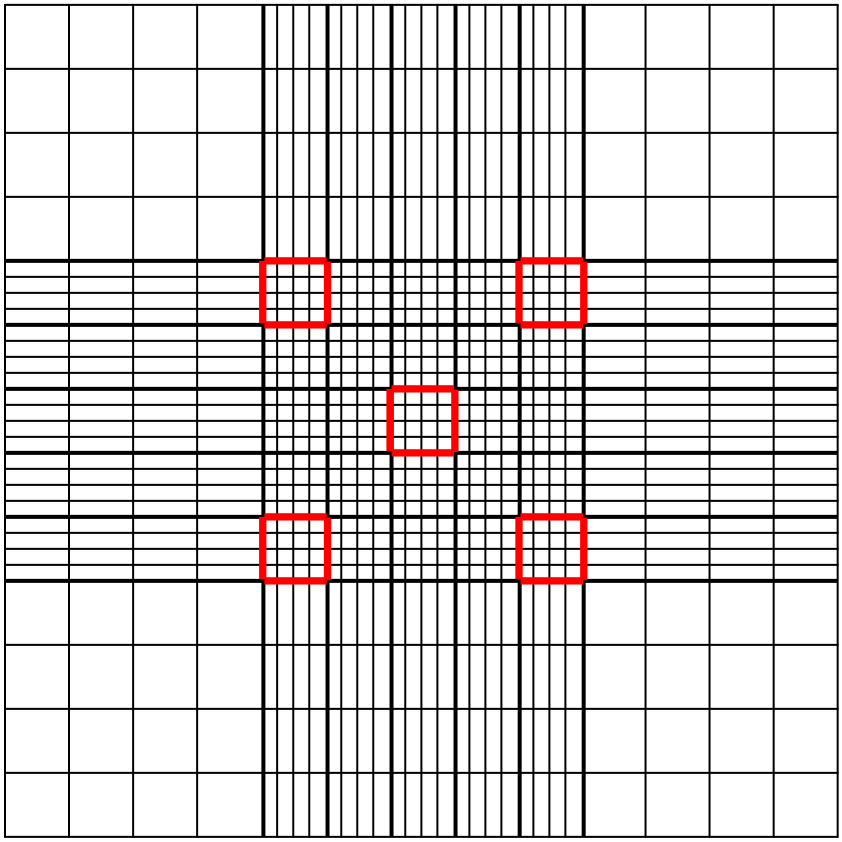


Figure 2.2 Hemocytometer diagram.

Platelets were counted only in the regions outlined in red, and then the total platelet count of the sample was estimated using the equation: **Cx5x10000x100=cells/mL,** where **C** is the number of cells counted in the 5 grids.

2.4 Western blotting

Work was performed as previously described.[73] To prepare samples for Western blotting, washed platelets were prepared, counted on a coulter counter, and diluted to $3.5x10^8$ cells/mL. Platelet suspensions were treated with 1mM aspirin and 1U/mL apyrase at room temperature for 20 minutes. 100uL volumes of platelet suspensions were stimulated with agonist and lightly vortexed for 10 seconds. At 0 seconds, 30 seconds, 1 minute, and 5 minutes, 100uL aliquots of the stimulated suspensions were added to tubes containing 100uL of 2X sample buffer (95% Laemmli buffer, 5% BME). These samples were then boiled for 5 minutes and frozen until they were run for the gel, usually the next day.

SDS-PAGE gels of a 10% polyacrylamide content were cast using buffers from Protogel, with a stacking gel cast above the running gel. All gels were cast in pre-made cassettes with combs inserted to make 10 sample wells. The gel cassettes were loaded into the running chamber, the chamber was filled with running buffer, and 40uL of each of the samples were loaded. 10uL of rainbow marker were also loaded (Fermentas,). Gels were run until the dyefront began to run off of the gel, at which point the gels were removed and placed into TBST, and the transfer apparatus was assembled. Transfer chamber was filled with ice-cold TBST and the transfer was run for 90 minutes.

At the end of the transfer, blots were blocked in 3% BSA or 5% milk (BSA was used if it was necessary to blot for phosphorylation) for 1 hour. Primary antibody solutions were made in the blocking solutions to a concentration of 1:1000, and each blot was placed into a sealed baggie with 3mL of the antibody solution. Primary antibodies against phosphorylated P38 and phosphorylated ERK1/2 were used during

the first blot. After stripping using stripping solution, an antibody against total P38 or total ERK1/2 was used as a primary antibody, as a control.

Baggies were kept at 4°C overnight. The next morning, these baggies were cut open and the blots were removed and washed in 1X TBST 3 times for 15 minutes each time. Blots were treated with secondary antibody for 1 hour, in TBST and at a concentration of 1:2000. An HRP-linked anti-rabbit IgG was used, and the blots were then developed in a darkroom using 20X LumiGLO reagent, 20X Peroxide (both from Cell Signaling), and HyBlot CL autoradiography film.

All antibodies used were purchased from Cell Signaling in Boston, MA.

2.5 Aggregometry

Testing of the ability of platelets to aggregate was performed using a lumiaggregometer (Chronolog corp., Havertown, Pa). Aggregation was performed using protocols established in the lab and in the field.[85,87]

Both PRP and washed platelets were used. Counting of platelets was done on a Coulter counter. Platelet suspensions diluted using Tyrode's buffer to a platelet concentration of $2x10^8$ cells/mL was aliquoted in small glass cuvettes to a volume of 250uL. A small siliconized stir bar was added to each cuvette to allow the suspension to be in motion during aggregation. Small quantities of the same PRP used for aggregation were centrifuged at high speeds to pellet all platelets out of the plasma, creating PPP. This PPP was then removed and added to cuvettes to be used as the blank in the aggregometer (representing 100% aggregation). PLK3-/- PRP was always compared to PLK3-/- PPP, and WT PRP was always compared to WT PPP. A single PPP sample for each genotype served as the blanks for all experiments. If

washed platelets were used, the Tyrode's buffer in which the platelets were suspended was used as a blank instead.

When an experiment was to be performed, a spacer was added to the bottom of the cuvette to bring the liquid level above the optical sensor inside the machine. The sample was then placed in the testing well and allowed to rest in the machine for 1 minute, to bring the temperature of the sample to 37°C. During this time the software was set to record readings from the tube, and the baseline of the PRP sample was set using the appropriate button on the aggregometer itself. This baseline then represented 0% aggregation. A small quantity (2-5uL) of the chosen agonist was then added to the cuvette (still inside the testing well) using a micropipettor fitted with a 1-200uL gel-loading pipette tip. Immediately after stimulation, the door covering the testing well was quickly shut. If luminescence was to be tested, 12.5uL of CHRONO-LUM solution (Chrono-Log corp., Havertown, Pa) was added to the cuvette just before agonist was added. The reaction was then recorded by the machine, and allowed to run until completion, which was usually defined as 1 minute after all curves ceased to show any changes (curves were essentially flatlined). At this point the recording of the reaction data was stopped. The sample cuvettes were removed from the testing well and discarded.

Extent of maximal aggregation was assessed at the same time point for both curves in a single test. This time point varied slightly between tests. WT and *Plk3*$^{-/-}$ platelets were always used together in a single test, and tests were run in triplicate. The triplicate tests were averaged together and these averaged values were compared using a student's t-test.

2.6 Flow cytometry

Flow cytometry was performed as previously described, with minor modifications.[88] For flow cytometric analysis of surface molecule expression, washed platelets were obtained and diluted to a concentration of $6x10^7$ cells/mL. Counting was done on a hemocytometer. For testing of the expression of $\alpha_{IIb}\beta_3$ and PECAM-1, 100uL aliquots were made, and platelets were incubated with these antibodies for 30 min, then fixed for 15 minutes by adding 900uL of a 1% paraformaldehyde solution. For testing of the expression of P-Selectin, platelets had to first be stimulated (P-selectin is contained in platelet alpha-granules and is not expressed on the surface unless the platelet has undergone granular secretion). In this case, platelets were stimulated for 20 minutes at room temperature prior to addition of antibody. Both PAR4 peptide (100uM) and ADP (10uM) were used for this stimulation. As expected, only the PAR4 produced stimulation of granular secretion.

After fixation, platelets were run on the flow cytometer. Separate treatments were run in triplicate to obtain the most accurate results. In all cases where fluorophore-conjugated antibodies were used, samples treated only with isotype IgG were also run as a control.

For testing of FITC-Fibrinogen binding, 100uL aliquots of the platelet suspensions were treated with various concentrations of convulxin to activate them. These aliquots were lightly vortexed and incubated at room temperature for 20 minutes. FITC-Fibrinogen (60uL) was then added, the platelets were again lightly vortexed, and then incubated at 37°C for 20 minutes. To fix the platelets, 900uL of ice cold paraformaldehyde was added to each 100uL aliquot and they were incubated a final time at room temperature for 15 minutes. Three different concentrations of

convulxin (50, 75, and 100 ng/mL) were used for stimulation of platelets for FITC-Fibrinogen binding experiments.

Samples were run on the flow cytometer and the mean fluorescence intensities of the samples were recorded. WT and *Plk3*$^{-/-}$ platelets were always used together in a single test, and tests were run in triplicate. The triplicate tests were averaged together and these averaged values were compared using a student's t-test.

2.7 Serotonin secretion

All work involving the use of radioactive materials was conducted in an appropriate fume hood with radiation shielding, or in other equipment designated for that purpose. Work performed, materials used, and waste generated were all handled in accordance with the University of Delaware's radiation safety policies.

The procedure used to carry out testing of secretion using radiolabeled serotonin was adapted from established methods used in the field[89,90], with minor modifications.

Platelets were isolated using the procedure already stated for obtaining washed platelets, but with the addition of a labeling step performed before the platelets were pelleted and the plasma removed. After spinning whole blood at 150 rcf for 7 minutes and removing the PRP to new 5 ml polypropylene tubes, 2uL of the PRP was removed for addition to 198uL of Tyrode's. These diluted samples were used for counting on hemocytometer. Radiolabeled serotonin was added to the original PRP to a concentration of 0.5uCi/mL. 15uL of the PRP was removed immediately after addition of serotonin and placed into an LSC vial with 5mL of LSC fluid. The tubes of PRP were then incubated for 1 hour at 37°C, with light mixing every 15 minutes. At the end of the hour the tubes were removed and 1uM of imipramine was added to each,

followed by another 10 minute incubation. Imipramine is a serotonin reuptake inhibitor, and prevents serotonin from crossing the cell membrane. Adding it to our platelets helps ensure that they do not slowly "leak" the radioactive serotonin. The labeled PRP was then centrifuged at 400 rcf for 10 minutes. The supernatant was removed and saved, and the pellet was resuspended in Tyrode's containing 1uM imipramine. This solution was then split into the necessary number of 100uL aliquots. Each sample was stimulated with a varying concentration of thrombin for 5 minutes. Immediately after the end of the 5 minute period, 100uL of a stopping solution containing 1 part 37% formaldehyde, 5 parts 77mM EDTA (pH 7.5), and 4 parts 1x PBS, was added to the 100uL aliquots. They were then spun at 13k rpm for 2 minutes to completely remove the platelets from suspension. 150uL of the supernatant from each aliquot was removed and added to 5mL of LSC fluid in LSC vials. Some samples were not stimulated and were not centrifuged, but instead the platelets were lysed with 100uL of a 0.5% Triton X-100 stopping solution. These samples served as controls for the total amount of radioactivity present in the aliquots before stimulation and pelleting. Other samples were not stimulated with agonist, but were immediately treated with stopping solution, pelleted, and the supernatant added to LSC vials (this was done before all other samples were processed). These samples served as controls for the amount of radioactivity present in the supernatant before stimulation (expected to be very low).

The vials were then counted on an LSC counter to determine how much radioactivity was present in the supernatant. This machine returns values for each vial that correspond to the number of scintillations (flashes of light caused by the interaction of released radioactive particles with the LSC fluid) that the machine

counted during the reading period. In the LSC vials which contained only the supernatant from the aliquots after pelleting, the values recorded represented the amount of radioactivity released from the platelets during stimulation. Any radioactive serotonin still present in the platelets when stopping solution was added would be excluded from this reading, thus giving us a very accurate reading for how much secretion had occurred. The values recorded in the unstimulated samples were subtracted from the supernatant readings to reduce "background" radioactivity. These values were then divided by the total readings and multiplied by 100 to obtain the % of serotonin released into the supernatant.

To determine the amount of serotonin actually taken up by the platelets, samples were taken to be read on the LSC at two points: immediately after addition of serotonin and also just after platelets were resuspended in Tyrodes. The former reading represented the total radioactivity added to platelets, the latter reading represented the radioactivity actually taken into the platelets. These values were then compared to determine the % incorporation of the radiolabeled serotonin.

WT and *Plk3*$^{-/-}$ platelets were always used together in a single test, and tests were run in triplicate for each concentration of thrombin used and each control. The triplicate tests were averaged together and these averaged values were compared using a student's t-test.

2.8 Platelet spreading

Platelet spreading was performed as previously described, with minor modifications.[75] To perform platelet spreading, we first needed to thoroughly clean the glass coverslips on which the platelets would spread. Cover slips were gently scrubbed using a 0.2% Triton X-100 solution and then rinsed in ddH_2O. Cover slips

were removed from the ddH2O and placed directly into a container of isopropyl alcohol. The container was rotated briefly to wash the alcohol over all of the cover slips. Using tweezers, the cover slips were carefully removed from the alcohol and rinsed again with ddH2O. Cover slips were lightly blotted with a KimWipe to remove large water droplets, then allowed to dry overnight in a 37°C oven.

Platelet spreading was done in two different ways. In the first case, 250uL of 100ug/mL Fibrinogen or 3% BSA was placed onto the cover slips and allowed to settle for 1 hour at 37°C. This fluid was then pipetted off the cover slips and replaced by 250uL of 0.5% heat inactivated BSA, then incubated again at 37°C for 1 hour. At this point, the cover slips were ready to have platelets placed onto them, which in all cases was done immediately after the 0.5% BSA was removed.

Alternatively, the cover slips were removed from the oven and an 8 well Secure-Seal sticker was placed directly onto the cover slip. Only the paper backing of the sticker was removed; the plastic backing was kept. The resulting wells that were formed on the cover slip were 9mm across and 0.12 mm deep. Compared to the former method, this allowed us to precisely control the area in which platelets were allowed to spread, so that the total area covered by spread platelets could be precisely measured by capturing images from 5 different points in each well. Wells were filled with 50uL of 100ug/mL Fibrinogen (or 150uL of 3% BSA for controls) and incubated in a 37°C cell culture incubator for one hour. At the end of the incubation the Fibrinogen solution was removed by pipetting and 50uL of 0.5% heat inactivated BSA was added to the wells. This solution was allowed to coat the wells for 1 hour in the cell culture incubator. After incubation the blocking solution was removed before platelets were added to the well.

Platelets were prepared the same way in both cases. During the first coating step, blood was drawn from mice and washed platelets were prepared as previously described. Platelets were counted on a Coulter counter and diluted to a concentration of $5x10^6$ cells/mL. Aliquots of the washed platelets were made. If an aliquot was to be stimulated with agonist, a certain quantity of agonist was added to the aliquot and the aliquot was tapped briefly to ensure mixing of the agonist in the suspension. 50uL of the suspension was then immediately placed onto the coated wells if the Secure-Seal sticker was used. If it was not used, 250uL of platelets were added directly to the cover slips. Platelets were allowed to settle for 1 hour at 37°C in the tissue culture incubator.

After settling, the excess platelet solution was pipetted off of the wells and 50uL of a 4% paraformaldehyde solution was added for fixing. This fixing was done for 20 minutes at room temperature. Excess paraformaldehyde was removed by pipetting and the coverslips were dipped 4 times in a small beaker of 1X PBS to rinse away the fixative and any unattached platelets. The plastic backing was then peeled away from the Secure-Seal sticker and the cover slips were placed well-side down on cleaned glass microscope slides. 150uL of ddH20 was placed onto the glass slides before placing the cover slips down. One end of each slip was then placed on the slide, and the other end was slowly lowered down using the fine point of a needle. This was done to ensure that no air became trapped inside any of the wells. When the cover slips had been lowered completely, light pressure was applied to the areas where the adhesive of the sticker made contact with the slides, but not to the wells themselves. Images of the platelets were taken as soon as possible after affixing the cover slips to

the slides. One hour was usually taken to allow the adhesive to properly attach cover slips to slides.

DIC microscopy was performed at the Delaware Biotechnology Institute's Bioimaging Center. Under 630X magnification, images of each well were taken. An initial image was taken in the very center of the well (as determined by visual inspection of the location of the objective on the well), and then the position of the stage was noted using the single micrometer rulings on the side of the stage. Moving the stage 2um distances at a time, subsequent pictures were taken at 2um distances above, below, to the left, and to the right of the central image. Platelets adhered in these 5 fields were counted, and the total area of all platelets appearing in each image was measured to determine an average. This average was taken to represent the extent of spreading in each well. Extent of spreading of stimulated and unstimulated WT and Plk3-/- platelets was assessed by obtaining the average surface area of the platelets in all 5 images taken of a single well. The averages for each well were then compared.

WT and *Plk3*$^{-/-}$ platelets were always compared to each other in a single test, and slides of a particular agonist concentration were made in triplicate for each genotype. The results of the triplicate slides were averaged together and these averaged values were compared using a paired t-test.

Chapter 3

RESULTS

3.1 Aggregation testing of WT and *Plk3*$^{-/-}$ platelets

One of the most basic tests one can perform for the activation of the $\alpha_{IIb}\beta_3$ integrin is a test of how well platelets in suspension are able to aggregate once stimulated and under flow conditions. The integrin is the major factor in the platelet-platelet interactions that govern this process. If stimulation by an agonist is able to initiate proper inside-out signaling, and there is sufficient fibrinogen in the suspension, platelets should follow a normal aggregation curve. Therefore, aggregation is primarily a test of inside-out signaling.

When the samples are inserted into the machine and "blanked", the tracings begin in a flat line around the zero mark on the graph. After sufficient stimulation, the tracings begin a characteristic pattern of "shape change", in which the tracings actually fall below the zero mark. This represents the fact that the platelets in the sample are changing from their discoid shape to a more spherical shape, thus scattering more of the light passing through the sample then they did when it was blanked. Shape change usually peaks quickly, and the curve begins to fall as true aggregation begins. As the platelets clump together they scatter less light. As the aggregates in the sample get larger and larger, they cause a characteristic "shaking" effect in the tracing by passing in front of the photosensor and scattering varying amounts of light in a seemingly

random pattern. However, overall the curve follows a steady pattern representative of how fast the platelets are aggregating in suspension.

3.1.1 *Plk3*$^{-/-}$ platelets exhibit hyperaggregation in response to certain agonists

Platelets in PRP were stimulated with varying doses of collagen (0.5, 1 and 2 ug/mL), and the aggregation tracings were monitored. Tracings of both genotypes followed a pattern of slow, extended shape change before a more gradual aggregation tracing. As a consequence of a more extended shape change phase and delayed aggregation, the tracing would occasionally rise beyond the threshold of the recording software, producing a cut-off shape change peak.

The resulting data from this experiment revealed that *Plk3*$^{-/-}$ platelets show an increased extent of aggregation in response to lower doses of collagen. (Figure 3.1, A-B) Extent of aggregation was assessed as the point where the tracing becomes flat and does not continue to descend further, indicating that the platelets have stopped aggregating. Overall data showed that in response to 0.5ug/mL of collagen, *Plk3*$^{-/-}$ would typically aggregate to the point where the sample reached 30-40% light transmittance. By contrast, WT platelets typically exhibited less than half that extent. Increasing doses of collagen resulted in both genotypes increasing in extent of aggregation, but PLK3-/- platelets nearly always aggregating to a greater extent. As the dose was increased, the gap between WT and PLK3-/- lessened until it was minimal or non-existent at approximately 2ug/mL of collagen. (Figure 3.1C)

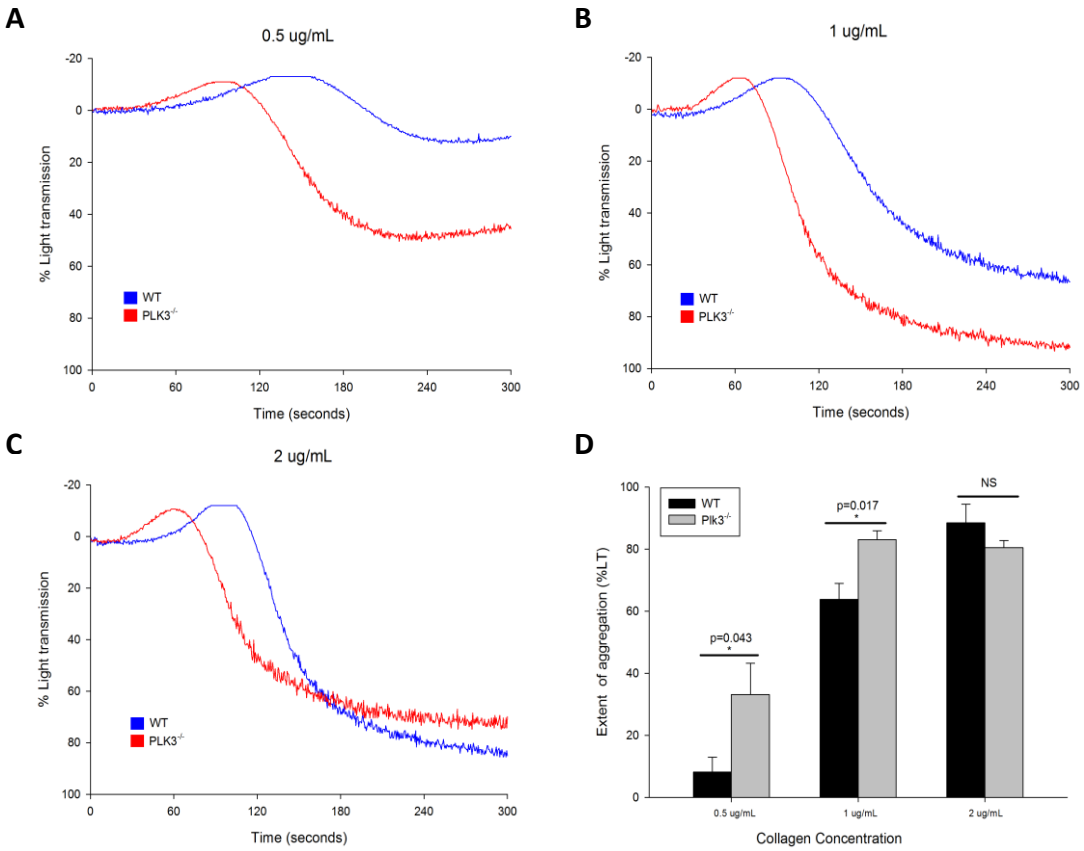


Figure 3.1 *Plk3*$^{-/-}$ platelets hyperaggregate when stimulated with low doses of collagen.

Panels A,B, and C show representative tracings of aggregation experiments, using various concentrations of collagen. Platelets in PRP diluted with Tyrode's buffer were stimulated and light transmittance of the sample was recorded, under stirring. Panel D shows the accumulated data of these experiments. A clear dose-dependent response and a strong increase in the extent of aggregation of *Plk3*$^{-/-}$ platelets can be seen. Extent of aggregation was assessed at the point when the tracing of one of the two samples would not aggregate any further, and either ran flat or began to draw upwards (indicating disaggregation).

A similar result was seen using PAR4 peptide as an agonist. This small peptide (A-Y-P-G-K-F) mimics the tethered ligand that results from thrombin cleavage of the receptor's amino terminus. It therefore activates the exact same receptors and pathways as thrombin, but can be used in situations where thrombin's other effects (fibrinogen cleavage to make fibrin, for example) would be undesirable. This agonist produces a very different aggregation tracing from collagen. Shape change occurs much faster, and aggregation begins quickly after if a sufficient dose is administered, producing a much shorter shape change peak.

My results indicated that low doses of PAR4 were sufficient to stimulate WT and *Plk3*$^{-/-}$ platelets to begin the process of aggregation, but that *Plk3*$^{-/-}$ platelets had a tendency to hyperaggregate. (Figure 3.2, A-D) In the lowest dose used (35uM), WT platelets exhibited only 20-25% increase in light transmission, while KO platelets showed a much higher (~60%) increase. At higher doses of PAR4 (40uM and 60uM) there was no significant difference.

Aggregation was also performed using washed platelets and 40uM PAR4 peptide as an agonist. Some samples had fibrinogen added to them, while others did not. In the samples with Fibrinogen, no difference was seen in aggregation. However without fibrinogen added, *Plk3*$^{-/-}$ platelets exhibited an approximately 20% increase in their extent of aggregation. (Figure 3.2, E-G)

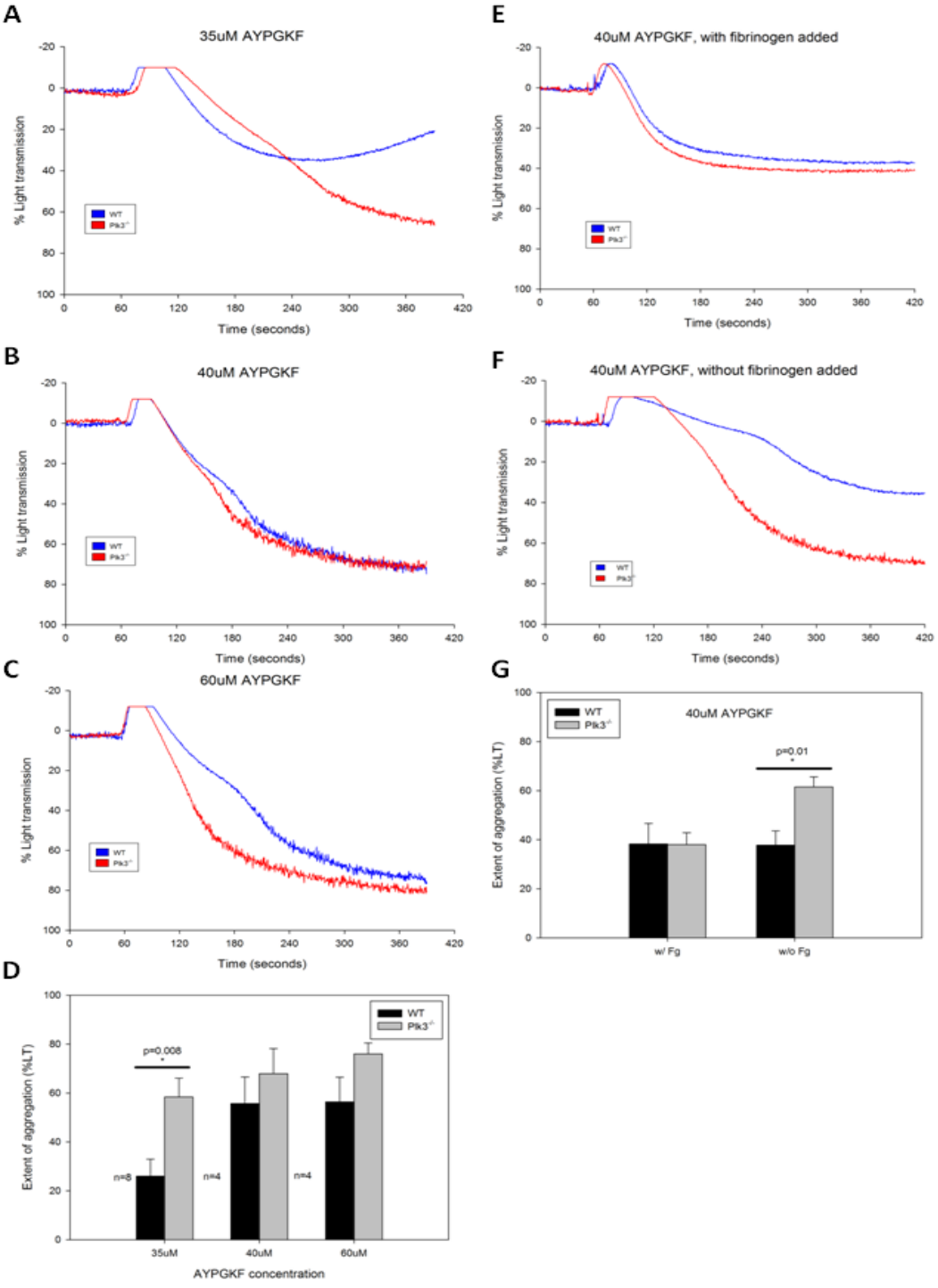

A
35uM AYPGKF
% Light transmission
Time (seconds)
WT
Plk3-/-
B
40uM AYPGKF
% Light transmission
Time (seconds)
C
60uM AYPGKF
% Light transmission
Time (seconds)
D
Extent of aggregation (%LT)
p=0.008
n=8
n=4
n=4
35uM
40uM
60uM
AYPGKF concentration
E
40uM AYPGKF, with fibrinogen added
% Light transmission
Time (seconds)
F
40uM AYPGKF, without fibrinogen added
% Light transmission
Time (seconds)
G
40uM AYPGKF
Extent of aggregation (%LT)
p=0.01
w/ Fg
w/o Fg

Figure 3.2 Plk3-/- platelets show hyperaggregation in response to AYPGKF, but only when stimulated with low doses of the peptide.

When testing platelets in PRP diluted with Tyrode's buffer, *Plk3*$^{-/-}$ platelets aggregated significantly more than WT platelets when the lowest concentration of AYPGKF was used (panels A,B, C and D). In washed platelets, this effect was only seen when platelets were allowed to aggregate without added fibrinogen (panels E, F, and G). Aggregation was carried out under stirring in both cases.

3.1.2 There is no difference in stimulation with 2-Me-S-ADP or U46619

Although aggregation using both collagen and PAR4 as agonists revealed hyperaggregation of *Plk3*$^{-/-}$ platelets, I was not able to uncover such a difference when using 2-Me-S-ADP (Figure 3.3, A-C) or the thromboxane A2 mimetic known as U46619 (Figure 3.4, A-C). This mimetic is necessary since TxA2 is unstable and quickly breaks down into thromboxane B2 after it is synthesized. U46619 on the other hand, is stable, although it has its own difficulties in handling and storage.

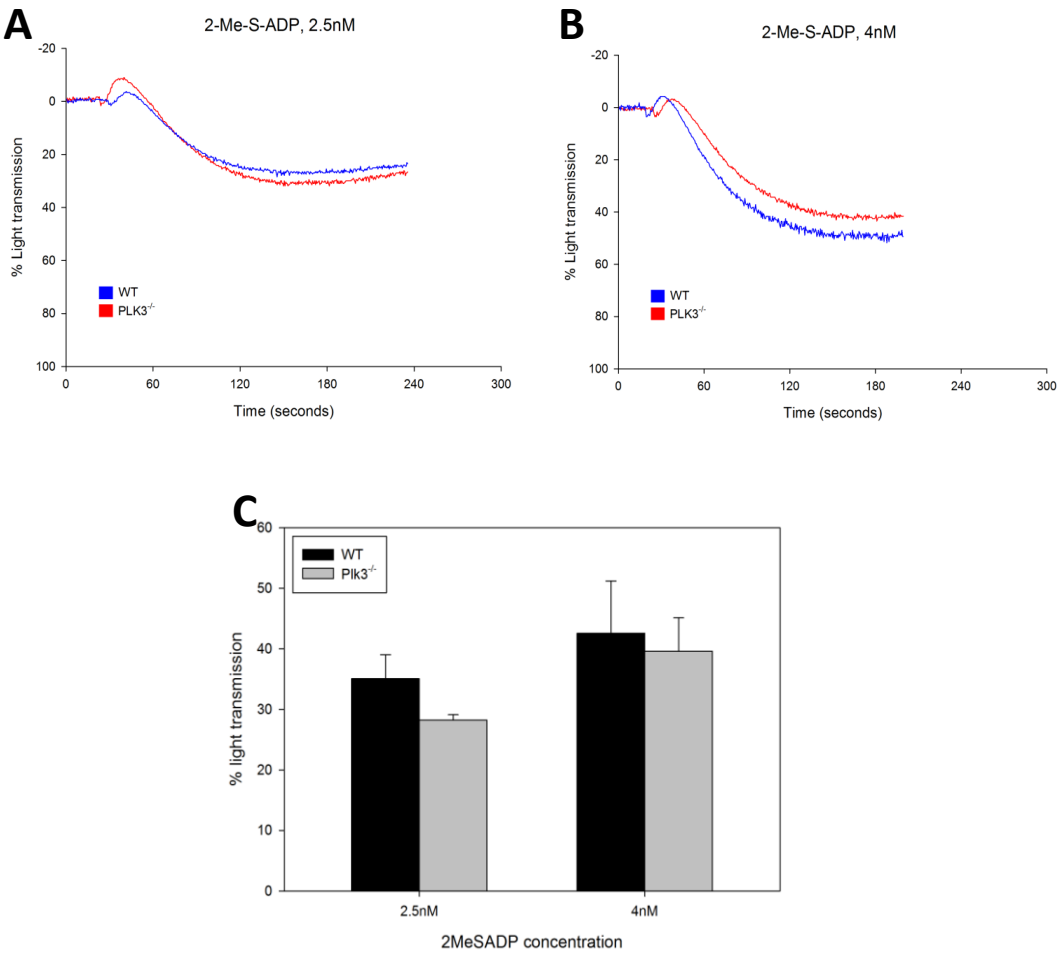


Figure 3.3 *Plk3*$^{-/-}$ platelets show a normal response when stimulated with 2MeSADP.

Platelets in PRP diluted with Tyrode's buffer were stimulated using 2MeSADP and light transmittance of the sample was recorded, under stirring. There was no significant difference in aggregation between *Plk3*$^{-/-}$ and WT platelets. Extent of aggregation increased slightly when the dose was increased, but still produced no difference between the two genotypes. Panels A and B show representative tracings. Panel C shows the cumulative data of all experiments.

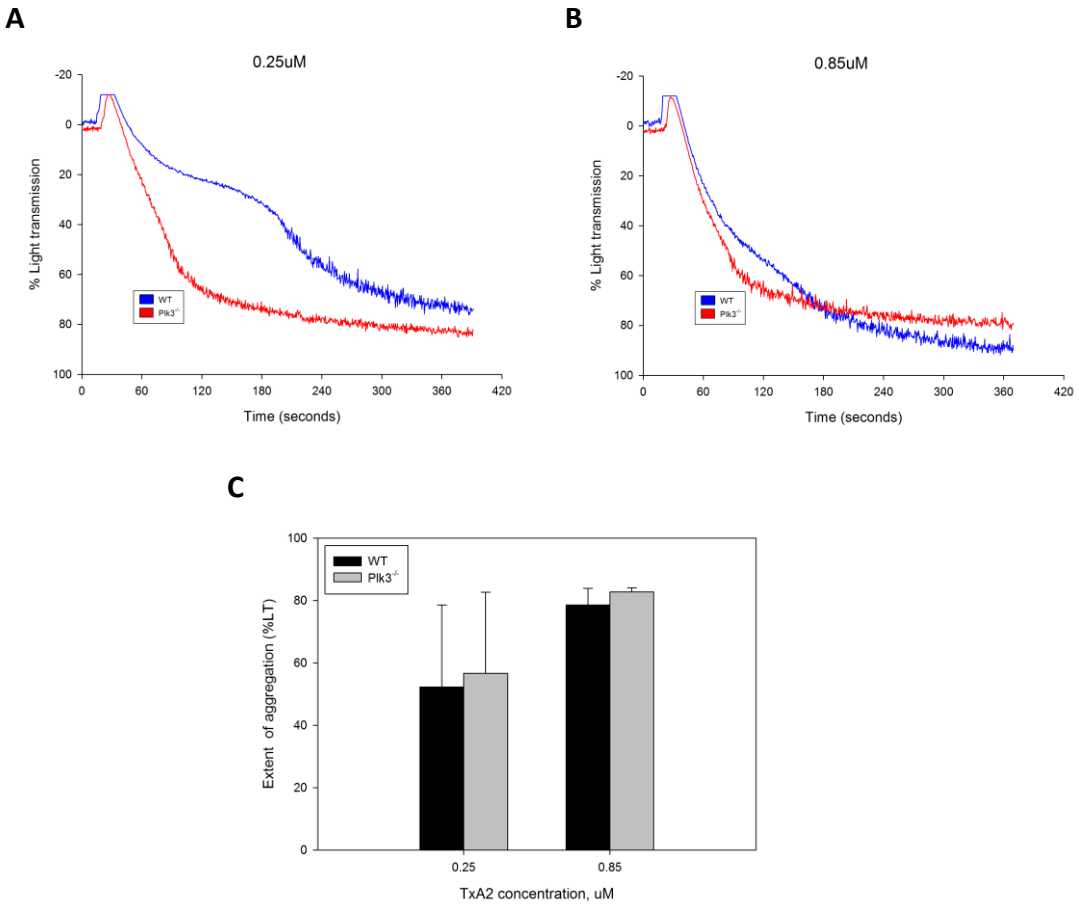


Figure 3.4 *Plk3*$^{-/-}$ platelets show a normal response when stimulated with U46619, a TxA2 mimetic.

Platelets in PRP diluted with Tyrode's buffer were stimulated using U46619 and light transmittance of the sample was recorded, under stirring. There was no significant difference in aggregation between *Plk3*$^{-/-}$ and WT platelets. Panels A and B show representative tracings. Panel C shows the cumulative data of all experiments.

3.2 *Plk3*$^{-/-}$ platelets bind more fibrinogen than WT platelets when stimulated with convulxin.

To test if the differences seen in aggregation were due to *Plk3*$^{-/-}$ platelets having a more active integrin $\alpha_{IIb}\beta_3$, we stimulated platelets with convulxin to activate them and then incubated them with FITC-conjugated fibrinogen. After running WT and *Plk3*$^{-/-}$ platelets on the flow cytometer, we could determine if one genotype bound more fibrinogen than the other.

The results of this experiment showed clearly that *Plk3*$^{-/-}$ platelets do bind more FITC-fibrinogen than WT platelets in response to convulxin (Figure 3.5), suggesting that they may have a more active integrin $\alpha_{IIb}\beta_3$ than WT platelets.

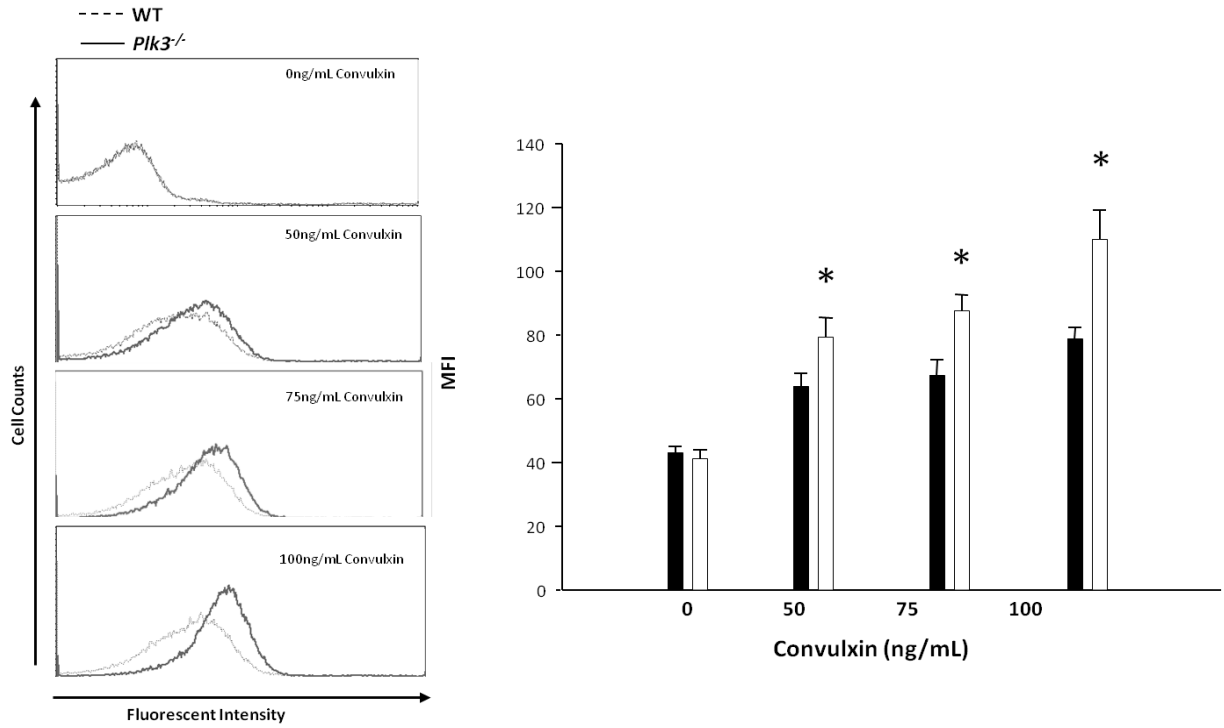


Figure 3.5 Plk3-/- platelets show increased binding of FITC-fibrinogen when stimulated with convulxin.

Plk3$^{-/-}$ and WT platelets were isolated from mice, washed, and stimulated with varying concentrations of convulxin. After this stimulation they were incubated for 20 minutes with FITC-fibrinogen and then fixed using a paraformaldehyde solution. When analyzed using a flow cytometer, *Plk3*$^{-/-}$ platelets clearly show increased binding of fibrinogen compared to WT platelets analyzed in the same manner.

3.3 There is no difference in the surface expression of $\alpha_{IIb}\beta_3$ integrin or PECAM-1 between *Plk3*$^{-/-}$ and WT platelets

In order to assess whether or not the differences in aggregation that I observed were related in any way to over or under expression of surface molecules involved in platelet-platelet interactions, I performed flow cytometry analysis of WT and *Plk3*$^{-/-}$

platelets. The first molecule that we investigated was the integrin $\alpha_{IIb}\beta_3$. Hyper aggregation in *Plk3*$^{-/-}$ platelets could be caused by an over-expression of the integrin, thus leading to more active integrin on the surface after stimulation. However, our results revealed that there was no difference in the level of surface expression of the integrin between WT and KO platelets (Figure 3.6, A-B).

The protein known as Platelet endothelial cell adhesion molecule 1 (or PECAM-1) is also expressed on the surface of mature platelets. Our purpose for investigating the expression of this molecule, however, was the inverse of investigation of the integrin; under expression of PECAM-1 on the surface of *Plk3*$^{-/-}$ platelets could lead to a hyperaggregation phenotype, since PECAM-1 can actually inhibit aggregation. However, once again we did not find any difference in the expression of this molecule between WT and *Plk3*$^{-/-}$ (Figure 3.6, C-D).

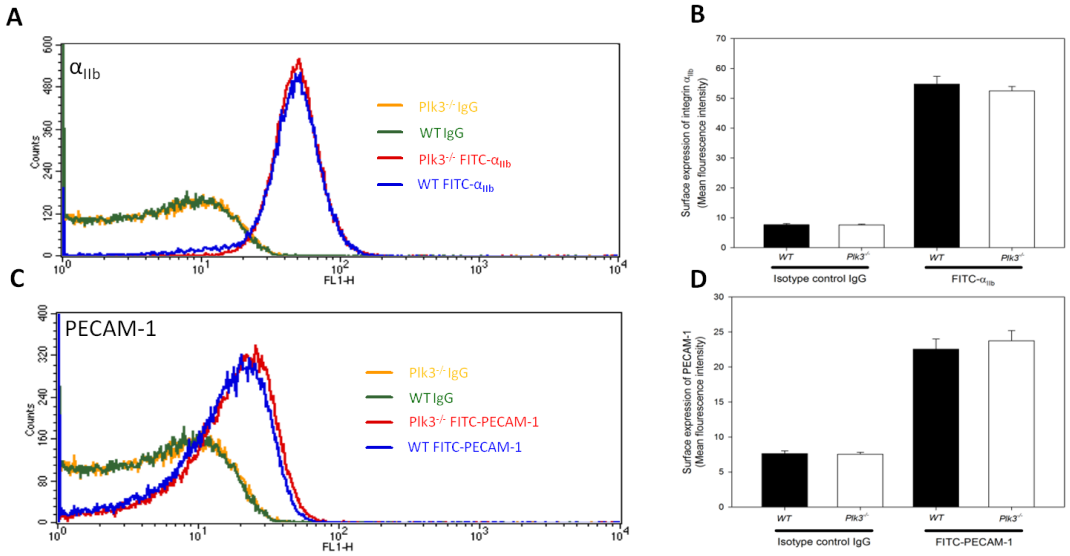


Figure 3.6 There is no difference in the surface expression of two crucial platelet surface molecules.

Unstimulated platelets were treated with flourophore-conjugated antibodies against α_{IIb} and PECAM-1, and analyzed on a flow cytometer. This analysis resulted in no difference in the surface expression of the two molecules between *Plk3*$^{-/-}$ and WT platelets.

3.4 *Plk3*$^{-/-}$ platelets show decreased secretion of alpha and dense granules

As mentioned earlier, granular secretion is an important step in aggregation and thrombus formation as a whole. Many of the granular constituents are themselves strong platelet activators, serving to amplify the coagulation response in flowing blood and leading eventually to stabilization of the forming clot. Since secreted factors can have such an effect on platelet activity, we decided to test the expression of a marker of alpha granule secretion, P-selectin. In unstimulated platelets, P-selectin is not expressed on the platelet membrane, and is found only in the lipid membrane of alpha granules. Upon the initiation of secretion, the membranes of the alpha granules fuse with the outer platelet membrane to release their contents, and it is after this point that P-selectin can be found on the platelet surface.

Plk3$^{-/-}$ platelets and WT platelets were stimulated with agonist, exposed to an antibody for P-selectin, and analyzed on a flow cytometer. Our results in this area were intriguing. We had expected that if a difference were to be found, *Plk3*$^{-/-}$ platelets would show an increased level of expression, suggesting increased secretion and a potential explanation of the aggregation phenotype that had been observed. However, we saw the opposite; *Plk3*$^{-/-}$ platelets actually had a decreased level of P-selectin expression as compared to WT platelets (Figure 3.7, A-B). This seems to imply that *Plk3*$^{-/-}$ platelets secrete less than their WT counterparts, but this finding does nothing to explain the hyperaggregation phenotype.

In light of the results seen in the flow cytometric analysis of P-selectin expression, I wanted to investigate granular secretion of *Plk3*$^{-/-}$ platelets as compared to the WT.

One of the best assays to use for doing this is the serotonin secretion assay. Serotonin, besides being an important neurotransmitter, is a key component of platelet dense granules and plays a significant role in platelet activation and signaling. Platelets do not synthesize their own serotonin, but instead absorb serotonin from the blood stream as they circulate. They incorporate serotonin by active transport using the SERT membrane transporter. For this reason, platelets will readily incorporate any serotonin dissolved in a platelet suspension. If this serotonin is radioactively labeled, we have an easily quantifiable and highly accurate method for determining just how much of that serotonin is secreted upon platelet stimulation.

Thrombin, being one of the stronger agonists that can be used to stimulate platelets, is also a good initiator of granular secretion. Therefore, we used it to test the ability of *Plk3*$^{-/-}$ platelets to secrete their dense granular contents. We found that *Plk3*$^{-/-}$ platelets were deficient in their ability to secrete their dense granular contents. They secreted about 25% less of the absorbed serotonin compared to WT platelets. As in aggregation, this difference was decreased or eliminated when higher concentrations of thrombin were used (Figure 3.7C). Similar to what was seen in aggregation testing, this seems to indicate that whatever deficiency is caused by the genetic ablation of *Plk3*, it is overcome when larger quantities of agonist are used.

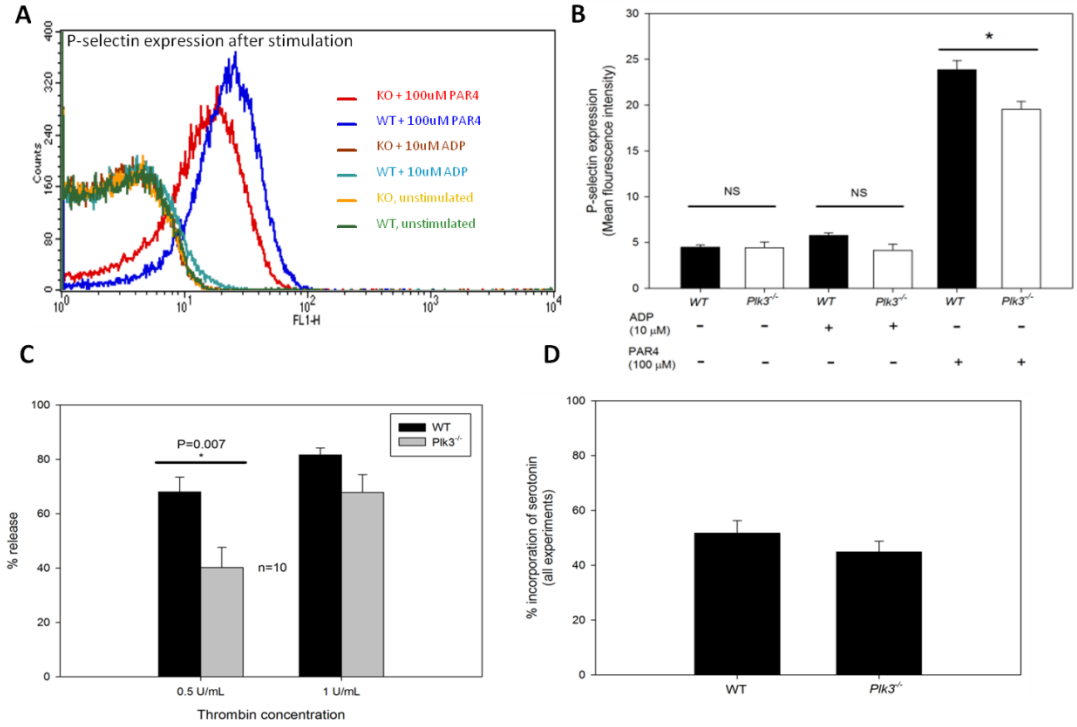


Figure 3.7 *Plk3*$^{-/-}$ platelets show decreased granular secretion.

Panels A and B show the results of flow cytometric analysis of *Plk3*$^{-/-}$ and WT platelets after stimulation with ADP or AYPGKF. There was a significant decrease in the level of expression of P-selectin after stimulation with AYPGKF, but not ADP. This indicates reduced alpha granule secretion. Panel C shows the results of a dense granule secretion assay using radioactively labeled serotonin. After stimulation for 5 minutes, *Plk3*$^{-/-}$ platelets exhibited less secretion than WT platelets. Secretion was assessed as the amount of radioactivity left in the supernatant after platelets were removed. Panel D shows % incorporation of serotonin into platelets.

3.5 WT and *Plk3*$^{-/-}$ platelets do not show any difference in the extent of platelet spreading.

Platelet spreading is an assay that is primarily used to assess the outside-in signaling capability of platelets. This is due to the fact that, unlike aggregation, the signaling events that govern platelet spreading are largely dependent on proper outside-in signaling. As platelets adhere to the immobilized fibrinogen, they undergo outside in signaling when the integrin binds to the fibrinogen. This binding causes unique signaling events that lead eventually to spreading.

My results revealed that there was no difference in platelet spreading between WT and *Plk3*$^{-/-}$ platelets when stimulated with PAR4 peptide or ADP. Platelets bound to the fibrinogen did not spread in any greater percentage in either genotype (Figure 3.8, A-C). However, *Plk3*$^{-/-}$ platelets did adhere to the fibrinogen in greater number than WT platelets. A difference was seen when platelets were treated with PAR4 peptide, but not when they were treated with ADP. Perhaps more telling is the fact that this difference was also seen when platelets were not stimulated at all, but simply allowed to settle on the fibrinogen (Figure 3.8D). This small yet significant difference may indicate that these platelets have a more active integrin $\alpha_{IIb}\beta_3$ than their WT counterparts.

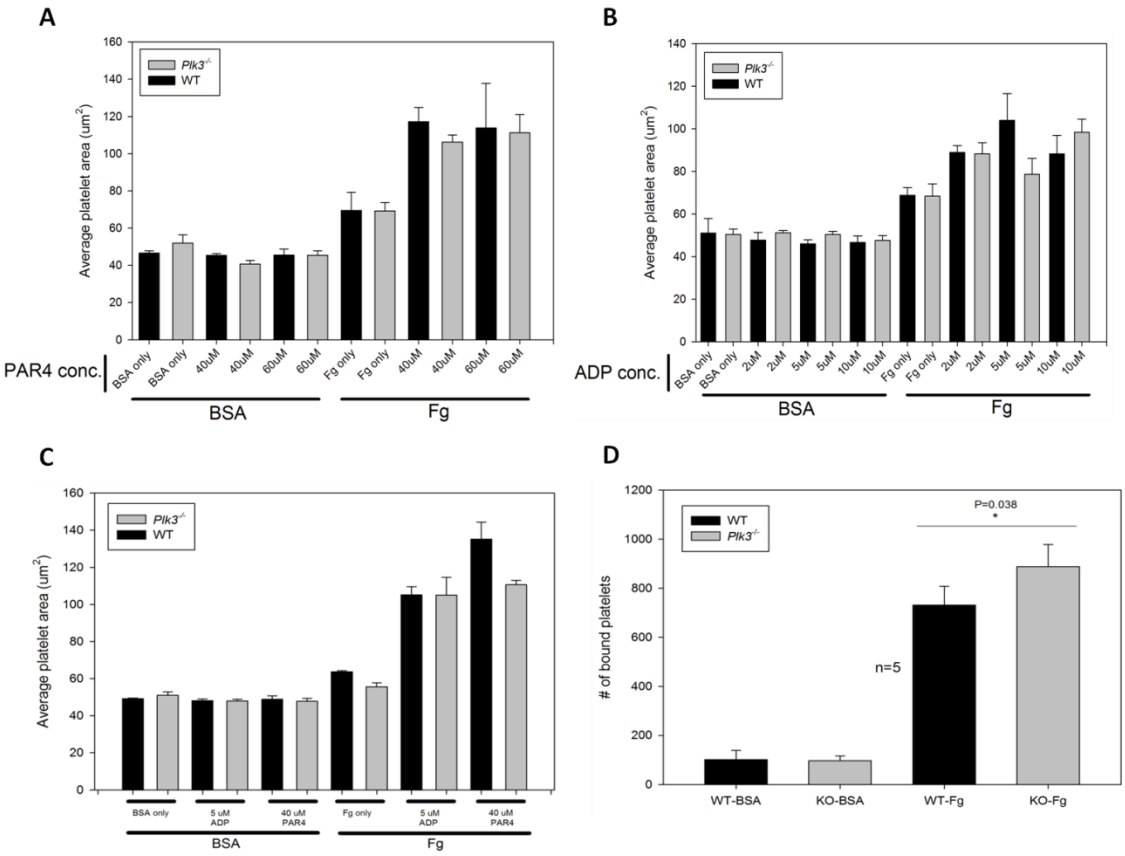


Figure 3.8 There is no difference in platelet spreading between *Plk3*$^{-/-}$ and WT platelets, but binding to fibrinogen may be increased.

Panels A and B show the results of tests for which the area used on the slide was not controlled for. Panel C shows the result of tests in which the area over which platelets were spread was strictly controlled for. Panel D shows the quantification of the number of platelets bound to the slides used in the experiments shown in Panel C.

3.6 There is no difference in the phosphorylation state of two platelet signaling proteins

In order to assess what might be causing the differences seen in WT and KO platelets following stimulation, Western blotting was performed on two proteins that are known to be important signal transducers in platelets: P38 and ERK1/2. Antibodies were used that recognized known phosphorylation sites on these proteins. If phosphorylated, it would represent that these proteins were active. A higher level of phosphorylated protein in KO platelets compared to WT would indicate that these proteins were more active, and possibly correlate to an overall increased level of activation.

However, I found no difference in the phosphorylation state of these proteins. In a preliminary Western blot, both WT and *Plk3*$^{-/-}$ yielded phosphorylated P38 and ERK1/2 protein in the same quantities, indicating that the differences seen were not due to any increase in the active levels of these signal transducers (Figure 3.9). However, this blot was only performed once, so a level of significance cannot yet be determined.

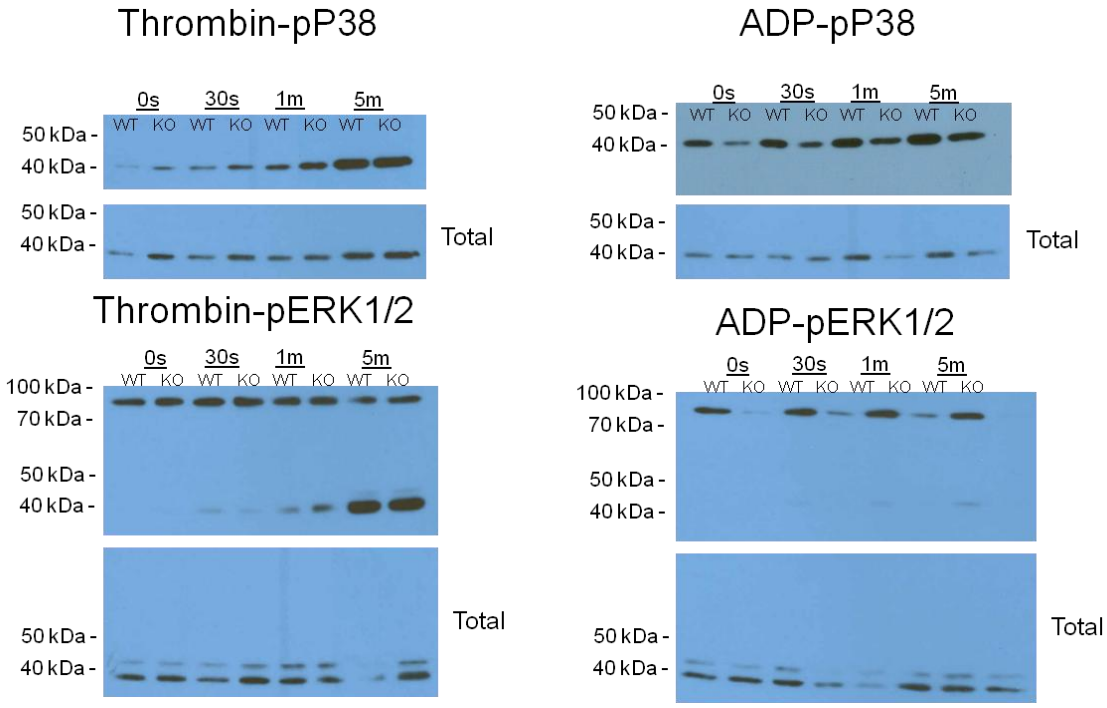


Figure 3.9 There is no difference in the level of phosphorylation of P38 and ERK1/2 between WT and *Plk3*$^{-/-}$ platelets.

WT and *Plk3*$^{-/-}$ platelets were stimulated with thrombin or ADP for various time periods and then lysed in 2x lysis buffer. Samples were run on a gel and blotted using antibodies detecting phosphorylation. No difference was seen for either signaling protein between WT and *Plk3*$^{-/-}$.

Chapter 4

DISCUSSION

My results clearly indicate that Plk3 plays an important role in the regulation of platelet function. This regulation appears to take place in two very different pathways. The first effect I was able to identify appears to be a negative control on the activity of the integrin $\alpha_{IIb}\beta_3$. *Plk3*$^{-/-}$ platelets show a definite increase in their ability to aggregate when stimulated with low levels of agonist. They also bind more fibrinogen than WT platelets, as seen using flow cytometry to quantify the amount of FITC-fibrinogen bound by both genotypes in response to convulxin stimulation. This appears to hold true for *Plk3*$^{-/-}$ platelets even when they are not stimulated by exogenous agonist, as evidenced by the fact that more *Plk3*$^{-/-}$ platelets bind to glass slides coated in fibrinogen.

Signaling through the integrin $\alpha_{IIb}\beta_3$ is subject to regulation primarily through the cytoplasmic tail of the β_3 subunit.[61] One way by which this regulation might be achieved is by phosphorylation of a threonine residue near a crucial protein binding site. This residue, Thr-753, is proximal to a site on the β_3 tail at which the protein known as talin binds, and phosphorylation of this site is inhibitory for integrin activation.[64,91] Talin has been shown to be crucial for integrin function, and it is likely responsible for separating the cytoplasmic tails of the integrin, a step required for inside-out signaling.[55] Plk3 is known to associate with CIB1[72], and CIB1 is known in turn to associate with the integrin $\alpha_{IIb}\beta_3$[73]. Plk3 is a serine/threonine kinase, and could be directly responsible for phosphorylating the Thr753 residue and thus providing a

check against $\alpha_{IIb}\beta_3$ activation. CIB1 might then be responsible for inhibiting the function of Plk3 once platelet activation has begun, allowing this check to be removed and for talin binding to proceed.

While the possibility exists that Plk3 is a direct inhibitor of integrin function, this is by no means the only way in which Plk3 might exert that effect. A recent study has implicated Plk3 as a negative regulator of the PI3K/Akt pathway. This pathway has long been known as important to both inside-out[92,93] and outside-in[94] signalling in platelets. In this study, Plk3 was shown to directly phosphorylate PTEN in murine embryonic fibroblasts. When active, PTEN inhibits the PI3K/Akt pathway by removing crucial phosphate groups placed on phospatidyl inositols by PI3K.These membrane bound PI 3-phosphates activate PDK1, which in turn activates Akt. The phosphorylation by Plk3 stabilizes PTEN, preventing its destruction .[95] This would result in a greater quantity of active PTEN and thus a greater removal of the crucial phosphate groups required for the activation of this pathway. If Plk3 is a significant antagonist of the Akt pathway in murine platelets, then *Plk3*$^{-/-}$ platelets would contain a more active Akt; this could lead to platelets that aggregated to a greater degree and were more sensitive to stimulation, as I saw in my experiments.

The close association of Plk3 with CIB1 may also provide an explanation as to why *Plk3*$^{-/-}$ platelets show hyperaggregation. Another recent study has suggested that the interaction of CIB1 with the α subunit of the integrin $\alpha_{IIb}\beta_3$ may be another way in which the active conformation of the integrin is stabilized, in much the same way as talin does so by binding to the β_3 cytoplasmic tail.[96] Since Plk3 is a binding partner for CIB1, knock out of the *Plk3* gene might result in more available CIB1 to bind to the

integrin and result in more integrin activation. This would represent an indirect effect of genetic ablation of Plk3.

Another, more direct effect may be proposed when considering the possibility that Plk3 is a regulator of the Akt pathway. If CIB1 is a direct regulator of Plk3 in platelets, it may be that upon platelet activation and subsequent calcium rise, CIB1 becomes active and binds to Plk3, inactivating it. As has been shown, this would negatively affect PTEN stability and PTEN activity would drop, causing a rise in the phosphoinosotols that lead to Akt activation. An increase in Akt activity would lead to an increase in integrin activation, and the phenotype that we see in in *Plk3*$^{-/-}$ platelets could be explained in this way.

Despite the fact that *Plk3*$^{-/-}$ platelets exhibit greater extent of aggregation and more binding of fibrinogen than WT platelets, *Plk3*$^{-/-}$ mice do not exhibit any extreme cardiovascular defects. As previously discussed, unpublished data obtained by other members of the Naik lab indicated that *Plk3*$^{-/-}$ mice exhibit longer tail bleeding times and take longer to form occlusive thrombi in carotid artery injury tests. While not severe enough to cause a significant health problem for the mouse, this phenotype would seem to be at odds with my findings that *Plk3*$^{-/-}$ platelets aggregate more easily and bind fibrinogen to an increased extent. This was at first very difficult to explain, but the results provided by my secretion studies provides a potential explanation. In the carotid artery injury test, many of the *Plk3*$^{-/-}$ mice failed to form a stable thrombus. Failure to form stable thrombi could be caused by a secretion defect[97], and the secretion defect in *Plk3*$^{-/-}$ mice may even be enough to cover up the hyperaggregation that we see when testing *Plk3*$^{-/-}$ platelets in vitro. *Plk3*$^{-/-}$ platelets were not different in terms of aggregation than WT platelets when higher concentrations of agonists were

used, and these low concentrations under laboratory conditions may not replicate a situation we would reasonably expect to find *in vivo.* Therefore, despite this potential for *Plk3*$^{-/-}$ mice to have thrombi that form faster than WT, the secretion defect they possess may be causing them to form smaller and less stable thrombi, leading to the in vivo phenotype that we see. The effect of the increase in aggregation and fibrinogen binding would be difficult to discern in the context of in vivo clot formation if there is a concurrent secretion defect, as appears to be the case. The former may even attenuate the latter, reducing its effect *in vivo*.

What then, could be causing this rather disparate effect that we see in *Plk3*$^{-/-}$ platelets? A potential answer may come from what is currently known about Plk3's role in cell types other than the platelet. Plk3 and its family members are known to function in the cell cycle, and Plk3 itself is believed to be responsible for changes in microtubule organization.[71] Furthermore, interfering with microtubules, and specifically inhibiting the reorganization of microtubules with substances that stabilize them, cause reduced secretion.[98,99] Plk3 may be responsible for changes in the platelet microtubule coil that are necessary for proper secretion, and in the absence of Plk3, microtubules are unnaturally stabilized and secretion is somehow inhibited.

Alternatively, Plk3 may play a role in the preparation and packaging of granules into platelets during megakaryocyte development. As platelet granules develop in megakaryocytes, they inherit some of their protein constituents from the MK trans-Golgi network. Platelet α granules at least appear to develop from vesicles originating directly from the the trans-Golgi.[100,101] Recently, Plk3 has been implicated as crucial for the fragmentation of the Golgi apparatus that must occur for it to be distributed to daughter cells during cell division. Studies have identified MEK1 as an

activator of Plk3, leading to microtubule changes and Golgi fragmentation (two processes that are closely linked).[102,103] Plk3 has been shown to interact with and directly phosphorylate a protein known as VRK1, and this phosphorylation was shown to be necessary for proper Golgi fragmentation to occur. Loss of kinase activity of either Plk3 or VRK1 blocked Golgi fragmentation, as did mutation of the serine residue on VRK1 that Plk3 phosphorylates.[104] Genetic ablation of Plk3 may disrupt the proper formation of granules in developing MKs by disrupting this MEK1/Plk3/VRK1 pathway, thereby significantly altering secretion in the platelets that are produced.

Additionally, the possibility exists that since the technique used to delete the *Plk3* gene resulted in a global knockout, the in-vivo phenotype we see may be contributed to by another cell type. Specifically, vascular endothelial cells also secrete vesicles when activated, known as Weibel-Palade bodies. These vesicles contain many proteins and factors, but chief among them are vWF and P-selectin.[105] A decrease in the amount of circulating vWF could seriously impact initial clot formation, leading to clots that do not form as quickly as they would in WT mice. A reduction in available P-selectin can affect clot stability as well. It has been shown that if the gene for P-selectin is knocked out in mice, clot stabilization and retraction is reduced following vascular injury.[106] If vascular endothelial cells of *Plk3*$^{-/-}$ mice exhibit a minor secretion defect as platelets do, it may contribute to, and help to explain, the in-vivo phenotype.

The data presented here in this report clearly identifies a role for Plk3 as a broad regulator of platelet function. I have demonstrated that absence of Plk3 in murine platelets results in enhanced aggregation through an as-yet undetermined effect on the integrin $\alpha_{IIb}\beta_3$. In order to establish the method by which Plk3 is able to

influence integrin activation, further studies will need to focus on avenues of signaling not explored here. Western blotting for active Akt and PDK1 in *Plk3*$^{-/-}$ platelets is needed to determine if this pathway is indeed more active, which would have wide-ranging implications for platelet function beyond just the integrin itself.

The aggregation phenotype alone would be sufficient cause to investigate Plk3 further, but I also was able to determine that Plk3 has another, seemingly disparate effect on platelet secretion that may be manifesting itself as the dominant phenotype in *Plk3*$^{-/-}$ mice. It is interesting to note that although *Plk3*$^{-/-}$ mice exhibit a definite (but mild) bleeding tendency, they have no outward signs of ill cardiovascular health. This may suggest that if the means by which Plk3 regulates secretion were properly elucidated, this understanding could yield subtle yet effective drug targets for the treatment of cardiovascular disease. Future studies in this area are thus highly warranted and should focus on the morphology, quantity, and protein contents of *Plk3*$^{-/-}$ platelet granules to determine if there is indeed a defect in vesicular trafficking that may occur during MK development. Additionally, it may be pertinent to examine microtubule integrity before, during, and after platelet activation in the event that a possible defect in microtubule reorganization in the platelet itself may be observed.

These previously unknown roles for Plk3 hint at just how much is still not understood regarding platelet function. My results clearly indicate that Plk3 is important for regulation of multiple aspects of this function, but I was not able to go so far as to determine exactly how this regulation might take place. It is even possible that Plk3 is involved in aspects of platelet signaling that have not yet been identified. By investigating Plk3 and its interacting partners more closely in the future we should be able to shed more light on this ever so crucial aspect of the cardiovascular system.

REFERENCES

1. Roger, V.L., *et al.* Heart disease and stroke statistics--2012 update: a report from the American Heart Association. *Circulation* **125**, e2-e220 (2012).
2. Bessis, M. *Living blood cells and their ultrastructure,* (Springer-Verlag, Berlin, New York,, 1973).
3. Fernandez-Real, J.M., Vendrell, J., Richart, C., Gutierrez, C. & Ricart, W. Platelet count and interleukin 6 gene polymorphism in healthy subjects. *BMC medical genetics* **2**, 6 (2001).
4. Ogawa, M. Differentiation and proliferation of hematopoietic stem cells. *Blood* **81**, 2844-2853 (1993).
5. Lordier, L., *et al.* Megakaryocyte endomitosis is a failure of late cytokinesis related to defects in the contractile ring and Rho/Rock signaling. *Blood* **112**, 3164-3174 (2008).
6. Odell, T.T., Jr., Jackson, C.W. & Friday, T.J. Megakaryocytopoiesis in rats with special reference to polyploidy. *Blood* **35**, 775-782 (1970).
7. Paulus, J.M. DNA metabolism and development of organelles in guinea-pig megakaryocytes: a combined ultrastructural, autoradiographic and cytophotometric study. *Blood* **35**, 298-311 (1970).
8. Behnke, O. An electron microscope study of the megacaryocyte of the rat bone marrow. I. The development of the demarcation membrane system and the platelet surface coat. *Journal of ultrastructure research* **24**, 412-433 (1968).
9. Radley, J.M. & Haller, C.J. The demarcation membrane system of the megakaryocyte: a misnomer? *Blood* **60**, 213-219 (1982).
10. Jones, O.P. Origin of megakaryocyte granules from Golgi vesicles. *The Anatomical record* **138**, 105-113 (1960).
11. Youssefian, T. & Cramer, E.M. Megakaryocyte dense granule components are sorted in multivesicular bodies. *Blood* **95**, 4004-4007 (2000).
12. Radley, J.M. & Scurfield, G. The mechanism of platelet release. *Blood* **56**, 996-999 (1980).
13. Italiano, J.E., Jr., Lecine, P., Shivdasani, R.A. & Hartwig, J.H. Blood platelets are assembled principally at the ends of proplatelet processes produced by differentiated megakaryocytes. *J Cell Biol* **147**, 1299-1312 (1999).
14. Lichtman, M.A., Chamberlain, J.K., Simon, W. & Santillo, P.A. Parasinusoidal location of megakaryocytes in marrow: a determinant of platelet release. *American journal of hematology* **4**, 303-312 (1978).

15. Gewirtz, A.M. Megakaryocytopoiesis: the state of the art. *Thrombosis and haemostasis* **74**, 204-209 (1995).
16. Eaton, D.L. & de Sauvage, F.J. Thrombopoietin and the humoral regulation of thrombocytopoiesis. *Current opinion in hematology* **2**, 167-171 (1995).
17. Kuter, D.J., Beeler, D.L. & Rosenberg, R.D. The purification of megapoietin: a physiological regulator of megakaryocyte growth and platelet production. *Proceedings of the National Academy of Sciences of the United States of America* **91**, 11104-11108 (1994).
18. Fielder, P.J., *et al.* Regulation of thrombopoietin levels by c-mpl-mediated binding to platelets. *Blood* **87**, 2154-2161 (1996).
19. Yang, Y.C., *et al.* Human IL-3 (multi-CSF): identification by expression cloning of a novel hematopoietic growth factor related to murine IL-3. *Cell* **47**, 3-10 (1986).
20. George, J.N. Platelets. *Lancet* **355**, 1531-1539 (2000).
21. Ruggeri, Z.M. & Mendolicchio, G.L. Adhesion mechanisms in platelet function. *Circulation research* **100**, 1673-1685 (2007).
22. Schneider, S.W., *et al.* Shear-induced unfolding triggers adhesion of von Willebrand factor fibers. *Proceedings of the National Academy of Sciences of the United States of America* **104**, 7899-7903 (2007).
23. Savage, B., Almus-Jacobs, F. & Ruggeri, Z.M. Specific synergy of multiple substrate-receptor interactions in platelet thrombus formation under flow. *Cell* **94**, 657-666 (1998).
24. Watson, S.P., Auger, J.M., McCarty, O.J. & Pearce, A.C. GPVI and integrin alphaIIb beta3 signaling in platelets. *Journal of thrombosis and haemostasis : JTH* **3**, 1752-1762 (2005).
25. Blomback, B., Blomback, M., Hessel, B. & Iwanaga, S. Structure of N-terminal fragments of fibrinogen and specificity of thrombin. *Nature* **215**, 1445-1448 (1967).
26. Springer, T.A. & Wang, J.H. The three-dimensional structure of integrins and their ligands, and conformational regulation of cell adhesion. *Advances in protein chemistry* **68**, 29-63 (2004).
27. Kulkarni, S., *et al.* A revised model of platelet aggregation. *The Journal of clinical investigation* **105**, 783-791 (2000).
28. Vu, T.K., Hung, D.T., Wheaton, V.I. & Coughlin, S.R. Molecular cloning of a functional thrombin receptor reveals a novel proteolytic mechanism of receptor activation. *Cell* **64**, 1057-1068 (1991).
29. Ishihara, H., *et al.* Protease-activated receptor 3 is a second thrombin receptor in humans. *Nature* **386**, 502-506 (1997).
30. Xu, W.F., *et al.* Cloning and characterization of human protease-activated receptor 4. *Proceedings of the National Academy of Sciences of the United States of America* **95**, 6642-6646 (1998).
31. Molino, M., Bainton, D.F., Hoxie, J.A., Coughlin, S.R. & Brass, L.F. Thrombin receptors on human platelets. Initial localization and subsequent

redistribution during platelet activation. *The Journal of biological chemistry* **272**, 6011-6017 (1997).
32. Leon, C., *et al.* The P2Y1 receptor is an ADP receptor antagonized by ATP and expressed in platelets and megakaryoblastic cells. *FEBS letters* **403**, 26-30 (1997).
33. Hollopeter, G., *et al.* Identification of the platelet ADP receptor targeted by antithrombotic drugs. *Nature* **409**, 202-207 (2001).
34. Hechler, B., Eckly, A., Ohlmann, P., Cazenave, J.P. & Gachet, C. The P2Y1 receptor, necessary but not sufficient to support full ADP-induced platelet aggregation, is not the target of the drug clopidogrel. *British journal of haematology* **103**, 858-866 (1998).
35. Ohlmann, P., *et al.* The human platelet ADP receptor activates Gi2 proteins. *The Biochemical journal* **312 (Pt 3)**, 775-779 (1995).
36. Jackson, S.P., Yap, C.L. & Anderson, K.E. Phosphoinositide 3-kinases and the regulation of platelet function. *Biochemical Society transactions* **32**, 387-392 (2004).
37. Kim, S., Jin, J. & Kunapuli, S.P. Akt activation in platelets depends on Gi signaling pathways. *The Journal of biological chemistry* **279**, 4186-4195 (2004).
38. Hamm, H.E. How activated receptors couple to G proteins. *Proceedings of the National Academy of Sciences of the United States of America* **98**, 4819-4821 (2001).
39. Williams, A.G., *et al.* Identification of the pertussis toxin-sensitive G proteins in platelets, megakaryocytes, and human erythroleukemia cells. *Blood* **76**, 721-730 (1990).
40. Klages, B., Brandt, U., Simon, M.I., Schultz, G. & Offermanns, S. Activation of G12/G13 results in shape change and Rho/Rho-kinase-mediated myosin light chain phosphorylation in mouse platelets. *J Cell Biol* **144**, 745-754 (1999).
41. Hubbard, K.B. & Hepler, J.R. Cell signalling diversity of the Gqalpha family of heterotrimeric G proteins. *Cellular signalling* **18**, 135-150 (2006).
42. Hartwig, J.H. & DeSisto, M. The cytoskeleton of the resting human blood platelet: structure of the membrane skeleton and its attachment to actin filaments. *J Cell Biol* **112**, 407-425 (1991).
43. Stossel, T.P., *et al.* Filamins as integrators of cell mechanics and signalling. *Nature reviews. Molecular cell biology* **2**, 138-145 (2001).
44. Kowit, J.D., Linck, R.W. & Kenney, D.M. Isolated cytoskeletons of human blood platelets: dark-field imaging of coiled and uncoiled microtubules. *Biology of the cell / under the auspices of the European Cell Biology Organization* **64**, 283-291 (1988).
45. Schwer, H.D., *et al.* A lineage-restricted and divergent beta-tubulin isoform is essential for the biogenesis, structure and function of blood platelets. *Current biology : CB* **11**, 579-586 (2001).

46. Berridge, M.J. Inositol trisphosphate and diacylglycerol as second messengers. *The Biochemical journal* **220**, 345-360 (1984).
47. Janmey, P.A. Phosphoinositides and calcium as regulators of cellular actin assembly and disassembly. *Annual review of physiology* **56**, 169-191 (1994).
48. Yin, H.L. & Stossel, T.P. Control of cytoplasmic actin gel-sol transformation by gelsolin, a calcium-dependent regulatory protein. *Nature* **281**, 583-586 (1979).
49. Falet, H., *et al.* Importance of free actin filament barbed ends for Arp2/3 complex function in platelets and fibroblasts. *Proceedings of the National Academy of Sciences of the United States of America* **99**, 16782-16787 (2002).
50. Machesky, L.M. & Gould, K.L. The Arp2/3 complex: a multifunctional actin organizer. *Current opinion in cell biology* **11**, 117-121 (1999).
51. Burridge, K. & Fath, K. Focal contacts: transmembrane links between the extracellular matrix and the cytoskeleton. *BioEssays : news and reviews in molecular, cellular and developmental biology* **10**, 104-108 (1989).
52. Nachmias, V.T., Kavaler, J. & Jacubowitz, S. Reversible association of myosin with the platelet cytoskeleton. *Nature* **313**, 70-72 (1985).
53. Hynes, R.O. Integrins: bidirectional, allosteric signaling machines. *Cell* **110**, 673-687 (2002).
54. Takagi, J., Petre, B.M., Walz, T. & Springer, T.A. Global conformational rearrangements in integrin extracellular domains in outside-in and inside-out signaling. *Cell* **110**, 599-511 (2002).
55. Tadokoro, S., *et al.* Talin binding to integrin beta tails: a final common step in integrin activation. *Science* **302**, 103-106 (2003).
56. Calderwood, D.A., *et al.* The phosphotyrosine binding-like domain of talin activates integrins. *The Journal of biological chemistry* **277**, 21749-21758 (2002).
57. Crittenden, J.R., *et al.* CalDAG-GEFI integrates signaling for platelet aggregation and thrombus formation. *Nature medicine* **10**, 982-986 (2004).
58. Han, J., *et al.* Reconstructing and deconstructing agonist-induced activation of integrin alphaIIbbeta3. *Current biology : CB* **16**, 1796-1806 (2006).
59. Watanabe, N., *et al.* Mechanisms and consequences of agonist-induced talin recruitment to platelet integrin alphaIIbbeta3. *J Cell Biol* **181**, 1211-1222 (2008).
60. Plow, E.F., Haas, T.A., Zhang, L., Loftus, J. & Smith, J.W. Ligand binding to integrins. *The Journal of biological chemistry* **275**, 21785-21788 (2000).
61. Shattil, S.J. & Newman, P.J. Integrins: dynamic scaffolds for adhesion and signaling in platelets. *Blood* **104**, 1606-1615 (2004).
62. Torti, M., Festetics, E.T., Bertoni, A., Sinigaglia, F. & Balduini, C. Clustering of integrin alphaIIb-beta3 differently regulates tyrosine phosphorylation of pp72syk, PLCgamma2 and pp125FAK in concanavalin A-stimulated platelets. *Thrombosis and haemostasis* **81**, 124-130 (1999).

63. Haling, J.R., Monkley, S.J., Critchley, D.R. & Petrich, B.G. Talin-dependent integrin activation is required for fibrin clot retraction by platelets. *Blood* **117**, 1719-1722 (2011).
64. Lerea, K.M., Cordero, K.P., Sakariassen, K.S., Kirk, R.I. & Fried, V.A. Phosphorylation sites in the integrin beta3 cytoplasmic domain in intact platelets. *The Journal of biological chemistry* **274**, 1914-1919 (1999).
65. Kirk, R.I., Sanderson, M.R. & Lerea, K.M. Threonine phosphorylation of the beta 3 integrin cytoplasmic tail, at a site recognized by PDK1 and Akt/PKB in vitro, regulates Shc binding. *The Journal of biological chemistry* **275**, 30901-30906 (2000).
66. de Carcer, G., Manning, G. & Malumbres, M. From Plk1 to Plk5: functional evolution of polo-like kinases. *Cell Cycle* **10**, 2255-2262 (2011).
67. Li, B., *et al.* Prk, a cytokine-inducible human protein serine/threonine kinase whose expression appears to be down-regulated in lung carcinomas. *The Journal of biological chemistry* **271**, 19402-19408 (1996).
68. Simmons, D.L., Neel, B.G., Stevens, R., Evett, G. & Erikson, R.L. Identification of an early-growth-response gene encoding a novel putative protein kinase. *Molecular and cellular biology* **12**, 4164-4169 (1992).
69. van Vugt, M.A., *et al.* Polo-like kinase-1 is required for bipolar spindle formation but is dispensable for anaphase promoting complex/Cdc20 activation and initiation of cytokinesis. *The Journal of biological chemistry* **279**, 36841-36854 (2004).
70. Lee, K.S., Grenfell, T.Z., Yarm, F.R. & Erikson, R.L. Mutation of the polo-box disrupts localization and mitotic functions of the mammalian polo kinase Plk. *Proceedings of the National Academy of Sciences of the United States of America* **95**, 9301-9306 (1998).
71. Wang, Q., *et al.* Cell cycle arrest and apoptosis induced by human Polo-like kinase 3 is mediated through perturbation of microtubule integrity. *Molecular and cellular biology* **22**, 3450-3459 (2002).
72. Kauselmann, G., *et al.* The polo-like protein kinases Fnk and Snk associate with a Ca(2+)- and integrin-binding protein and are regulated dynamically with synaptic plasticity. *The EMBO journal* **18**, 5528-5539 (1999).
73. Naik, U.P., Patel, P.M. & Parise, L.V. Identification of a novel calcium-binding protein that interacts with the integrin alphaIIb cytoplasmic domain. *The Journal of biological chemistry* **272**, 4651-4654 (1997).
74. Yamniuk, A.P., Nguyen, L.T., Hoang, T.T. & Vogel, H.J. Metal ion binding properties and conformational states of calcium- and integrin-binding protein. *Biochemistry* **43**, 2558-2568 (2004).
75. Naik, U.P. & Naik, M.U. Association of CIB with GPIIb/IIIa during outside-in signaling is required for platelet spreading on fibrinogen. *Blood* **102**, 1355-1362 (2003).
76. Naik, M.U., Pham, N.T., Beebe, K., Dai, W. & Naik, U.P. Calcium-dependent inhibition of polo-like kinase 3 activity by CIB1 in breast cancer cells.

International journal of cancer. Journal international du cancer **128**, 587-596 (2011).
77. Yang, Y., *et al.* Polo-like kinase 3 functions as a tumor suppressor and is a negative regulator of hypoxia-inducible factor-1 alpha under hypoxic conditions. *Cancer research* **68**, 4077-4085 (2008).
78. Morgan, A. Exocytosis. *Essays in biochemistry* **30**, 77-95 (1995).
79. White, J.G. & Krumwiede, M. Further studies of the secretory pathway in thrombin-stimulated human platelets. *Blood* **69**, 1196-1203 (1987).
80. Coppinger, J.A., *et al.* Characterization of the proteins released from activated platelets leads to localization of novel platelet proteins in human atherosclerotic lesions. *Blood* **103**, 2096-2104 (2004).
81. Lawson, J.H., Kalafatis, M., Stram, S. & Mann, K.G. A model for the tissue factor pathway to thrombin. I. An empirical study. *The Journal of biological chemistry* **269**, 23357-23366 (1994).
82. Frojmovic, M.M. & Milton, J.G. Human platelet size, shape, and related functions in health and disease. *Physiological reviews* **62**, 185-261 (1982).
83. Holmsen, H. & Weiss, H.J. Secretable storage pools in platelets. *Annual review of medicine* **30**, 119-134 (1979).
84. Offermanns, S. Activation of platelet function through G protein-coupled receptors. *Circulation research* **99**, 1293-1304 (2006).
85. Naik, M.U., *et al.* CIB1 deficiency results in impaired thrombosis: the potential role of CIB1 in outside-in signaling through integrin alpha IIb beta 3. *Journal of thrombosis and haemostasis : JTH* **7**, 1906-1914 (2009).
86. Cazenave, J.P., *et al.* Preparation of washed platelet suspensions from human and rodent blood. *Methods Mol Biol* **272**, 13-28 (2004).
87. Jarvis, G.E. Platelet aggregation: turbidimetric measurements. *Methods Mol Biol* **272**, 65-76 (2004).
88. Kostyak, J.C. & Naik, U.P. Calcium- and integrin-binding protein 1 regulates endomitosis and its interaction with Polo-like kinase 3 is enhanced in endomitotic Dami cells. *PloS one* **6**, e14513 (2011).
89. Lapetina, E.G., Silio, J. & Ruggiero, M. Thrombin induces serotonin secretion and aggregation independently of inositol phospholipids hydrolysis and protein phosphorylation in human platelets permeabilized with saponin. *The Journal of biological chemistry* **260**, 7078-7083 (1985).
90. Crosby, D. & Poole, A.W. Platelet dense-granule secretion: the [3H]-5-HT secretion assay. *Methods Mol Biol* **272**, 95-96 (2004).
91. Garcia-Alvarez, B., *et al.* Structural determinants of integrin recognition by talin. *Molecular cell* **11**, 49-58 (2003).
92. Joo, S.J. Mechanisms of Platelet Activation and Integrin alphaIIbeta3. *Korean circulation journal* **42**, 295-301 (2012).
93. Woulfe, D.S. Akt signaling in platelets and thrombosis. *Expert review of hematology* **3**, 81-91 (2010).

94. Banfic, H., *et al.* A novel integrin-activated pathway forms PKB/Akt-stimulatory phosphatidylinositol 3,4-bisphosphate via phosphatidylinositol 3-phosphate in platelets. *The Journal of biological chemistry* **273**, 13-16 (1998).
95. Xu, D., Yao, Y., Jiang, X., Lu, L. & Dai, W. Regulation of PTEN stability and activity by Plk3. *The Journal of biological chemistry* **285**, 39935-39942 (2010).
96. Huang, H. & Vogel, H.J. Structural basis for the activation of platelet integrin alphaIIb-beta3 by Calcium- and Integrin- Binding protein 1. *Journal of the American Chemical Society* (2012).
97. Holmsen, H. & Weiss, H.J. Hereditary defect in the platelet release reaction caused by a deficiency in the storage pool of platelet adenine nucleotides. *British journal of haematology* **19**, 643-649 (1970).
98. Berry, S., Dawicki, D.D., Agarwal, K.C. & Steiner, M. The role of microtubules in platelet secretory release. *Biochimica et biophysica acta* **1012**, 46-56 (1989).
99. White, J.G. & Rao, G.H. Effects of a microtubule stabilizing agent on the response of platelets to vincristine. *Blood* **60**, 474-483 (1982).
100. Cramer, E.M., *et al.* Uncoordinated expression of alpha-granule proteins in human megakaryocytes. *Progress in clinical and biological research* **356**, 131-142 (1990).
101. Hegyi, E., Heilbrun, L.K. & Nakeff, A. Immunogold probing of platelet factor 4 in different ploidy classes of rat megakaryocytes sorted by flow cytometry. *Experimental hematology* **18**, 789-793 (1990).
102. Xie, S., *et al.* MEK1-induced Golgi dynamics during cell cycle progression is partly mediated by Polo-like kinase-3. *Oncogene* **23**, 3822-3829 (2004).
103. Ruan, Q., *et al.* Polo-like kinase 3 is Golgi localized and involved in regulating Golgi fragmentation during the cell cycle. *Experimental cell research* **294**, 51-59 (2004).
104. Lopez-Sanchez, I., Sanz-Garcia, M. & Lazo, P.A. Plk3 interacts with and specifically phosphorylates VRK1 in Ser342, a downstream target in a pathway that induces Golgi fragmentation. *Molecular and cellular biology* **29**, 1189-1201 (2009).
105. Lowenstein, C.J., Morrell, C.N. & Yamakuchi, M. Regulation of Weibel-Palade body exocytosis. *Trends in cardiovascular medicine* **15**, 302-308 (2005).
106. Smyth, S.S., *et al.* Beta(3)-integrin-deficient mice but not P-selectin-deficient mice develop intimal hyperplasia after vascular injury: correlation with leukocyte recruitment to adherent platelets 1 hour after injury. *Circulation* **103**, 2501-2507 (2001).

Appendix

PERMISSION LETTERS AND INTERNET SOURCES

Internet sources:

Figure 1.1: A: http://upload.wikimedia.org/wikipedia/commons/8/82/SEM_blood_cells.jpg
B: http://www.ouhsc.edu/platelets/Platelet%20Pics/Platelets3.jpg
C: http://www.bolandcell.co.za/images/plateltex_2/images/Platelets%202007%20001_jpg.jpg
D: http://faculty.augie.edu/~mklarson/Research_Interests_files/image001.jpg

Figure 1.3: http://scienceblogs.com/scientificactivist/2009/10/two_new_papers_on_integrin_act.php

Permission to use Figure 2.1: Disruption of mouse *Plk3* locus:

Hello Brendan,
RE: Cancer Research, 2008;68 #11; 4077-85; Yang et al – figure 1
It is expected that third parties should obtain the approval of the authors before corresponding with the AACR Permissions Department.
AACR does not grant permission in perpetuity and blanket permission for the reuse of a figure or portions of content will not be honored. The AACR expects a permission request for each subsequent form of reuse in connection with the reuse of the figure or portion of the journal content including the adaptation or revision and use in any media forms.
The AACR grants exclusively to you, the User, for onetime, non-exclusive use of Figure 1 for the purpose stated in your request and used only with a maximum distribution equal to the number you identified in the permission process. This permission may not be transferred. The content is designed for scientific use only. Should your intention to reuse be for commercial purposes, written permission is to be submitted to the AACR.
The new work must carry without modification the following copyright notice and appropriate journal citation format: Reprinted (or adapted if you are reproducing a modified version of an image) by permission from the American Association for Cancer Research: author, article title, journal name, publication date, vol. issue, page range, and **DOI** when available.
Kind regards,
Karola

~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
(Ms) Karola Rac, MLS
Assistant Director, Circulation & Fulfillment
Publications Division - Sales & Marketing
American ***A***ssociation for ***C***ancer ***R***esearch
615 Chestnut Street - 17th Floor, Philadelphia, PA 19106-4404,USA

NATURE PUBLISHING GROUP LICENSE TERMS AND CONDITIONS

Apr 10, 2013

This is a License Agreement between University of Delaware ("You") and Nature Publishing Group ("Nature Publishing Group") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by Nature Publishing Group, and the payment terms and conditions.

All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.

License Number	3125450429838
License date	Apr 10, 2013
Licensed content publisher	Nature Publishing Group
Licensed content publication	Nature
Licensed content title	Thrombin signalling and protease-activated receptors
Licensed content author	Shaun R. Coughlin
Licensed content date	Sep 14, 2000
Volume number	407
Issue number	6801
Type of Use	reuse in a thesis/dissertation
Requestor type	academic/educational
Format	print
Portion	figures/tables/illustrations
Number of figures/tables/illustrations	1
Figures	Figure 2 Mechanism of PAR1 activation
Author of this NPG article	no
Your reference number	
Title of your thesis / dissertation	DUAL ROLES FOR POLO-LIKE KINASE 3 IN THE REGULATION OF PLATELET FUNCTION
Expected completion date	Apr 2013
Estimated size (number of pages)	80
Total	0.00 USD
Terms and Conditions	

Terms and Conditions for Permissions

Nature Publishing Group hereby grants you a non-exclusive license to reproduce this material for this purpose, and for no other use, subject to the conditions below:

1. NPG warrants that it has, to the best of its knowledge, the rights to license reuse of this material. However, you should ensure that the material you are requesting is original to Nature Publishing Group and does not carry the copyright of another entity (as credited in the published version). If the credit line on any part of the material you have requested indicates that it was reprinted or adapted by NPG with permission from another source, then you should also seek permission from that source to reuse the material.

2. Permission granted free of charge for material in print is also usually granted for any electronic version of that work, provided that the material is incidental to the work as a whole and that the electronic version is essentially equivalent to, or substitutes for, the print version. Where print permission has been granted for a fee, separate permission must be obtained for any additional, electronic re-use (unless, as in the case of a full paper, this has already been accounted for during your initial request in the calculation of a print run). NB: In all cases, web-based use of full-text articles must be authorized separately through the 'Use on a Web Site' option when requesting permission.

3. Permission granted for a first edition does not apply to second and subsequent editions and for editions in other languages (except for signatories to the STM Permissions Guidelines, or where the first edition permission was granted for free).

4. Nature Publishing Group's permission must be acknowledged next to the figure, table or abstract in print. In electronic form, this acknowledgement must be visible at the same time as the figure/table/abstract, and must be hyperlinked to the journal's homepage.

5. The credit line should read:
   Reprinted by permission from Macmillan Publishers Ltd: [JOURNAL NAME] (reference citation), copyright (year of publication)
   For AOP papers, the credit line should read:
   Reprinted by permission from Macmillan Publishers Ltd: [JOURNAL NAME], advance online publication, day month year (doi: 10.1038/sj.[JOURNAL ACRONYM].XXXXX)

   Note: For republication from the *British Journal of Cancer*, the following credit lines apply.
   Reprinted by permission from Macmillan Publishers Ltd on behalf of Cancer Research UK: [JOURNAL NAME] (reference citation), copyright (year of publication) For AOP papers, the credit line should read:
   Reprinted by permission from Macmillan Publishers Ltd on behalf of Cancer Research UK: [JOURNAL NAME], advance online publication, day month year (doi: 10.1038/sj.[JOURNAL ACRONYM].XXXXX)

6. Adaptations of single figures do not require NPG approval. However, the adaptation should

be credited as follows:

Adapted by permission from Macmillan Publishers Ltd: [JOURNAL NAME] (reference citation), copyright (year of publication)

Note: For adaptation from the *British Journal of Cancer*, the following credit line applies.
Adapted by permission from Macmillan Publishers Ltd on behalf of Cancer Research UK: [JOURNAL NAME] (reference citation), copyright (year of publication)

7. Translations of 401 words up to a whole article require NPG approval. Please visit http://www.macmillanmedicalcommunications.com for more information. Translations of up to a 400 words do not require NPG approval. The translation should be credited as follows:

   Translated by permission from Macmillan Publishers Ltd: [JOURNAL NAME] (reference citation), copyright (year of publication).

   Note: For translation from the *British Journal of Cancer*, the following credit line applies.
   Translated by permission from Macmillan Publishers Ltd on behalf of Cancer Research UK: [JOURNAL NAME] (reference citation), copyright (year of publication)

We are certain that all parties will benefit from this agreement and wish you the best in the use of this material. Thank you.

Special Terms:

v1.1

If you would like to pay for this license now, please remit this license along with your payment made payable to "COPYRIGHT CLEARANCE CENTER" otherwise you will be invoiced within 48 hours of the license date. Payment should be in the form of a check or money order referencing your account number and this invoice number RLNK500996991. Once you receive your invoice for this order, you may pay your invoice by credit card. Please follow instructions provided at that time.

Make Payment To:
Copyright Clearance Center
Dept 001
P.O. Box 843006
Boston, MA 02284-3006

For suggestions or comments regarding this order, contact RightsLink Customer Support: customercare@copyright.com or +1-877-622-5543 (toll free in the US) or +1-978-646-2777.

Gratis licenses (referencing $0 in the Total field) are free. Please retain this printable license for your reference. No payment is required.

University of Delaware
Institutional Animal Care and Use Committee
Request to Amend an Animal Use Protocol

(Please complete below using Arial, size 12 Font.)

Title of Protocol: The investigation of novel protein functions in vascular biology	
AUP Number: 1094-2011-A	**← (4 digits only)**
Principal Investigator: Dr. Ulhas P. Naik	

Requested Changes

I am requesting a change to: ***(Check all that apply)***

- ☐ Animal Species *(Complete Section 1)*
- ☐ Animal Numbers *(Complete Section 2)*
- x☐ Animal Procedures *(Complete Section 3)*
- ☐ Therapeutic or Experimental Agents *(Complete Section 4)*
- ☐ Pain Category *(Complete Section 5)*
- ☐ Use of Biological Material, Hazardous Agents or Radiation *(Complete Sections 4 & 6)*
- ☐ Other *(Specify)* _____ *(Complete Section 7)*

Changes MUST NOT be initiated until IACUC approval is granted

Official Use Only

IACUC Approval Signature: Steven D Brass

Date of Approval: 19 Nov 2010

Principal Investigator Assurance

1. I agree to abide by all applicable federal, state, and local laws and regulations, and UD policies and procedures.
2. I understand that deviations from an approved protocol or violations of applicable policies, guidelines, or laws could result in immediate suspension of the protocol and may be reportable to the Office of Laboratory Animal Welfare (OLAW).
3. I understand that the Attending Veterinarian or his/her designee must be consulted in the planning of any research or procedural changes that may cause more than momentary or slight pain or distress to the animals.
4. I declare that all experiments involving live animals will be performed under my supervision or that of another qualified scientist listed on this AUP. All listed personnel will be trained and certified in the proper humane methods of animal care and use prior to conducting experimentation.
5. I understand that emergency veterinary care will be administered to animals showing evidence of discomfort, ailment, or illness.
6. I declare that the information provided in this application is accurate to the best of my knowledge. If this project is funded by an extramural source, I certify that this application accurately reflects all currently planned procedures involving animals described in the proposal to the funding agency.
7. I assure that any modifications to the protocol will be submitted to the UD-IACUC and I understand that they must be approved by the IACUC prior to initiation of such changes.
8. I understand that the approval of this project is for a maximum of one year from the date of UD-IACUC approval and that I must re-apply to continue the project beyond that period.
9. I understand that any unanticipated adverse events, morbidity, or mortality must be reported to the UD-IACUC immediately.
10. I assure that the experimental design has been developed with consideration of the three Rs: reduction, refinement, and replacement, to reduce animal pain and/or distress and the number of animals used in the laboratory.
11. I assure that the proposed research does not unnecessarily duplicate previous experiments. *(Teaching Protocols Exempt)*
12. I understand that by signing, I agree to these assurances.

Laura P. Nail

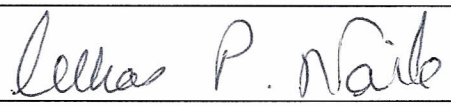
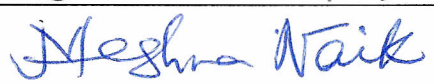
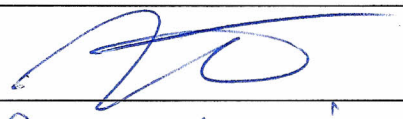
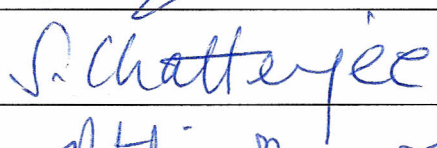
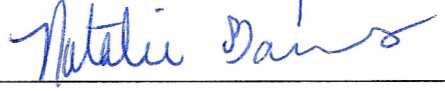
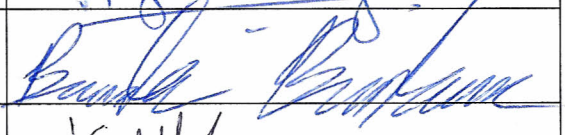
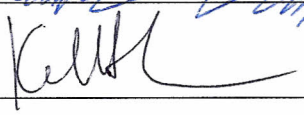
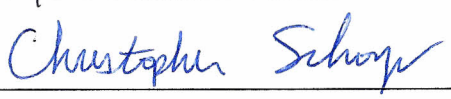
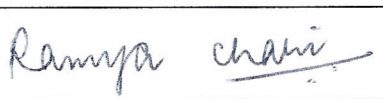
Signature of Principal Investigator

10/6/10

Date

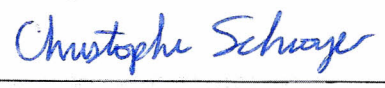
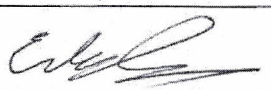
SIGNATURE(S) OF ALL PERSONS LISTED ON THIS PROTOCOL

I certify that I have read this protocol, accept my responsibility and will perform only the procedures that have been approved by the IACUC.

Name	Signature
1. Ulhas P. Naik	
2. Meghna Naik	
3. John Kostyak	
4. Sharmila Chatterjee	
5. Natalie Barnes	
6. Arjit Nigam	
7. Brendan Bachman	
8. Kelvin Lee	
9. Chris Schmoyer	
10. Erhe Gao	
11. Ramya Chari	
12.	
13.	
14.	
15.	

SIGNATURE(S) OF ALL PERSONS LISTED ON THIS PROTOCOL

I certify that I have read this protocol, accept my responsibility and will perform only the procedures that have been approved by the IACUC.

Name	Signature
1. Chris Schmoyer	
2. Erhe Gao	
3.	
4.	
5.	
6.	
7.	
8.	
9.	
10.	
11.	
12.	
13.	
14.	
15.	