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Dr. Trevor Creamer, Director of Graduate Studies

CONTROLLING PLATELET SECRETION TO MODULATE HEMOSTASIS AND THROMBOSIS

Dissertation

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

By

Smita Joshi

Lexington, Kentucky

Director: Dr. Sidney W. Whiteheart, Professor of Molecular and Cellular Biochemistry

Lexington, Kentucky

2018

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Abstract

CONTROLLING PLATELET SECRETION TO MODULATE HEMOSTASIS AND THROMBOSIS

Upon vascular injury, activated blood platelets fuse their granules to the plasma membrane and release cargo to regulate the vascular microenvironment, a dynamic process central to platelet function in many critical processes including hemostasis, thrombosis, immunity, wound healing, angiogenesis *etc.* This granule-plasma membrane fusion is mediated by a family of membrane proteins- Soluble *N*-ethyl maleimide Attachment Receptor Proteins(SNAREs). SNAREs that reside on vesicle (v-SNAREs) /Vesicle-Associated Membrane Proteins(VAMPs) interact with target/t-SNAREs forming a trans-bilayer complex that facilitates granule fusion. Though many components of exocytic machinery are identified, it is still not clear how it could be manipulated to prevent occlusive thrombosis without triggering bleeding. My work addresses this question by showing how the rates and extents of granule secretion could be regulated by various v-SNAREs. We also show that the granule cargo decondensation is an intermediate to secretion that also contributes to rates of cargo release.

Platelets contain four major VAMP isoforms (-2, -3, -7, and -8), however, VAMP-8 and -7 play a primary role while VAMP-2 and -3 are ancillary in secretion. To exploit this heterogeneity in VAMP usage, platelet-specific $V-2/3^{-/-}$ and $V-2/3/8^{-/-}$ mouse models were generated and characterized to understand how secretion influences hemostasis. We found that each VAMP isoform differentially contributes by altering the rates and extents of cargo release. The loss of VAMP-2 and -3 had a minimal impact while the loss of VAMP-2, -3 and -8 significantly reduced the granule secretion. Platelet activation and aggregation were not affected though the spreading was reduced in $V-2/3/8^{-/-}$ platelets indicating the importance of secretion in spreading. Though coagulation pathways were unaltered, PS exposure was reduced in both $V-2/3^{-/-}$ and $V-2/3/8^{-/-}$ platelets suggesting diminished procoagulant activity. *In vivo* experiments showed that $V-2/3/8^{-/-}$ animals bled profusely upon tail transaction and failed to form occlusive thrombus upon arterial

injury while V-2/3^{-/-} animals did not display any hemostatic deficiency. These data suggest that about 40-50% reduction in secretion provides protection against thrombosis without compromising hemostasis and beyond 50% secretion deficiency, the animals fail to form functional thrombi and exhibit severe bleeding. Additionally, detailed structural analysis of activated platelets suggests that the post-stimulation cargo dissolution depends on an agonist concentration and stimulation duration. This process is VAMP-dependent and represents intermediate steps leading to a full exodus of cargo. Moreover, we also show that VAMP-8 is important for compound fusion events and regulates fusion pore size.

This is a first comprehensive report that shows how manipulation of the exocytic machinery have an impact on secretion and ultimately on hemostasis. These animals will be instrumental in future investigations of platelet secretion in many other vascular processes.

Keywords: Platelet SNAREs, granule exocytosis, cargo release, hemostasis, thrombosis, decondensation

Smita Joshi

March 1st, 2018

CONTROLLING PLATELET SECRETION TO MODULATE HEMOSTASIS AND THROMBOSIS

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Dr. Sidney W. Whiteheart

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March 1st, 2018

To my family

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Abbreviations

ADP- Adenosine Diphosphate

ARC- Arthrogyriposis, Renal Dysfunction, And Cholestasis Syndrome

ATP- Adenosine Triphosphate

BLOC- Biogenesis of Lysosome-Related Organelles Complex

BMCC-Bone Marrow-Derived Mast Cells

CHS- Chediak-Higashi Syndrome

CLEC-2- C-Type Lectin-Like Receptor 2

CO-IP- Co-Immunoprecipitation

DAG- Diacylglycerol

ELISA- Enzyme-Linked Immunosorbent Assay

FACS- Fluorescent-Activated Cell Sorting

FHL3- Familial Hemophagocytic Lymphohistiocytosis Type 3

FHL4- Familial Hemophagocytic Lymphohistiocytosis Type 4

FHL5 -Familial Hemophagocytic Lymphohistiocytosis Type 5

FIB-SEM- Focused Ion Beam-Scanning Electron Microscopy

GLUT- Glucose Transporter

GPCR-G-Protein-Coupled Receptor

GPS- Grey Platelet Syndrome

GSVs- GLUT Storage Vesicles

GWAS- Genome-Wide Association Studies

HPS- Hermansky Pudlack Syndrome

IP3- Inositol 3 Phosphate

ITAM-Immunoreceptor Tyrosine-Based Activation Motif

LAMP- Lysosomal-Associated Membrane Protein

MIP-1 α - Macrophage Inflammatory Protein

MVB- Multi-Vesicular Body

NSF- N- Ethylmaleimide Sensitive Factor

OCS- Open Canalicular System

PAGE -Polyacrylamide Gel Electrophoresis

PAR-Protease Activated Receptors

PF4- Platelet Factor IV

PGI₂- Prostaglandin I₂

PI3K- Phosphatidyl Inositol-3 Kinase

PLC γ 2- Phospholipase γ 2

PDI- Protein Disulfide Isomerase

RT- Room Temperature

SLPs- Synaptotagmin-Like-Proteins

SNAP- Soluble NSF Attachment Proteins

SNAP- Synaptosome Associated Proteins

SNARE- Soluble N-Ethylmaleimide- Sensitive Fusion Protein Attachment Receptor

SNP- Single Nucleotide Polymorphism

SPD- Storage Pool Deficiency

STXBP5- Syntaxin Binding Protein 5/ Tomosyn

TMD- Trans-Membrane Domain

TNF- Tumor Necrosis Factor

TXA₂- Thromboxane A2

VAMP-Vesicle Associated Membrane Protein

vWF- Von Willebrand Factor

WPB- Weibel- Palade body

WT- Wild Type

Chapter 1 Introduction

Platelet cell biology

Platelets are discoid, anucleate, cytoplasmic cell fragments that originate from megakaryocytes mainly within bone marrow and lungs (Lefrancais, Ortiz-Munoz et al. 2017). Platelets are minute in size, about 2-3 μm in diameter and 0.5 μm in thickness. They circulate in the blood for about 4-5 days for mouse (Manning, Novinger et al. 1996) and for about 7-10 days in humans before being eliminated in the spleen (Harker, Roskos et al. 2000). Platelets are the most abundant cell type, second only to RBCs (average human- $1.5\text{-}4.5 \times 10^5$ platelets/ μL , average mouse- $1.1\text{-}1.3 \times 10^6$ platelets/ μL). Platelets contribute to normal hemostasis and, recently, their additional roles in inflammation, infection, wound healing, angiogenesis, and metastasis is receiving increasing attention (Smyth, McEver et al. 2009, Tesfamariam 2016).

Platelet structure

Platelets are metabolically active and contain mitochondria, Golgi apparatus, endoplasmic reticulum (also called dense tubular system), granules or vesicles, endosomes, autophagosomes (Ouseph, Huang et al. 2015), and peroxisomes. The cytoskeleton of platelets includes spectrin-based skeleton, microtubules, and actin filaments. These elements maintain the discoid shape of resting platelets. Some of these important features of a resting platelet are depicted in a schematic in Figure 1.1. The platelet plasma membrane surface area is about $19 \mu\text{m}^2$ and the total surface area of their granules is about $14 \mu\text{m}^2$ (Fitch-Tewfik and Flaumenhaft 2013).

The surface of platelets is decorated with five types of integrins -three $\beta 1$ integrins facilitate platelet adhesion to matrix proteins- collagen, fibronectin, and laminin. Additionally, two $\beta 3$ integrins- $\alpha\text{IIb}\beta 3$, and $\alpha\text{v}\beta 3$ are also present on platelets. Considered as the most abundant integrin (about 80,000 copies/platelet) $\alpha\text{IIb}\beta 3$ binds to fibrinogen, von Willebrand Factor/vWF, and fibronectin (Bennett, Berger et al. 2009).

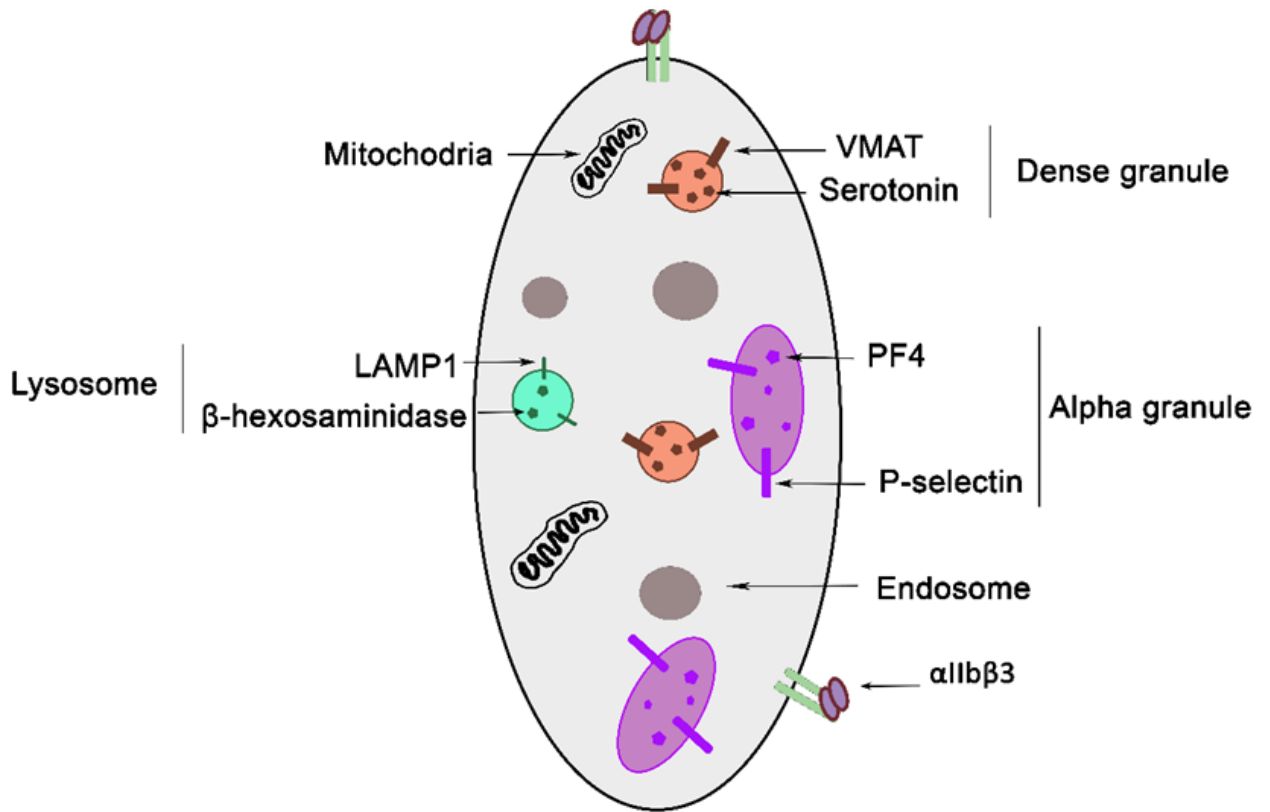


Figure 1.1. Schematic of a resting platelet

Schematic of a resting platelet containing various types of granules, endosomes, and mitochondria. Granules shown are- dense granules (membrane protein -vesicular monoamine transporter/VMAT, and soluble cargo- serotonin), alpha granules (membrane protein – P-selectin, and soluble cargo -Platelet Factor 4/PF4), and lysosome (membrane protein- LAMP1, and soluble cargo β-hexosaminidase). Only selective cargo is depicted for simplification.

The distinct feature of platelet cytoplasm is a presence of discrete types of granules (Figure 1.1). The release of these granular content is central to most platelet functions because it allows platelets to modulate the vascular microenvironment at sites where they are activated. Based on their morphology, contents, and functions, granules are classified into three main types- dense (δ -granules/dense bodies), alpha (α), and lysosomes. Platelets contain 7-10 dense granules per platelet which contain small molecules, e.g., ADP, ATP, serotonin, polyphosphate, and calcium (Eckly, Rinckel et al. 2016). Holmsen and Weiss measured the intragranular concentrations of these cargoes as 65 mM serotonin, 436 mM ATP, 653 mM ADP, 2.2 M calcium, and 326 mM pyrophosphate (Holmsen and Weiss 1979, McNicol and Israels 1999). Serotonin/5-hydroxytryptamine (5HT) is produced by enterochromaffin cells of the intestinal tract and is released in plasma. Platelets take up this plasma serotonin by serotonin receptors (SERT) present on the plasma membrane. This serotonin is then stored in dense granules through the action of vesicular monoamine transporter (VMAT2). It has been shown that pharmacological inhibition of SERT by selective serotonin reuptake inhibitors (SSRIs- paroxetine and citalopram) significantly diminishes 5HT contents in platelets. Carneiro, A.D., and colleagues showed that SERT potentially associates with an abundant integrin α IIb β 3 making integrin activation essential for SERT function. The group also showed that SERT deficiency significantly affects platelet aggregation. (Carneiro, Cook et al. 2008). Dense granule cargoes are immensely critical for amplification of the platelet activation signal and recruitment of platelets at the injury site.

Alpha granules are the largest (about 200-500 μ m in diameter) and the most numerous (40-45/platelet) granules in platelets (Eckly, Rinckel et al. 2016). Alpha granules contain a plethora of proteins with diverse functions, e.g., angiogenic (e.g. Vascular Endothelial Growth Factor/VEGF) and anti-angiogenic (endostatin) factors, tissue factors, growth factors (Platelet Derived Growth Factor/PDGF, Transforming Growth Factor β /TGF β), mitogens, cytokines (Platelet Factor 4/PF4), proteoglycans (serglycin, β - thromboglobulin), adhesive proteins (von Willebrand Factor/vWF,

fibronectin, vitronectin, thrombospondin, P-selectin), membrane proteins (α IIb β 3, GP-1b-IX) and coagulation factors (factor V, fibrinogen). Release from these granules facilitates platelets' role in angiogenesis, infection, inflammation, metastasis, wound healing among many other pathologies. The third type of granule is the lysosome. Lysosomes are intermediate in size – about 175-250 nm in diameter. Electron density of lysosomes is like alpha granules, making it a challenge to distinguish them. Specific cytochemical stains such as arylsulfatase or acid phosphatase are required to characterize them (Bentfeld-Barker and Bainton 1982). These degradative compartments contain acid hydrolyzes, cathepsins- D and E, carboxypeptidases- A and B, collagenase, acid phosphatase, heparinase, and proteases and membrane proteins (LAMP-1, LAMP-2) (Rendu and Brohard-Bohn 2001), which may contribute to clot remodeling.

Platelet biogenesis

Megakaryocytes are myeloid precursor cells that arise from pluripotent hematopoietic stem cells and constitute less than 0.1% of the total bone marrow cell population. During the maturation process, megakaryocytes undergo endomitosis, a chromosomal duplication process that results in increased ploidy without cell division (Thon and Italiano 2012). This increased ploidy is important for supporting carbohydrate, protein, and lipid synthesis requirements of a large cell (Bluteau, Lordier et al. 2009). Mature megakaryocyte contains platelet-specific cytoplasmic organelles including granules and cytoskeletal proteins. Upon maturation, these megakaryocytes migrate to vascular bed in bone marrow or in lungs (Lefrancais, Ortiz-Munoz et al. 2017). There they develop pseudopod-like thin, elongated projections, also known as proplatelets which mature and release platelets - either in bone marrow sinusoids (Junt, Schulze et al. 2007) or in pulmonary capillaries of lungs (Sharnoff and Scardino 1960).

Platelet granule biogenesis

A) Dense granule biogenesis

Dense granules belong to the family of lysosomal-related organelle that includes Weibel – Palade bodies in endothelial cells, melanosomes in melanocytes, lamellar

bodies in type II lung alveolar cells, lytic granules, chromaffin granules, basophil granules, and azurophil granules. These organelles are characterized by the nature of their cargoes- they contain acid-dependent hydrolases and an array of lytic enzymes as well as cell- type-specific cargo that has a specialized function (Dell'Angelica, Mullins et al. 2000).

Platelet dense granules contain small molecules such as polyphosphates, ATP, ADP, serotonin, and calcium that function as secondary agonists in platelet activation and recruitment. Dense granules also contain membrane proteins LAMP-1, LAMP-2, and CD63/LAMP3. Dense granules contain a vesicular ATPase proton pump that maintains the acidic pH of dense granules (~ 5.4 (Dean, Fishkes et al. 1984)). The multidrug transporter protein Multidrug resistance protein (MRP4) is thought to transport adenine nucleotides into the dense granules (Jedlitschky, Tirschmann et al. 2004). Vesicular monoamine transporter 2 (VMAT2) helps transport serotonin from the platelet cytosol to dense granules (Fukami, Holmsen et al. 1984).

Though many molecules have been identified that potentially play a role in dense granule biogenesis in megakaryocytes and platelets, the exact mechanism is still elusive. Platelet storage pool deficiencies/SPDs characterized by dense granule defects/alpha granule defects/dense and alpha granule defects implicate proteins that are involved in the biogenesis of respective granules and have been instrumental in illuminating these processes. Several genes/proteins have been identified that are linked to dense granule biogenesis defects include proteins that are crucial for trafficking, mainly Biogenesis of Lysosome-Related Organelles Complex (BLOC-1(Falcon-Perez, Starcevic et al. 2002), 2, 3), VPS 33A, AP-3, and LYST.

In a seminal paper (Hermansky and Pudlak 1959), Hermansky and Pudlak characterized a condition that exhibits oculocutaneous albinism and prolonged bleeding. At the time, they coined this condition pseudohemophilia considering that moderate bleeding deficiency was the main symptom. Since then, 8 proteins in humans and 15 proteins in mice have been implicated as causative in HPS pathology. Mice mutants have been named according to their coat color. The severity of hemostasis

defect is variable among these strains. The whole mount blood specimens from HPS patients show complete absence or diminished numbers of dense granules in platelets. HPS proteins are components of the BLOC complexes. There are three of these large multiprotein tethering complexes that play an important role in trafficking and exocytosis of granules. Mutations in any one or multiple of their components may affect dense granule biogenesis (Dell'Angelica, Mullins et al. 2000).

Chediak – Higashi syndrome was known long before it was characterized as dense granule SPD (Rendu, Breton-Gorius et al. 1983). CHS is an autosomal recessive disorder, responsible for severe bleeding, neurological abnormalities, hypopigmentation, immunodeficiency and the presence of giant lysosomes in granulocytes and leukocytes (Table 1.1). Several groups mapped the mutations in the genes encoding Lysosomal trafficking regulator/ LYST protein which are responsible for CHS (Barbosa, Nguyen et al. 1996, Nagle, Karim et al. 1996, Perou, Moore et al. 1996). LYST protein is the first identified and an extensively studied member of a large family of proteins that contain a BEACH (Beige and Chediak- Higashi) domain. The BEACH domain is a conserved domain of about 280 amino acids and is crucial for protein-protein interactions. Other important members of this family and their associated disorders include neurobeachin (NBEA)- autism , neurobeachin-like 1 (NBEAL1)- glioma, neurobeachin-like 2 (NBEAL2)- Gray Platelet Syndrome/ GPS, lipopolysaccharide-responsive, beige-like anchor protein (LRBA)-autoimmune disorders , WD and FYVE zinc finger domain containing protein 3 (WDFY3), WD and FYVE zinc finger domain containing protein 4 (WDFY4)- systemic lupus erythematosus/ SLE, neutral sphingomyelinase activation-associated factor (NSMAF), and WD repeat domain 81 (WDR81) – mental retardation (Cullinane, Schaffer et al. 2013). BEACH containing proteins differentially affect platelet granule biogenesis; while LYST is responsible for dense granule deficiency in CHS, another member NBEAL 2 is associated with an alpha-granule deficiency in GPS. The underlying theme of all these pathologies points to the role of BEACH- containing proteins' role in intracellular trafficking and vesicle

fission/fusion processes. Although the molecular details of interactions of these scaffold proteins are not fully understood.

In a recent study, Walker et al., showed that serum and glucocorticoid-inducible kinase 1 (SGK-1) deficient platelets are devoid of dense granules and have diminished dense granule cargo levels of serotonin and ATP, while numbers of alpha granules and their cargo levels were not affected. These platelets also had an aggregation defect that could be overcome with exogenous ADP. SGK-1^{-/-} animals showed delayed and diminished thrombosis in the absence of a bleeding defect. Mass spectroscopic analysis showed that VAMP-7 and Rab 27B levels were significantly lowered in these animals, suggesting a potential association with SGK-1 (Walker, Schmid et al. 2015). Apart from SGK-1, another potential protein associated with dense granule biogenesis is SLC35D3. Using immunofluorescence, Immunoelectron microscopy and subcellular fractionation of megakaryocytoid cells, Meng, and colleagues showed that SLC35D3, a dense granule related protein is associated with early endosomes, suggesting that these granules originate from an endocytic pathway (Meng, Wu et al. 2015). In addition to human and mice, other species affected by CHS are cats (Meyers, Seachord et al. 1981), cattle (Meyers, Seachord et al. 1981, Meyers, Seachord et al. 1983), Aleutian minks (Meyers, Holmsen et al. 1979), killer whales (Meyers, Holmsen et al. 1979, Meyers, Hopkins et al. 1982) and pigs (Daniels, Fass et al. 1986).

B) Alpha-granule biogenesis

Alpha granules are the most abundant and versatile granule type in platelets. According to various proteomic studies, alpha granules contain more than 300 proteins (Maynard, Heijnen et al. 2007) and in many ways, are like Weibel Palade bodies in endothelial cells. Both contain vWF, P-selectin, and CD63 and play crucial roles in hemostasis and inflammation (Heijnen and van der Sluijs 2015).

Though there are recent advances in proposed mechanisms, alpha-granule biogenesis is far from resolved. The current model is based on our understanding that alpha granules contain both endocytosed (Fibrinogen, albumin, IgG) as well as *de novo* synthesized cargo (Platelet Factor 4, vWF, β -thromboglobulin) (Blair P et al 2009). This

suggests that alpha granules probably originate from the trans-Golgi network/TGN and endocytic pathways. After budding from the TGN, the vesicles containing alpha-granule cargoes are trafficked to multi-vesicular bodies/MVBs (Heijnen, Debili et al. 1998). Subsequent cargo sorting takes place in MVBs and after maturation of various types of MVBs, (MVB I, containing only limiting membrane vesicles, MVB II, containing internal membrane vesicles along with vesicles containing electron- dense materials), alpha granules are formed (Heijnen, Debili et al. 1998). Alternatively, both synthesized and endocytosed cargoes could be delivered directly to alpha granules (Blair and Flaumenhaft 2009).

This understanding stems from studies of platelet storage pool deficiencies (SPDs), Two major platelet SPDs characterized by either defective or absent alpha granules are Gray Platelet Syndrome/ GPS and Arthrogryposis, Renal dysfunction, and Cholestasis /ARC syndrome (Table 1.1). GPS is a rare, autosomal recessive disorder characterized by macrothrombocytopenia, splenomegaly, myelofibrosis, and large platelets that appear gray with Giemsa staining under a light microscope. These platelets have an aggregation defect and patients with GPS show a broad range of bleeding diathesis (Nurden and Nurden 2011). Three different groups simultaneously found that mutations in Neurobeachin Like 2 /NBEAL2 protein cause GPS (Albers, Cvejic et al. 2011, Gunay-Aygun, Falik-Zaccari et al. 2011, Kahr, Hinckley et al. 2011). Though megakaryocytes and platelets from *Nbeal2*^{-/-} mice (Deppermann, Cherpokova et al. 2013) and GPS patients may have rudimentary alpha-granule precursors, they show lack of alpha-granule cargoes that are endogenously synthesized (e.g. PF4) or endocytosed by megakaryocytes/platelets (e.g. fibrinogen). These granules, however, contain the alpha-granule membrane marker P-selectin, suggesting that granules could form but are not filled in absence of NBEAL2.

Originally described in 1973, ARC syndrome is a rare autosomal recessive disorder. Patients affected by ARC syndrome manifest congenital joint contractures, renal tubular dysfunction, cholestasis, ichthyosis, central nervous system malformation, platelet anomalies, and severe failure to thrive. ARC syndrome is caused by mutations in

Vacuolar protein sorting 33 homolog B /VPS33B or VPS33B-interacting protein, apical-basolateral polarity regulator/VIPAR/VPS16B genes (Gissen, Johnson et al. 2004, Cullinane, Straatman-Iwanowska et al. 2010).

Table 1.1 Summary of major granulopathies affecting dense and alpha granules in platelets

Storage pool deficiency	Affected granule	Clinical features	Genes/proteins affected	References
Hermansky Pudlak Syndrome (HPS)	Dense	Severe bleeding, oculocutaneous albinism <i>etc.</i>	BLOC-1/pallid, BLOC-2, and BLOC-3. Adaptor protein 3 (AP-3/pearl) SLC35D3 (9 genes in humans and 15 genes in mice)	(Falcon-Perez, Starcevic et al. 2002, Chintala, Tan et al. 2007, Meng, Wu et al. 2015)
Chediak-Higashi Syndrome(CHS)	Dense	severe bleeding, neurological abnormalities, hypopigmentation, immunodeficiency <i>etc.</i>	LYST	(Barbosa, Nguyen et al. 1996)
Gray Platelet Syndrome (GPS)	Alpha	Macrothrombocytopenia, splenomegaly, myelofibrosis <i>etc.</i>	NBEAL2	(Albers, Cvejic et al. 2011, Gunay-Aygun, Falik-Zaccai et al. 2011, Kahr, Hinckley et al. 2011)
Arthrogryposis-renal dysfunction-cholestasis (ARC) syndrome	Alpha	Ichthyosis, central nervous system malformation, platelet anomalies, and severe failure to thrive <i>etc.</i>	VPS33B, VPS16B	(Gissen, Johnson et al. 2004, Cullinane, Straatman-Iwanowska et al. 2010)

Platelet receptors and intracellular signaling pathways

Platelets are decorated with a multitude of surface receptors that sense and reciprocate to the intravascular microenvironment. They participate in hemostasis by facilitating platelet adhesion and secretion upon vascular injury. Additionally, they also contribute to endocytosis and intracellular trafficking upon microbial and immunological challenges. A vast repertoire of these receptors include integrins (α IIb β 3, α 2 β 1, α 5 β 1, α 6 β 1, α V β 3), leucine-rich repeated (LRR) receptors (Glycoprotein [GP] Ib/IX/V, Toll-like receptors), G-protein coupled seven transmembrane receptors (GPCR) (PAR-1 and PAR-4 thrombin receptors, P2Y1 and P2Y12 ADP receptors, TPa and TPb TxA2 receptors), proteins belonging to the immunoglobulin superfamily (GPVI, Fc γ RIIA), C-type lectin receptors (P-selectin), tyrosine kinase receptors (thrombopoietin receptor, Gas-6, ephrins and Eph kinases) and a collection of other receptors (CD63, CD36, P-selectin ligand 1, TNF receptor type, *etc.*)(Rivera, Lozano et al. 2009).

Formation of a platelet plug at the site of vascular damage is a result of spatially and temporally concerted response by various components of the vascular system. The main objective of these dynamic events is to stop bleeding, prevent uncontrolled, occlusive thrombosis and maintain a platelet plug until wound healing occurs. This process could be divided into three stages that describe how platelets respond to injury and form a stable plug for hemostasis.

These stages are as the following:

Initiation phase

In this phase, platelets engage with exposed subendothelial adhesive proteins and adhere at the site of injury. These get activated, creating a monolayer of activated platelets. Upon vascular injury, platelet receptors interact with subendothelial proteins, mainly vWF, collagen, thrombospondin, fibronectin, and laminin. These interactions are highly dependent on the shear rate. At lower shear, platelet interactions with collagen, mediated by GPVI, are predominant while at high shear, platelet interactions with vWF, mediated by α IIb β 3, are critical. vWF interaction with another receptor, a major

glycoprotein [GP] Ib/IX/V which is critical for the initiation phase. The lack of or defect in this receptor leads to Bernard Soulier syndrome (BSS), a congenital bleeding disorder characterized by macrothrombocytopenia and inability of these platelets to aggregate.

In addition to vWF, the GPIb/IX/V complex also has multiple other ligands including other adhesive proteins (collagen, thrombospondin-1), alpha-thrombin and coagulation factors (kininogen, FXI, FXII). GP1b is indispensable for hemostasis while the loss of vWF could be compensated by other GP1b ligands (Bergmeier, Piffath et al. 2006, Dubois, Panicot-Dubois et al. 2007). The interaction between GP1b and vWF leads to platelet activation manifested as increased $[Ca^{++}]$, thromboxane A₂/TxA₂ synthesis, protein phosphorylation for downstream signaling, ADP release from dense granules and finally, activation of α IIb β 3.

Another critical ligand for platelet adhesion at the site of injury is collagen. Collagen is a strong and only stationary agonist. It binds with vWF in flowing blood to efficiently carry out platelet adhesion and activation. vWF facilitates initial engagement of platelets at the site of injury, while collagen forms stable bonds for firm adhesion (mediated through α 2 β 1 receptor) and causes platelet activation (mainly through GPVI). This binding of platelets to collagen is facilitated by two platelet surface receptors: GPVI and integrin α 2 β 1.

GPVI is complexed with FcR γ -chain dimers that have an immunoreceptor tyrosine-based activation motif/ ITAM as a signal transducing subunit of the receptor. Upon activation of GPVI, syk phosphorylated ITAM and downstream signaling results in PLC γ 2 activation. Diacylglycerol/DAG and inositol 1,4,5-triphosphate/IP3 are released, leading to increased intracellular $[Ca^{++}]$ levels that ultimately activate platelets. The lack of GPVI or the FcR γ - chain in mice leads to a defective response to collagen and impaired thrombosis (Poole, Gibbins et al. 1997, Kato, Kanaji et al. 2003). GPVI is an attractive target for antithrombotic therapies. Matsumoto and colleagues showed that the Fab fragment of anti-human GPVI antibody, OM4, affected aggregation but had a minor effect on bleeding time in cynomolgus monkeys as compared to the effects of

anti- α IIb β 3 antibody that significantly augmented the bleeding time (Matsumoto, Takizawa et al. 2006). These findings were subsequently validated by Li et al., who showed that OM4 inhibits thrombosis without causing bleeding in rats (Li, Lockyer et al. 2007). Another antibody, JAQ1, has also shown effective long-term protection from occlusive thrombosis by downregulating GPVI (Nieswandt, Schulte et al. 2001), however studies with similar antibodies against different epitopes by the same group showed that targeting the collagen binding site on GPVI may downregulate the receptors without affecting platelet activation (Schulte, Rabie et al. 2003).

The α 2 β 1 integrin is crucial for platelet adhesion and activation. Polymorphisms within this gene and receptor density on platelets are associated with the kinetics of platelet adhesion on collagen underlining its importance for platelet function (Kritzik, Savage et al. 1998, Corral, Gonzalez-Conejero et al. 1999). Mouse models that are deficient in either α 2 or β 1 subunits show minor defects in platelet adhesion and aggregation on collagen and have slightly increased bleeding times (Holtkotter, Nieswandt et al. 2002, Gruner, Prostedna et al. 2003) which is incongruent with the human data where subjects with genetically decreased expression of α 2 β 1 integrin display diminished platelet responses to collagen and mild bleeding defect (Nieuwenhuis, Akkerman et al. 1985). However, *in vivo* thrombosis studies in mice deficient in α 2 β 1 gave conflicting results. Using fluorescence microscopy on the injured carotid artery, Gruner et al., showed that these mice do not have any thrombosis effect (Gruner, Prostedna et al. 2003). On the other hand, using photochemically induced endothelial injury to the carotid artery, He et al., showed the thrombus formation was delayed and decreased in these animals, possibly due to defective collagen interaction (He, Pappan et al. 2003, Kuijpers, Pozgajova et al. 2007). Combined deficiency of both collagen receptors (α 2 β 1 and GPVI) leads to complete inhibition of thrombus formation unlike the partial defects observed in absence of either of these receptors (Sarratt, Chen et al. 2005). This suggests that α 2 β 1 and GPVI work synergistically to achieve the optimal level of platelet adhesion and activation.

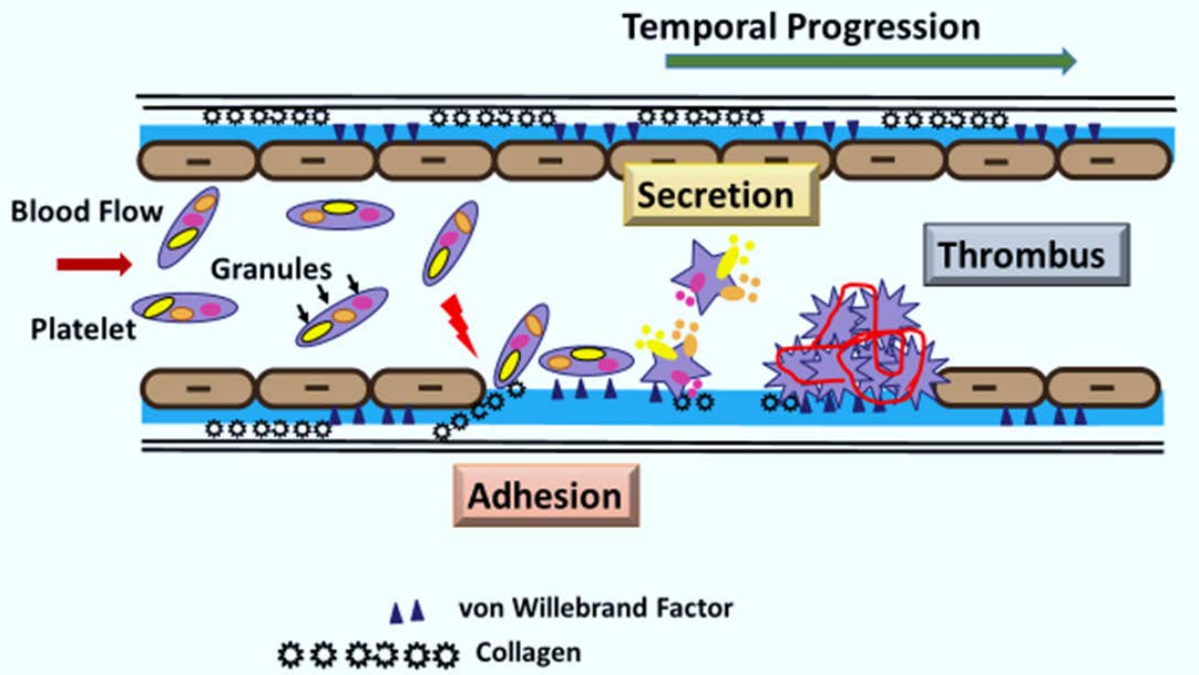


Figure 1.2. Platelet secretion affects the vascular microenvironment

Resting, discoid platelets flow in blood while endothelial cells prevent direct contact between platelets and subendothelial adhesive proteins such as vWF and collagen. Upon injury, these proteins interact with activate platelets. Platelet activation leads to platelet shape change, granule secretion and ultimately, aggregation. Platelet aggregation results in thrombus formation.

Extension phase

In this phase, activated platelets recruit and activate more platelets by releasing secondary agonists such as ADP. Once a platelet monolayer is formed on exposed vWF and collagen in the vascular wall, the subsequent process of recruiting and activating more platelets is initiated. The major players in this phase include platelet surface integrin $\alpha\text{IIb}\beta\text{3}$, GPCRs, and secondary agonists from platelet dense granules (ADP, TxA₂, epinephrine, thrombin, serotonin, calcium *etc*). that stimulate other platelets. These soluble agonists stimulate platelets and form microaggregates. Finally, activation of $\alpha\text{IIb}\beta\text{3}$ leads to conformational changes that facilitate its binding to fibrinogen and vWF. These interactions lead to stable contacts among platelets and a platelet plug is formed. Integrin $\alpha\text{IIb}\beta\text{3}$ is the most abundant (40,000-80,000 copies/platelet) integrin on the platelet surface and can be activated by inside out or outside in signaling. Platelet activation by any agonists leads to PLC activation that increases intracellular calcium levels. Subsequent PKC and PI3K activation alter cytoskeletal reorganization and talin, a crucial cytoskeletal protein gets activated and binds to a cytoplasmic domain of β3 subunit affecting the conformation of $\alpha\text{IIb}\beta\text{3}$ that facilitates fibrinogen binding to the receptor.

GPCRs facilitate the most stable platelet plug formation by propagating signals from potent agonists such as thrombin, PAR peptides, and Thromboxane A₂. GPCRs are seven – transmembrane spanning signaling molecules that activate heterotrimeric guanine nucleotide binding proteins. Many downstream effectors get subsequently activated including adenylyl cyclase, PLC, and PI3K to finally result in platelet activation. GPCRs acts in three ways:

1. First, through $G_{q\alpha}$, they stimulate PLC β isoforms. This leads to increased concentration of cytosolic Ca^{++} and PKC activation.
2. Through $G_{12\alpha}$ and $G_{13\alpha}$, they reorganize the actin cytoskeleton which leads to filopodia and lamellipodia formation and platelet shape change.
3. Through $G_{i\alpha}$, they inhibit cAMP synthesis by suppressing adenylyl cyclase

Thus, GPCRs facilitate platelet plug formation through various pathways. ADP, TxA₂, and thrombin are the major agonists that activate platelets through GPCR coupled receptors. ADP binds to two classes of purinergic GPCRs- P2Y1 (coupled to G_{qα}) and P2Y12 (coupled to G_{iα}) while TxA₂ binds to the TxA₂ receptor. Thrombin is one of the most potent platelet agonists and is also crucial for generating fibrin from precursor fibrinogen. It acts through Protease-Activated Receptor/PAR1 and PAR4 in humans and PAR3 and PAR4 in mice.

ADP, released from platelet dense granules as well as RBCs, is an important agonist capable of causing the full range of platelet activation. P2Y1 receptors are present in a comparatively lower amount (150/platelet (Baurand, Raboisson et al. 2001) compared to PAR 1, which is 1000-2000/platelet) (Gachet 2008). Leon et al., showed that P2Y1 deficiency leads to defective platelet aggregation and shape change. Additionally, P2Y1 participates in collagen-induced platelet activation. This defective platelet activation in absence of P2Y1 does not, however, lead to any significant bleeding diathesis. Though it mediates the weak response to ADP, it is still a vital component for ADP/collagen-induced platelet activation (Leon, Hechler et al. 1999). P2Y12 is an extensively studied ADP receptor and an appealing anti-platelet target since subjects lacking this receptor have defective ADP induced platelet aggregation (Cattaneo 2011). P2Y12 deficient platelets do not aggregate upon ADP stimulation and their response to other agonists is also defective. Mice with this deficiency, have a severe bleeding deficiency and are protected from arterial thrombosis (Andre, Delaney et al. 2003). P2Y12 plays a central role in the amplification of aggregation from various agonists in different pathways including thrombin, collagen, TxA₂, immune complexes, adrenaline, and serotonin. This amplification is achieved by inhibition of cAMP production and PI3K and small GTPase Rap 1B activation (Gachet 2008). This pivotal role of ADP in platelet activation by multiple agonists makes it an important target. Thienopyridine compounds such as clopidogrel and prasugrel as well as competitive antagonists of P2Y12 such as AZD6140 and cangrelor are some of the prominent antiplatelet drugs. The third receptor for ADP is P2X1, a ligand-gated cation channel that

is important for a fast calcium entry upon agonist stimulation. This receptor is important in high shear conditions and participates in collagen and shear-induced aggregation.

TxA₂, a potent vasoconstrictor and platelet agonist synthesized by activated platelets, is generated by cyclooxygenase (COX) and TxA₂ synthase enzymes. It acts as a secondary agonist by diffusing across the platelet membrane and activating other platelets. TxA₂ receptor-deficient mice (TP^{-/-}) have increased bleeding time and defective responses to TxA₂ analogs and collagen (Nieswandt, Aktas et al. 2005). Inhibition of TxA₂ by aspirin has proven to be a valuable treatment for managing high cardiovascular risk patients (Eidelman, Hebert et al. 2003), however, its efficiency is still controversial at high shear conditions (Barstad, Orvim et al. 1996, Valerio, Tran et al. 2016).

Thrombin is a potent, soluble agonist generated on the surface of activated platelets (Offermanns 2006). Thrombin is generated from the prothrombinase complex that is comprised of coagulation factor Xa and factor Va that together stimulate factor II to generate thrombin. Translocation of phosphatidylserine from the inner to the outer leaflet of the platelet plasma membranes provides a procoagulant surface for thrombin generation. Thrombin activates platelets through GP1b and other GPCRs-Protease-activated receptors/PARs. Human platelets have PAR1 and PAR4 while murine platelets contain PAR3 and PAR4. Thrombin can stimulate platelets at very low concentration and generate the full range of activation (increased intracellular calcium, granule secretion, protein phosphorylation, shape change *etc.*). The role of GP1b in thrombin response is illustrated by the patients with Bernard Soulier syndrome. These patients lack GP1b and have a defective response to thrombin and increased bleeding time (Lopez, Andrews et al. 1998). PAR receptors are activated by a unique mechanism in which thrombin cleaves the N terminal sequence exposing a peptide that acts as a tethered ligand. Peptides with similar sequences as the uncovered region could be used to stimulate PAR receptors. PAR receptors differ in their thrombin response. PAR1 mediates platelet activation at low thrombin concentration while PAR4 requires high thrombin concentration and calcium mobilization is slower in a PAR4 mediated response. PAR4 deficient mice have a defective response to thrombin even at high concentrations

(Sambrano, Weiss et al. 2001). Both PAR3 KO and PAR4 KO mice are protected against thrombosis and show a similar increase in bleeding time (Sambrano, Weiss et al. 2001, Weiss, Hamilton et al. 2002). For downstream signaling, PAR1 couples to $G_{q\alpha}$, $G_{12/13\alpha}$, and $G_{i\alpha}$ and PAR4 couples to $G_{q\alpha}$, and $G_{12/13\alpha}$. This wide network of G proteins provides thrombin a broader access for platelet activation (Rivera, Lozano et al. 2009).

Epinephrine is a weak agonist that plays a role in potentiating an activation signal from other agonists mainly by inhibiting cAMP production. Epinephrine receptor $\alpha 2A$ deficient mice show a defective response to epinephrine but not to other agonists and the tail bleeding time and thrombosis are not affected. These mice are protected against pulmonary thromboembolism induced by collagen and epinephrine suggesting that epinephrine may play role in the stability of the thrombus (Pozgajova, Sachs et al. 2006).

Stabilization phase

In this phase, the platelet plug is stabilized to prevent premature dissolution to facilitate wound healing. Integrin $\alpha IIb\beta 3$ plays a central role in the stabilization phase by making potent contacts among neighboring platelets. It is a major receptor for fibrinogen, vWF, fibronectin, and vitronectin. Deficiency of $\alpha IIb\beta 3$ leads to a severe bleeding disorder called Glanzmann's thrombasthenia (Bennett 2005). Platelet activation leads to "inside-out" signaling that alters the conformation of $\alpha IIb\beta 3$ making binding to fibrinogen more accessible. Alternatively, ligand binding (predominantly fibrinogen) to $\alpha IIb\beta 3$ results in "outside in" signaling that leads to cytoskeletal rearrangements, platelet shape change, aggregation and clot retraction. Furthermore, activated platelets also provide a procoagulant surface for prothrombinase complex where thrombin is generated and proceeds to cleave fibrinogen to form fibrin fibers. Fibrin maintains the integrity of thrombus till blood loss is ceased. Apart from integrin $\alpha IIb\beta 3$, many other molecules promote stability of the thrombus. These molecules include junctional adhesion molecules (JAM-A and JAM-C), Ephrin family of receptor tyrosine kinases (EphA4, EphB1, and ephrin B), and CD 40 Ligand (Rivera, Lozano et al. 2009).

Negative regulation of platelet activation and thrombus growth

Thrombus growth is a dynamic process with multiple factors working concurrently to facilitate thrombus formation to stop blood loss. This process, however, must be controlled to prevent occlusive thrombosis that prevents blood flow to tissues downstream. Many mechanisms exist to control this process. First, nitric oxide and prostaglandin I₂ /PGI₂ are secreted by endothelial cells that inhibit adenylyl cyclase and increase cAMP levels to prevent platelet activation (Radomski, Palmer et al. 1987). Nitric oxide activates guanylyl cyclase and inhibits PI3K and cyclooxygenase-1, preventing platelet activation, adhesion, and aggregation. Platelets are also an important source of NO and this platelet pool is a deciding factor in the recruitment of platelets to the platelet-rich thrombus (Loscalzo 2001). Second, a nucleosidase is secreted that cleaves ATP and ADP to AMP inhibiting platelet activation by reducing the intracellular nucleoside stock. Additionally, some surface receptors such as Platelet endothelial cell adhesion molecule-1 (PECAM-1), a glycoprotein, expressed on platelets and endothelial cells play an inhibitory role in platelet aggregation (Falati, Patil et al. 2006).

The degree of platelet activation varies depending on the nature of stimulation - strong vs weak agonist (thrombin/collagen vs epinephrine), duration of activation (short vs long) and quantity/dose of agonist (low vs high- minor vs major vascular damage) and alters the rate and extent of platelet secretion (Hechler, Nonne et al. 2010). This is manifested in diverse platelet morphologies. In resting conditions, platelets are discoid. Upon activation, they become spherical and form cellular projections, in the form of filopodia and lamellipodia. In early stages of activation, platelets undergo cytoskeletal changes that result in shape change but they do not release any cargo. Dense granule release is responsive to weaker stimulation since these granules are docked before stimulation (Chicka, Ren et al. 2016), while alpha granule release needs a stronger stimulation. At a higher agonist concentration, platelets release all their cargoes (Jonnalagadda, Izu et al. 2012).

Subsection Two: Hemostasis and thrombosis

Hemostasis is a process by which the vascular system maintains blood in a fluid state under physiological conditions. Upon vascular injury, it responds rapidly to stop blood loss by forming a clot/ thrombus. Thrombosis is a process of unrestricted thrombus formation in the absence of natural anticoagulant mechanisms or when these mechanisms fail to control thrombosis due to the severity of the injury. Hemostasis and thrombosis are mediated by dynamic interactions among vascular cells, extracellular matrix components, platelets, and the coagulation system and the synchronization among all these components is fundamental for achieving hemostatic balance.

Coagulation cascade

The coagulation cascade is traditionally described as an extrinsic pathway (tissue factor-initiated) and intrinsic pathway which activates a common downstream pathway which ultimately leads to fibrin formation delivering the necessary “glue” to aggregated platelets providing a mechanical support to a growing thrombus. According to a widely used current model, coagulation is divided into three phases:

1. Initiation phase
2. Amplification phase
3. Propagation phase

1. Initiation phase-

The initiation phase is also referred to as the extrinsic pathway (Figure 1.3) and starts when a vascular injury occurs. This phase is marked by the generation of low amounts of active coagulation factors. Disruption of the vasculature exposes a critical coagulation factor, Tissue factor (TF), which resides on endothelium and on extravascular tissues like fibroblasts and smooth muscle cells (Versteeg, Heemskerk et al. 2013). TF binds and activates factor VII(FVII) to FVIIa by proteolysis. This TF\FVIIa complex then cleaves FIX and FX into FIXa and FXa respectively. FXa associates with FVa to form a prothrombinase complex that cleaves prothrombin to thrombin, a strong platelet agonist.

2. Amplification phase-

In this phase, the levels of active coagulation factors are significantly increased. Thrombin activates platelets, converts FV to FVa, and FVIII to FVIIIa. FVIIIa is a cofactor for FIXa generation that ultimately leads to FXa generation, thus thrombin amplifies prothrombinase activity by providing positive feedback. Additionally, thrombin also converts FXI into FXIa.

3. Propagation Phase-

In this phase, coagulation factors bind to procoagulant membranes of activated platelets and fibrin clots are formed.

Role of platelets in coagulation

Platelet activation and blood coagulation are mutually dependent processes in hemostasis and thrombosis (Heemskerk, Bevers et al. 2002). Platelets contain some (*i.e.* FV) and interact with multiple coagulation factors. Thrombin, generated by prothrombinase complex activates platelets leading to platelet granule secretion and aggregation. Increased cytosolic calcium levels in platelets also lead to exposure of phosphatidylserine (PS) on their outer membrane providing a procoagulant surface for the assembly of coagulation factors. PS exposure mainly facilitates the assembly and activation of tenase (TF-FVIIa) and prothrombinase (FVa-FXa) complexes, leading to thrombin and fibrin generation.

PS exposure in platelets

The plasma membrane has asymmetric lipid composition. The outer layer is composed of phosphatidylcholine/PC and sphingolipids while the inner/cytosolic leaflet contains phosphatidylethanolamine/PE, phosphatidylinositol/PI, phosphoinositides and PS. This gradient of phospholipids where neutral lipids are on the outer leaflet and mostly anionic lipids on the inner leaflet is crucial for membrane protein interactions with the membrane and signaling. PS exposure on the outer leaflet is an apoptotic signal for clearance of dying cells whereas PS exposure on RBCs and platelets is critical for

thrombosis. The absence of PS exposure leads to a rare bleeding disorder known as Scott syndrome.

First described in 1979 (Weiss, Vivic et al. 1979), Scott syndrome is characterized as a bleeding disorder caused due to lack of PS exposure rendering feeble thrombosis (Toti, Satta et al. 1996). These platelets show normal morphology and functions, including secretion, aggregation, metabolism, granule content and adhesion to subendothelium. Moreover, coagulation factor levels are not altered. Using annexin V antibody, the authors reported that PS exposure was affected in these platelets that interfered with fibrin formation and ultimately thrombosis (Toti, Satta et al. 1996).

Three classes of proteins are important for the formation, maintenance, and dissolution of this membrane asymmetry: flippases, floppases, and scramblases. Flippases catalyze the movement of phospholipids from outer to inner leaflet while floppases are responsible for the same movement in reverse directions. Both flippases and floppases function in ATP dependent manner, unlike scramblases which are ATP-independent and randomly distribute lipids in the inner or outer leaflet (Hankins, Baldrige et al. 2015). Following agonist stimulation, cytosolic calcium levels are increased in platelets leading to inhibition of flippases and stimulating scramblase activity (Lhermusier, Chap et al. 2011). Multiple reports suggested that store-operated calcium entry (SOCE) machinery including the Orai 1 channel and the STIM1 calcium sensors are important for PS exposure; although non-SOCE mechanisms may also participate in PS exposure (Gilio, van Kruchten et al. 2010). Calpain activity (Siljander, Farndale et al. 2001) and cholesterol-rich rafts are also important for PS exposure (Kunzelmann-Marche, Freyssinet et al. 2002).

Since PS exposure has dual roles as an apoptotic signal and a component of the procoagulant surface, it was unclear if these processes are related to each other. In an elegant study, Schoenwaelder and colleagues showed that PS exposure inducing an apoptotic signal is different from the one aiding in the formation of the procoagulant surface (Schoenwaelder, Yuan et al. 2009). Inhibition of one pathway by pharmacological or signaling inhibitors does not have any impact on the other pathway.

These findings are consistent with normal PS exposure in apoptotic lymphocytes whereas PS exposure in calcium-stimulated platelets is absent (Williamson, Christie et al. 2001). Suzuki and coworkers showed that TMEM16F also known as anoctamin 6 is a transmembrane protein, crucial for calcium-dependent phospholipid scrambling activity and is muted in Scott syndrome (Suzuki, Umeda et al. 2010). Subsequent studies identified two new mutations in TMEM16F in another Scott syndrome patient (Castoldi, Collins et al. 2011).

Despite this insight, many questions are still unanswered. What is the trafficking route of flippases/flippases and scramblases that play roles in PS exposure? Phospholipid synthesis occurs in ER. In addition to the vesicle budding and fusion, other pathways also contribute to their transport to their respective destination (Kaplan and Simoni 1985) (Vance, Aasman et al. 1991). Some reports suggest a role of lipid transfer proteins (LTPs) in PS trafficking to the plasma membrane/PM in *S. cerevisiae* (Maeda, Anand et al. 2013), but it is not clear if this process is unidirectional and how the PS concentration in PM is maintained. Also, is PS trafficking in platelets LTP dependent? Finally, is it possible that these enzymes may get transferred from megakaryocytes to platelets, possibly packaged in granules or some other intracellular organelle such as endosomes? Does platelet granule secretion affect the release of PS exposure? PS exposure is affected in *Nbeal2*^{-/-} animals (Deppermann, Cherpokova et al. 2013) suggesting that alpha granules play a role in PS exposure leading to procoagulant nature of platelets. However, it remains to be seen if dense granules are also important for PS exposure.

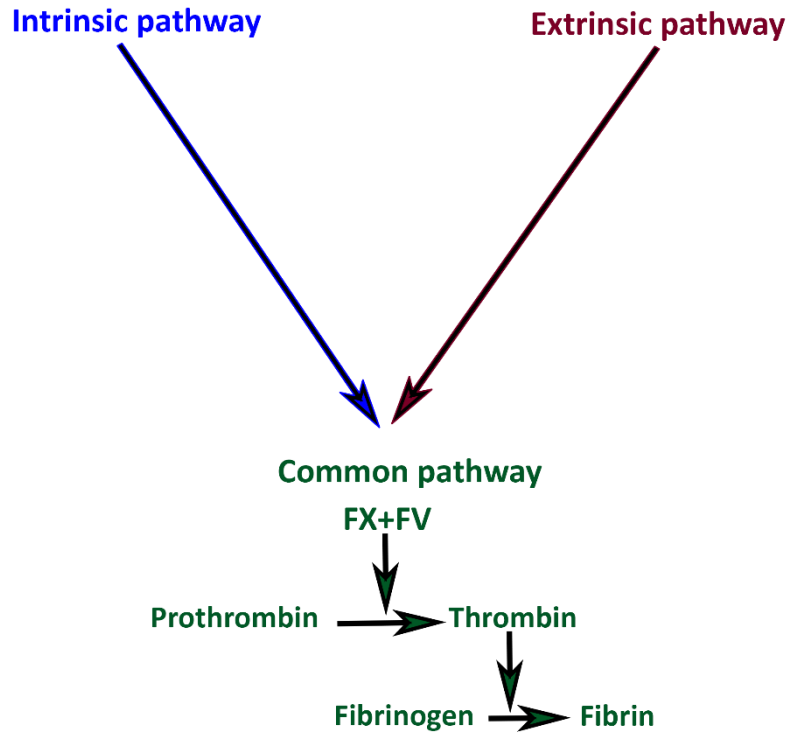


Figure 1 3. Intrinsic and extrinsic coagulation pathways of coagulation

The intrinsic pathway is a sequence of proteolytic events that lead to activation of FIX. Tissue factor exposure upon vascular injury initiates the extrinsic pathway. Both pathways ultimately lead to the assembly of prothrombinase complex (FX and FV) that results in cleavage of fibrinogen into fibrin forming a blood clot.

Thrombus architecture

Upon vascular injury, exposure to adhesive proteins in the endothelial cortex and to soluble agonists leads to platelet activation as described above and ultimately platelet shape change, granule secretion, and aggregation. Several *in vivo* studies using genetic and pharmacologic approaches have demonstrated that platelet activation during thrombus formation is a heterogeneous process where the most activated platelets form a core/central part of the thrombus while less activated platelets form the outer shell (van Gestel, Heemskerk et al. 2002) (Stalker, Traxler et al. 2013) (Hechler, Nonne et al. 2010) (Hayashi, Mogami et al. 2008). Multiple distinct pathways of activation are synchronized in a temporal and spatial manner to achieve an optimal response to form a stable thrombus. A myriad of platelet agonists is present at the site of vascular injury including collagen, thrombin, ADP, TxA₂, serotonin, and calcium among others which vary in their potency and concentration (Hechler, Nonne et al. 2010). Strong, potent platelet stimulation from all these agonists will result in a robust release of platelet granule cargo as a bolus. Considering the shear stress of blood flow, the cargo molecules may wash away downstream of the flow and will not be able to engage at the site of injury. Similarly, weak stimulation of platelets may delay thrombus formation altogether leading to severe bleeding. Hence the balance between unwanted thrombosis and required hemostasis is crucial and should be met by an optimal structure of the thrombus.

Using a systems approach, Brass et al., demonstrated how thrombus structure is influenced by various factors including the distribution of platelet agonists, the porosity of the region, permeability, the ease of solute transport within the platelet plug and platelet density (Stalker, Welsh et al. 2014, Tomaiuolo, Stalker et al. 2014). Using a novel thrombin biosensor and a thrombus porosity probe, the authors reported that molecular transport is slow in the shell region and even slower in the core which overlaps with high packing in the core region (Welsh, Stalker et al. 2014).

Sema4D KO mice are deficient in collagen-mediated platelet activation (Wannemacher, Zhu et al. 2010). Using these mice, the authors showed that platelet

accumulation at the site of injury and gradual reduction in gaps among platelets impede agonist transport in the region leading to robust activation on platelets in the core and progressively weaker activation in the shell region (Figure 1.4). They also showed that thrombin is a central agonist responsible for core formation while ADP plays a crucial role in shell formation (Stalker, Traxler et al. 2013). These studies and multiple other reports clearly demonstrate that platelet activation and granule secretion shapes thrombus composition. Hyperactive platelets lead to occlusive thrombosis while hypoactive/secretion deficient platelets contribute to bleeding.

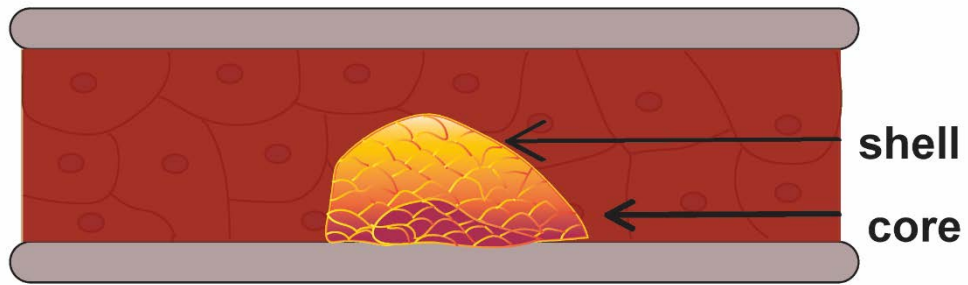


Figure 1 4. Platelet activation influences thrombus architecture

Thrombus composition is influenced by the level of platelet activation. Platelets exposed to subendothelial adhesive proteins get activated and forms a core region while less activated platelets form an shell region.

Platelets influence vascular microenvironment

How do platelets influence vascular microenvironment? The content of the platelet release and how its composition is controlled have been subjects of great interest. Are platelets able to release only subsets of their cargo or is the process stochastic? Both unbiased proteomics and directed antibody array systems have been used to monitor the release of multiple cargoes simultaneously. Several groups suggest that specific classes of cargo (*e.g.*, pro-angiogenic and anti-angiogenic factors) can be released in response to specific agonists (Ma, Perini et al. 2005, Italiano, Richardson et al. 2008, Chatterjee, Huang et al. 2011). However, other studies detected no thematic patterns in cargo release. Jonnalagadda et al., using a custom microELISA array and four agonists (thrombin, convulxin, PAR1 and PAR4), showed that agonist potency influences the kinetics and extent of secretion, but there were no “functionally thematic” patterns in the release process (Jonnalagadda, Izu et al. 2012). Broader proteomic studies confirmed the lack of thematic patterns in the release of granule cargo (Wijten, van Holten et al. 2013, van Holten, Bleijerveld et al. 2014); however, there are distinct kinetic patterns with cargo release occurring in waves. These findings suggest that platelets can use distinct release rates to temporally affect their microenvironment. The kinetics of release may be governed by the degree of stimulation as discussed earlier, the chemical properties of the cargo, the locale where the cargo is packaged in a granule and/or the machinery used (Sehgal and Storrie 2007, Kamykowski, Carlton et al. 2011, Jonnalagadda, Izu et al. 2012). A newly emerging perspective is the presence of a granule scaffold that acts as a platform to contain densely packed granule cargo inside the granule. It may also facilitate the release of these cargoes once fusion occurs, releasing contents closer to the fusion pore first. It remains to be seen if serglycin shown to be present in alpha granules (Woulfe, Lillieandahl et al. 2008) is important for controlling the rate of alpha-granule cargo release.

Since most of these past studies were done in suspension, it is unclear if there are also spatial constraints on platelet secretion. Early electron microscopy studies

suggested that the ventral platelet surface is different from the distal surface, consistent with some degree of polarity (White and Krumwiede 1994). Furthermore, a recent study reported that platelets actively migrate to collect bacteria (Gaertner, Ahmad et al. 2017) suggesting that at least some degree of polarity must be present in platelets guiding their movement in the vasculature.

Subsection Three: Mechanisms of secretion

Secretion or exocytosis is an event of fusion between a granule/vesicle to the plasma membrane to release cargo content. This process can be variable in different cell types. Types of fusion events include simple fusion (granule fuses to plasma membrane), compound fusion (granule fuses to another granule before or after fusing to plasma membrane), piecemeal degranulation (selective vesicular transport of cargo from the cytoplasmic secretory granules to the cell surface), and total granule extrusion/cytolysis (release of intact granules that coincides with the cell death).

Simple fusion is the most common fusion event. Compound fusion events are observed in mast cells (Hide, Bennett et al. 1993), eosinophils (Scepek, Moqbel et al. 1994), neutrophils (Lollike, Lindau et al. 2002), and platelets (Morgenstern 1995). Piecemeal degranulation/PMD has been identified as the predominant mode of secretion from eosinophils (Melo and Weller 2010), and to some extent, by mast cells (Crivellato, Candussio et al. 2002) while cytolysis is a mode of fusion mainly used by tissue eosinophils, secondary to PMD (Erjefalt, Greiff et al. 1999).

Apart from the type of fusion, these various secretion events also differ in their SNARE usage. v- and t- SNARE expression alters according to tissue type as does the SNARE complex composition. While VAMP-2/syntaxin-1/ -SNAP-25 complex is important for chromaffin granule release (Fitch-Tewfik and Flaumenhaft 2013), VAMP-8/syntaxin-11/SNAP23 complex is crucial for platelet granule release (Joshi and Whiteheart 2017).

Mechanism of platelet secretion

Platelet secretion is a textbook example of regulated secretion since there are stores of cargoes in granules that are released upon stimulation with an agonist (often called a secretagogue). Platelets respond to many agonists; including some, such as

thrombin and convulxin, considered strong and cause robust release while others, *e.g.*, ADP and epinephrine are weak secretagogues. In physiological conditions, these various agonists stimulate platelets at various stages and at various concentrations to shape thrombus architecture.

Secretion of dense granules is generally monitored by measuring ATP or serotonin release. Alpha-granule exocytosis is monitored by measuring the release of soluble cargo proteins *e.g.*, PF4, thromboglobulin, vWF, or the surface exposure of P-selectin, an abundant alpha granule membrane protein. Hydrolytic enzymes, such as β -hexosaminidase, are used as metrics of lysosome release. In many studies, single time points or agonist doses are used; however, this obscures the complexity of the platelet exocytosis process. More detailed studies demonstrate that the rates and extents of platelet exocytosis are directly related to stimulation strength which could be from strong vs weak agonist or shorter vs longer stimulation time (Jonnalagadda, Izu et al. 2012). Dense granule release is the fastest, most sensitive process; lysosome release is the slowest and requires greater stimulation. The alpha-granule release is kinetically the most diverse. Together, these three exocytic processes form the platelet releasate, which has been shown to contain hundreds of bioactive components (Coppinger, Cagney et al. 2004, Maynard, Heijnen et al. 2007, van Holten, Bleijerveld et al. 2014). It is this releasate that affects the microenvironment around activated platelets and contributes to platelet function.

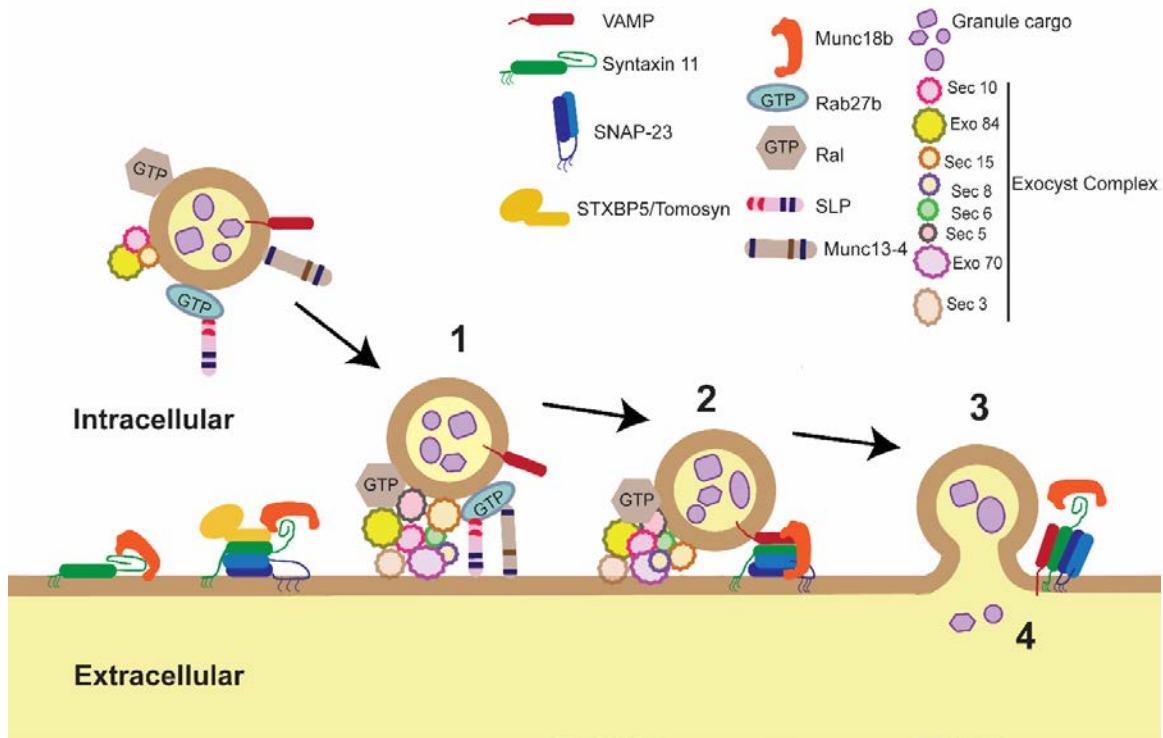


Figure 1.5. Pathway of SNARE-mediated platelet granule release

Platelet exocytosis is a pathway of protein-protein interactions leading from 1) granule docking, 2) SNARE engagement to 3) membrane fusion and 4) cargo release. The interactions and their proposed order of occurrence are depicted in the schematic. The machinery, which could be on the granules, includes v-SNAREs (Vesicle-Associated Membrane Protein/VAMPs); Munc13-4 and synaptotagmin-like protein (SLP); small GTPases Rab and Ral; and the Exocyst complex. The machinery on the plasma membrane includes t-SNARE heterodimer of syntaxin-11 and SNAP23; the Syntaxin-chaperone Munc18b; and the t-SNARE regulator STXBP5/Tomosyn 1. Also depicted are domains in each protein, which contribute to protein function and thus platelet exocytosis: blue lines, C2 domains in Munc13-4 and SLP; brown line, Munc homology domain in Munc13-4; red lines, SLP homology domain in SLP; rounded rectangles, SNARE domain.

Granule-plasma membrane fusion

The penultimate step of exocytosis is the fusion of cargo- containing granules with the plasma membranes/PM. Platelet granules also fuse with each other, in a process called compound fusion, which may or may not precede fusion with the PM (Morgenstern 1995). Membrane fusion is mediated by proteins called Soluble N-ethylmaleimide-sensitive factor Attachment Protein Receptor (SNARE) proteins (Figure 1.4). This process is highly conserved in all eukaryotes and the family of SNARE proteins is quite large (Kloepper, Kienle et al. 2007). SNAREs are classified into two groups based on their relative locations: v- for vesicle/granule localized and t- for target membrane-localized SNAREs. They are also classified based on the amino acid at the center of their SNARE domains, either R (Arg) or Q (Glu). Vesicle-Associated Membrane Proteins (VAMPs) constitute a large group of the known v-SNAREs (R-SNAREs). t- SNAREs (Q-SNAREs) consist of two classes of proteins – Syntaxins and Synaptosome Associated Proteins/SNAP-23/25 proteins (Fasshauer, Sutton et al. 1998). All SNAREs contain one or two amphipathic, heptad- repeat-containing, SNARE domains of ~60 amino acids; which are typically adjacent to a C-terminal tail-anchored membrane segment. Cognate v- and t- SNAREs interact, through these domains, to form a stable transmembrane complex that promotes membrane fusion (Jahn and Scheller 2006). Four SNARE domains, one each from v- SNARE (R-SNARE) and syntaxin (Q_a-SNARE) and two from SNAP23/25 (Q_{bc} SNARE), form a coiled-coil structure that buries the hydrophobic residues and draws the two membranes together for fusion.

v-SNAREs in platelets

Though there are nine VAMPs found in the human genome (VAMP-1-8 excluding VAMP-6, Sec22, and Ykt6p) (Bock, Matern et al. 2001), only six of them are expressed in platelets (Graham, Ren et al. 2009, Burkhardt, Schumbrutzki et al. 2012, Joshi and Whiteheart 2017). VAMP-2, VAMP-3, VAMP-7, and VAMP-8 are the major ones and VAMP-4 and VAMP-5 have been found only in trace amounts. VAMPs are divided into two groups based on their structure. Brevin VAMPs are smaller with a short cytoplasmic domain, a transmembrane domain, and an N terminal SNARE domain. This class includes

VAMP-2, VAMP-3, and VAMP-8. The second group is called longins, since the members of this group contain a unique longin domain on the N terminus, downstream of a SNARE domain. VAMP-7, Sec22, Ykt6 and the yeast protein Nyv1p are contained within this group (Chaineau, Danglot et al. 2009).

Synaptobrevin/VAMP-2/V-2

First identified in synaptic vesicles and named as a homolog of VAMP-1 (Elferink et al, 1989), VAMP-2 is expressed in the central nervous system/CNS and in peripheral neurons. V-2^{-/-} animals die after birth though neuronal development is normal and they show defective secretion upon Ca⁺⁺ stimulation in the neuronal synapse (Schoch, Deak et al. 2001). These findings support previous reports from Schiavo and colleagues suggesting that upon tetanus toxin treatment, which cleaves VAMP-1, -2, and -3, synaptic transmission is severely affected (Schiavo, Benfenati et al. 1992). Since VAMP-1 is present in trace amounts and VAMP-3 is not detectable in neurons, VAMP-2 must be essential for synaptic fusion events. Apart from the nervous system, VAMP-2 is also expressed in adipocytes, skeletal muscles and in hematopoietic lineage cells such as mast cells, neutrophils and eosinophils (Stow, Manderson et al. 2006). VAMP-2 is important for gelatinase-containing granule secretion from neutrophils and secretory vesicles from eosinophils. It also plays a role in insulin-mediated GLUT4 translocation in cultured 3T3-L1 adipocytes, and in L6 muscle cell line (Martin, Shewan et al. 1998, Randhawa, Bilan et al. 2000).

Cellubrevin/VAMP-3/V-3-

VAMP-3 is a homolog of VAMP-2 and is expressed in a wide variety of tissues. In fact, its ubiquitous expression led to naming this protein “cellubrevin” (McMahon et al, 1993). VAMP-3 is expressed in neuroendocrine cells (Chilcote, Galli et al. 1995), glial cells (Bezzi, Gundersen et al. 2004), macrophages, heart, adipose tissue, skeletal muscles, brain, liver, testis, kidneys, lungs, mast cells, chromaffin granule, and pancreatic beta cells (Yang, Mora et al. 2001). Like VAMP-2, VAMP-3 is also a substrate for proteolytic cleavage by tetanus toxin light chain (McMahon, Ushkaryov et al. 1993, Galli, Chilcote et al. 1994). VAMP-3 has been implicated in many trafficking events. It is

important for guanosine-59-O-(3-thiotriphosphate) (GTP γ S)-stimulated GLUT4 translocation (Millar, Shewan et al. 1999), phagosome formation (Bajno, Peng et al. 2000), phagocytosis (Hackam, Rotstein et al. 1998, Murray, Kay et al. 2005), and transferrin recycling through endosomes (Galli, Chilcote et al. 1994, Daro, van der Sluijs et al. 1996). VAMP-3 contributes to apical transport of H⁺-ATPase (Breton, Nsumu et al. 2000). It is also important for migration of epithelial cells and recycling of β 1 integrins (Proux-Gillardeaux, Gavard et al. 2005).

Tetanus toxin insensitive VAMP / TI-VAMP/VAMP-7

VAMP-7 is structurally distinct from other, shorter VAMP isoforms (VAMP-2, -3, and -8). It contains a characteristic domain at its N terminus, called a Longin domain. The longin domain is important for regulation of SNARE complex formation and targeting VAMP-7 to late endosomes through interaction with an adaptor protein, AP-3 (Martinez-Arca, Rudge et al. 2003). Recently, it was shown to interact with cytoskeleton regulators, specifically with VPS9 and ankyrin repeat containing protein (VARP) (Schafer, Hesketh et al. 2012, Koseoglu, Peters et al. 2015).

Galli et al., first identified this VAMP isoform in epithelial cells. They found that VAMP-7 contributes to apical membrane exocytosis and it is protected from the tetanus toxin light chain that cleaves VAMP-1, -2 and -3 (Galli, Zahraoui et al. 1998). Apart from epithelial cells, VAMP-7 is expressed in neurons, neuroendocrine cells, heart, kidney, liver, spleen, and thymus (Advani, Bae et al. 1998). VAMP-7 has been implicated in inhibition of neurite outgrowth (Martinez-Arca, Alberts et al. 2000) and in intracellular trafficking from endosomes to lysosomes (Advani, Yang et al. 1999). VAMP-7 contributes to lysosomal secretion in Normal Rat Kidney Epithelial/NRK cells (Rao, Huynh et al. 2004), FcR stimulated exocytosis and phagocytosis in macrophages (Braun, Fraiser et al. 2004), and Glut4 trafficking in adipocytes (Williams and Pessin 2008). A recent report by Molino, et al., show that the transport of endogenous and exogenous GPI anchored proteins was altered in VAMP-7 deficient fibroblasts. The authors suggested that VAMP-7 contributes to Golgi homeostasis by facilitating trafficking of GPI anchored proteins to the cell surface (Molino, Nola et al. 2015).

Endobrevin\VAMP-8\V-8-

VAMP-8 was first identified by Wong, et al., as an endosomal v- SNARE in cultured cells and in rat liver and thus named endobrevin. Its presence in HeLa cells, MDCK cells, and normal rat kidney cells/NRK was reported (Wong, Zhang et al. 1998). VAMP-8 is broadly expressed in all membranes connected with the endosomal system including the plasma membrane, clathrin-coated pits, late endosomes, and membranes of the *trans*-Golgi network (Antonin, Holroyd et al. 2000). It mediates fusion of early and late endosomes (Antonin, Holroyd et al. 2000). VAMP-8 forms a complex with syntaxin-7, syntaxin-8, and vti1b, and aids in the homotypic fusion of late endosomes or heterotypic fusion with lysosomes (Antonin, Holroyd et al. 2000). Williams, et al., showed that in adipocytes, siRNA mediated VAMP-8 knockdown affected GLUT4 endocytosis from plasma membranes (Williams and Pessin 2008). Using bone marrow-derived mast cells (BMCCs) from *V-8^{-/-}* mice, Tiwari, et al., showed that VAMP-8 plays a role in β -hexosaminidase and histamine release from mast cells. These animals, however, showed no defect in secretion of cytokines such as Tumor necrosis factor/TNF- α , IL-6, and Macrophage inflammatory protein/MIP-1 α . This indicates that VAMP-8 deficiency affects Fc ϵ RI induced degranulation but not cytokine and chemokine secretion (Tiwari, Wang et al. 2008).

Though important for endosomal trafficking, VAMP-8 is dispensable for endocytosis. Using global VAMP-8 deficient mice, Wang et al., showed that cultured embryonic *V-8^{-/-}* fibroblasts or primary hepatocytes do not have any endocytic defect. Furthermore, receptor-mediated endocytosis, intracellular trafficking of cathepsin D from TGN to lysosomes, degradation of low-density lipoproteins/LDL and recycling of the transferrin receptors all were normal (Wang, Ng et al. 2004). Wang et al., also reported that VAMP-8 is enriched in zymogen granule membranes and is important for their secretion from pancreatic acinar cells. In fact, VAMP-8 deficient pancreatic acinar cells harbored three times more zymogen granules than the control cells indicating accumulation of granules due to failure to secrete. Furthermore, *V-8^{-/-}* pancreas failed to

secrete digestive enzymes upon cholecystokinin stimulation indicating VAMP-8 is required for enzyme secretion from the exocrine pancreas.

Another elegant study by the same group further illuminated VAMP-8's broad role in encompassing regulated exocytosis of the entire exocrine system (Wang, Shi et al. 2007). In this report, the authors characterized the role of VAMP-8 in secretion from exocrine tissues. They found that VAMP-8 is widely expressed in an exocrine system including salivary glands, lacrimal glands, sweat glands, sebaceous glands, mammary glands and the prostate. Further evaluation indicated that VAMP-8 mediated secretion is vital in salivary and lacrimal glands. VAMP-8 is also expressed in kidneys and has been attributed to playing a role in the transport of a water channel aquaporin 2, to the plasma membrane (Wang, Ng et al. 2010).

Since these early reports, major *in vitro* studies have implicated VAMP-8 as a critical player in the terminal step of cytokinesis in mammalian cells along with syntaxin-2 (Low, Li et al. 2003) and also in the release of hexosaminidase from rat basophilic leukemia cells (Lippert, Ferrari et al. 2007).

Table 1.2 Secretory machinery in platelets

Protein	Gene	Aliases (in <i>Homo sapiens</i>)	Abundance in Mice	Abundance in Humans	Functional References
v- SNAREs (R-SNAREs)					
VAMP-2	VAMP2	SYB2, VAMP-2	7,600 † 1,012 *	<800 †	(Ren, Barber et al. 2007)
VAMP-3	VAMP3	CEB	2,200 † 35,813 *	4,600 † 4,200 ‡	(Flaumenhaft, Croce et al. 1999, Feng, Crane et al. 2002, Polgar, Chung et al. 2002, Schraw, Rutledge et al. 2003)
VAMP-4	VAMP4	VAMP-4, VAMP24	ND	ND	
VAMP-5	VAMP5		6,775 *	740 ‡	
VAMP-7	VAMP7	SYBL1, TIVAMP, VAMP-7, TI-VAMP	2,700 †	3,800 † 8,100 ‡	(Koseoglu, Peters et al. 2015)
VAMP-8	VAMP8	EDB, VAMP-8	8,400 † 37,751 *	6,600 † 7,100 ‡	(Ren, Barber et al. 2007, Graham, Ren et al. 2009)
t- SNAREs (Q-SNAREs)					
Syntaxin 2	STX2	EPM, EPIM, STX2A, STX2B, STX2C	1,800 § 1,738 *	1,600 § 4,100 ‡	(Chen, Bernstein et al. 2000, Chen, Lemons et al. 2000)
Syntaxin 4	STX4	STX4A, p35-2	1,600 § 9,892 *	9,600 § 3,800 ‡	(Lemons, Chen et al. 1997, Chen, Bernstein et al. 2000, Chen, Lemons et al. 2000,

						Lemons, Chen et al. 2000) (Flaumenhaft, Croce et al. 1999)
Syntaxin 6	STX6		522 *	1,400 ‡		
Syntaxin 7	STX7		3,700 §	3,900 §		
			10,468 *	7,100 ‡		
Syntaxin 8	STX8	CARB	1,077 *	<500 ‡		}(Golebiewska, Harper et al. 2015)
Syntaxin 11	STX11	FHL4, HLH4, HPLH4	47,000 §	52,000 §		(Ye, Karim et al. 2012)
			12,190 *	7,500 ‡		
SNAP-23	SNAP23	SNAP-23, SNAP23A, SNAP23B, HsT17016	5,400 §	19,000 §		(Lemons, Chen et al. 1997, Flaumenhaft, Croce et al. 1999, Chen, Lemons et al. 2000, Lemons, Chen et al. 2000)
			27,531 *	11,400 ‡		
SNAP-25	SNAP25	SUP, RIC4, SEC9, SNAP, CMS18, RIC-4, SNAP-2, bA416N4.2, dJ1068F16.2	ND	ND		
SNAP-29	SNAP29	CEDNIK, SNAP-29	13,969 *	3,400 ‡		(Williams, Savage et al. 2016)
SNARE REGULATORS						
Munc18a	STXBP1	P67 NSEC1, UNC18, RBSEC1, MUNC18-1	984 *	1,600 ‡		(Schraw, Lemons et al. 2003)
Munc18 b	STXBP2	FHL5, UNC18B, Hunc18b, UNC18-2, pp10122, MUNC18-2	18,722 *	15,800 ‡		(Al Hawas, Ren et al. 2012)
Munc18c	STXBP3	PSP, MUNC18C, UNC-18C, MUNC18-3	7,626 *	2,900 ‡		(Reed, Houg et al. 1999)
α-Synuclein	SNCA	PD1, NACP, PARK1, PARK4	42,331*	38,100 ‡		unpublished
STXBP5 /	STXBP5	LGL3, LLGL3, Nbla04300	6,910*	3,300 ‡		(Ye, Huang et al. 2014, Zhu,

Tomosyn 1						Yamakuchi et al. 2014)
SLP4 / Granuphilin	SYTL4	SLP4	18,116 *	9,200 ‡		(Hampson, O'Connor et al. 2013)
α-SNAP	NAPA	SNAPA	ND	8,900 ‡		unpublished
NSF	NSF	SKD2	7,162 *			(Polgar and Reed 1999)and unpublished
γ-SNAP	NAPG	GAMMASNAP	ND	3,500 ‡		
IKK-α	CHUK	IKK1, IKKA, IKBKA, TCF16, NFKBIKA, IKK-alpha	516 *	1,800 ‡		
IKK-β	IKBKB	IKK2, IKKB, IMD15, NFKBIKB, IKK-beta	603 *	1,300 ‡		(Karim, Zhang et al. 2013)
IKK-γ	IKBKG	IP, IP1, IP2, FIP3, IKKG, IPD2, NEMO, FIP-3, Fip3p, IMD33, AMCBX1, IKKAP1, ZC2HC9, IKK-gamma	ND	ND		

TETHERING FACTORS

Munc13-4	UNC13D	FHL3, HLH3, HPLH3, Munc13-4	320, 9,249 *	ND		(Ren, Wimmer et al. 2010)
Rab 27b	RAB27B	C25KG	55,593 *	35,900 ‡		(Tolmachova, Abrink et al. 2007)
SLP1	SYTL1	JFC1, SLP1	ND	ND		Neumuller, 2009 #75}

EXOCYST COMPONENTS

Sec3	EXOC1	SEC3, SEC3P, BM-102, SEC3L1	1,525 *	1000 ‡		
Sec5	EXOC2	SEC5, Sec5p, SEC5L1	ND	1800 ‡		(Kawato, Shirakawa et al. 2008)
Sec6	EXOC3	SEC6, Sec6p, SEC6L1	1,081 *	1600 ‡		
Sec8	EXOC4	SEC8, Sec8p, SEC8L1	ND	1900 ‡		

Sec10	EXOC5	SEC10, HSEC10, SEC10P, PRO1912, SEC10L1	1,331 *	1300 ‡	
Sec15	EXOC6	SEC15, EXOC6A, SEC15L, Sec15p, SEC15L1, SEC15L3	1,061 *	710 ‡	
Exo70	EXOC7	EXO70, EXO70, EXOC1, 2-5-3p, Exo70p, YJL085W	ND	1400 ‡	
Exo84	EXOC8	EXO84, SEC84, Exo84p	1,325 *	1100 ‡	
Ral A	RALA	RAL	2,755*	3400 ‡	(Kawato, Shirakawa et al. 2008)
Ral B	RALB	-	12,165 *	6,800 ‡	

*Determined by Stable Isotope Labeling in Cell Culture- Protein Epitope Signature Tag (SILAC- PrEST) method

† Determined by quantitative western blotting (Graham et al)

§ Determined by quantitative western blotting (Ye and Whiteheart unpublished)

‡ Determined by spectral counting

ND Not Determined

All numbers are rounded to nearest hundredth.

VAMPs/v-SNAREs in platelets

Quantitative western blotting and proteomic analysis (Graham, Ren et al. 2009, Burkhardt, Schumbrutzki et al. 2012) have shown that platelets contain multiple v-SNARE isoforms (e.g., VAMP-2, -3, -4, -5, -7, and -8; Table 1.2). VAMP-8 and -7 are most abundant in human platelets while VAMP-8, -2, and -7 are most abundant in mouse platelets. Although previous studies using permeabilized platelets showed a role for VAMPs, the assignment of specific isoforms was equivocal (Flaumenhaft, Croce et al. 1999, Feng, Crane et al. 2002, Polgar, Chung et al. 2002, Schraw, Rutledge et al. 2003). More definitive studies of platelets from knockout (KO) mice demonstrated roles for VAMP-8 and -7; however, in neither case does a single VAMP account for all the release (Ren, Barber et al. 2007, Koseoglu, Peters et al. 2015). Deletion of VAMP-3 or reduction of VAMP-2 had no effect when VAMP-8 and -7 were present (Ren, Barber et al. 2007). Interestingly, permeabilized V-8^{-/-} mouse platelets did show diminished secretion upon treatment with a tetanus toxin light chain fragment, which specifically cleaves VAMP-2 and -3 (Schiavo, Matteoli et al. 2000). VAMP-7 is resistant to the toxin peptidase (Galli, Zahraoui et al. 1998). The roles of VAMP-4 and -5 have not been addressed, but their levels are low compared to the other platelet VAMPs (Table 1.2). These data imply unique and dominant roles for VAMP-7 and -8 in platelet secretion and suggest that, at least in mice, VAMP-2 and -3 contribute to the process. This type of compensation or “ranked redundancy” in isoform usage has been reported in chromaffin cells (Borisovska, Zhao et al. 2005) and in mast cells (Sander, Frank et al. 2008, Tiwari, Wang et al. 2009) and may be related to some intrinsic property of each VAMP or to the amounts of each isoform. This distinction is not clear at present. Phenotypically, the loss of VAMP-8 causes defective thrombosis. Global deletion of VAMP-8 resulted in delayed and diminished thrombus formation (Graham, Ren et al. 2009). However, V-8^{-/-} mice failed to show any bleeding diathesis. Interestingly, loss of VAMP-7 caused no defect in platelet accumulation at the site of laser injury nor in the tail-bleeding assay, though the alpha-granule release is affected in the growing thrombus (Koseoglu, Peters et al. 2015). These data support the primacy of VAMP-8-mediated secretion in hemostasis and

suggest that VAMP-7 and -8 contribute distinctly to platelet function. Consistent with the primacy of its usage, the gene encoding VAMP-8 has been linked, by Genome-Wide Association Studies (GWAS), to early-onset myocardial infarction, which is indicative of hyper-reactive or perhaps “hyper-secretory” platelets (Shiffman, Rowland et al. 2006, Shiffman, O'Meara et al. 2008).

Additional genetic studies identified a microRNA (microRNA-96) that controls VAMP-8 expression and correlates with increased platelet response to epinephrine (Kondkar, Bray et al. 2010). This microRNA affected VAMP-8 levels in a tissue culture system, but the effects on secretion were not tested. From the mouse models and the implications of the human genetic data, it seems possible that VAMP-8 levels directly affect platelet secretion efficacy.

VAMP-7 positive structures localize to the periphery of spreading platelets, while VAMP-8 and -3 positive granules concentrate in the central granulomere (Peters, Michelson et al. 2012). These data imply that VAMP-7 mediates secretion at the periphery where it is needed for platelet spreading. VAMP-8 (and perhaps VAMP-2 or -3) mediates fusion in the centralized granulomere, which is needed for thrombus growth. However, while the platelet granulomere is readily visible in platelets bound to rigid surfaces (*i.e.*, glass coverslips and electron microscopy (Allen, Zacharski et al. 1979)), it is yet to be defined in a growing thrombus. VAMP-8's role in platelet spreading has not been directly tested. Similar studies in neutrophils (Mollinedo, Calafat et al. 2006), and mast cells (Puri and Roche 2008) also show a polarization of VAMP isoform-positive structures during the exocytosis process.

The spatial differences between VAMP-7 and -8 positive granules suggest the potential for differential cargo release mediated by the two isoforms. At present, it is unclear if the two VAMPs associate specifically with distinct cargo or mediate differential release in response to different secretagogues. However, the existing data offer intriguing hints about the spatial nature of platelet exocytosis. Future experiments

using microfluidics and/or enhanced-resolution imaging, *in vivo*, will be needed to resolve these questions.

t-SNAREs in platelets

Platelets contain syntaxin-2, -4, -6, -7, -8, -11 -12, -16, -17, and -18 and SNAP23, 25 and 29 (Table 1.2; (Ren, Ye et al. 2008, Burkhart, Schumbrutzki et al. 2012, Zeiler, Moser et al. 2014, Joshi and Whiteheart 2017). SNAP23 is thought to be the functionally relevant Qbc t-SNARE, based on studies using permeabilized platelets with inhibitory antibodies and peptides (Flaumenhaft, Croce et al. 1999, Chen, Bernstein et al. 2000, Chen, Lemons et al. 2000, Lemons, Chen et al. 2000). SNAP25 is much less abundant and platelets from SNAP29 KO mice show no significant secretion defect (Williams, Savage et al. 2016). The Qa t-SNAREs, syntaxin-2 and -4, were previously reported to be important (Flaumenhaft, Croce et al. 1999, Chen, Bernstein et al. 2000, Chen, Lemons et al. 2000, Lemons, Chen et al. 2000). However, these findings were not consistent with data from KO mice (Ye 2012, Ye, Karim et al. 2012). Secretion from syntaxin-2/4 double KO platelets is unaffected though endocytosis is defective (Ye 2012). Analysis of platelets from a Familial Hemophagocytic Lymphohistiocytosis type 4 (FHL-4) patient, lacking syntaxin-11, indicate its role in secretion from all three granules. Consistently, syntaxin-11 forms complexes with SNAP23 and VAMP-8. To explain previous data, Ye et al., showed that the original anti-syntaxin-2 antibodies, which inhibited release from permeabilized platelets, cross-reacted with syntaxin-11 (Ye, Karim et al. 2012). Recently, another report demonstrated that syntaxin-8 influences dense granule but not alpha granule or lysosome release (Golebiewska, Harper et al. 2015). The authors showed that syntaxin-8 interacts with syntaxin-11 but not with SNAP23. This shows that syntaxin-8's Qb SNARE motif forms different complexes with the Qa motif of syntaxin-11 than the complexes formed with SNAP23's Qbc motifs.

SNARE heterogeneity in exocytosis

A role of SNARE isoforms in trafficking and exocytosis of various intracellular vesicles has been a subject of intense investigation. Multiple studies indicate that SNARE isoforms may have overlapping functions in trafficking and exocytosis depending on the

cell type. First started with studies in *Saccharomyces cerevisiae*, these investigations soon were implemented in other model systems.

Early studies in *Drosophila* neurons showed compensatory mechanisms among SNAREs. Bhattacharya et al., elegantly showed that members of VAMP family compensate for each other (Bhattacharya, Stewart et al. 2002). *Drosophila* has two distinct VAMP family members: ubiquitously expressed synaptobrevin/syn and neuronal synaptobrevin (n-syb) which is expressed exclusively in the CNS. While syb is important for cell viability, n-syb is required for synaptic vesicle secretion. Deletion of syb leads to developmental defects while the loss of n-syb reduces spontaneous vesicle fusion and completely blocks action potential-evoked vesicle exocytosis. Interestingly, the expression of the n-syb in syb^{-/-} and syb in n-syb^{-/-} mutants effectively rescue these phenotypes. In fact, rat n-syb and cellubrevin/VAMP-3 also can compensate for the loss of syb. This indicates that despite different cellular locations, these VAMP isoforms could efficiently compensate for each other (Bhattacharya, Stewart et al. 2002). Another study in *Drosophila* suggested that the loss of the t-SNARE SNAP25 could be compensated for by another SNAP isoform, SNAP24, to facilitate synaptic transmission in *Drosophila* larval neuromuscular junction (Vilinsky, Stewart et al. 2002).

This concept of functionally interchangeable SNARE isoforms is heavily supported by vigorous studies in yeast. In yeast, two syntaxin homologs, Vam3p and Pep12p function as vacuolar and endosomal t- SNAREs respectively. Mutations in Vam3p affect vesicle fusion with vacuoles while mutations in the Pep12p manifest as defective vesicle trafficking from the late Golgi to the endosome. When overexpressed, both Pep12p and Vam3p can compensate for each other's loss and rescue the relevant phenotype (Darsow, Rieder et al. 1997, Gotte and Gallwitz 1997). Likewise, Liu et al., showed that a similar relationship exists between yeast Sec22p and Ykt6p. Sec22p is crucial for fusion of ER-derived vesicles with Golgi membranes and Ykt6p is important at late stages of the secretory pathway. Upon deletion of Sec22p, Ykt6p is up-regulated and compensates for the loss of Sec22p by facilitating ER- Golgi secretory pathway (Liu and Barlowe 2002).

In a seminal study in chromaffin cells, Borisovska and colleagues showed that synaptobrevin II/VAMP-2 is the major v- SNARE critical for catecholamine release from chromaffin cells. However, its loss could be efficiently compensated for by VAMP-3. Similarly, VAMP-2 could compensate in the absence of VAMP-3. The catecholamine secretion was completely abolished in V-2/3^{-/-} animals indicating that other residual VAMP isoforms could not efficiently override the effect of V-2/3 loss (Borisovska, Zhao et al. 2005).

This pattern of functional redundancy in SNAREs is also observed in neutrophils. The secretion of various types of cytoplasmic granules in human neutrophils is mediated by distinct combinatorial SNARE complexes. Specific and tertiary granules which are readily exocytosed upon stimulation are under the control of VAMP-1, VAMP-2, syntaxin-4, and SNAP23, while the release from azurophilic granules was mediated by VAMP-1, VAMP-7, and syntaxin-4. This suggests that differential mobilization of a distinct type of granule is facilitated by the quantitative and qualitative difference in combinatorial SNARE complexes formation. This diversity in SNARE complexes aids the rate and the degree of secretion (Mollinedo, Calafat et al. 2006).

In myoblasts, VAMP-2 but not VAMP-3 has been shown to mediate insulin-stimulated Glucose Transporter/GLUT4 translocation to the plasma membrane (Randhawa, Bilan et al. 2000). In adipocytes, GLUT4 is found in two pools of vesicles-endocytic pool (including trans-Golgi network) and non-endocytic pool that consists of special vesicles called GLUT4 storage vesicles (GSVs). Millar and colleagues showed that these different pools of vesicles release GLUT4 in agonist-dependent and VAMP isoform-dependent manner. VAMP-3 facilitates GLUT4 translocation from the endosomal pool in a guanosine-59-O-(3-thiotriphosphate) (GTPγS) dependent manner while VAMP-2 is important for insulin-stimulated GLUT4 translocation. VAMP-3 is also associated with both insulin and GTPγS stimulated GLUT1 translocation contrary to VAMP-2 which does not have any impact on GLUT1 translocation (Millar, Shewan et al. 1999).

In human mast cells, VAMP-7 and VAMP-8 translocate to the plasma membrane upon IgE stimulation and play central roles in the release of histamine granules while VAMP-2 and VAMP-3 are dispensable (Sander, Frank et al. 2008). The role of VAMP-8 in mast cell degranulation was further supported by another study. Using bone marrow-derived mast cells (BMCC) from VAMP-8^{-/-} mice, Tiwari et al., showed that VAMP-8 is crucial for mast cell degranulation but not for cytokine/chemokine release. However, there is some residual secretion in absence of VAMPs suggesting a compensatory mechanism. Co-immunoprecipitation studies indicated an increased presence of VAMP-2/SNAP23 complexes in VAMP-8 deficient platelets suggesting a compensatory role for VAMP-2 (Tiwari, Wang et al. 2008). However, the lack of direct evidence fails to support this possibility.

The data gleaned from all these studies could be interpreted as-

1. Various SNARE isoforms, both v- and t- SNAREs play redundant roles in secretion and trafficking.
2. Despite their different intracellular locations and dissimilar structures, they can compensate for each other *in vitro* and *in vivo*, albeit sometimes less efficiently.
3. Though they are thought to be important for vesicle fusion specificity, they might not be the only contributing factors.

However, it is not yet clear if this redundancy in the roles of SNAREs could be due to the quantity at which they are expressed in these tissues or if it is because of their structural attributes.

Recently, using V-2^{-/-}, V-3^{-/-} and V-2/3^{-/-} model systems, Schwarz, et al., showed that though both VAMP-2 and VAMP-3 are expressed in cultured hippocampal astrocytes, their expression is limited to distinct subcellular organelles. VAMP-2 is preferentially found at the cells' periphery while VAMP-3 is scattered throughout the cytoplasm. VAMP-2 is located on glutamatergic vesicles and is responsible for their release while VAMP-3 is associated with endogenous large dense core vesicles/LDCV and contributes to the release of marker protein neuropeptide Y/ NPY from them (Schwarz, Zhao et al. 2017). NPY secretion was unaltered in V-2^{-/-} astrocytes but was

completely abolished in V-3^{-/-} and V-2/3^{-/-} astrocytes. Conversely, glutamate release was affected in V-2^{-/-} and V-2/3^{-/-} astrocytes. Further investigation showed that neuronal glutamatergic signaling was influenced by both VAMP dependent pathways. This report points to a possibility that VAMP isoforms may facilitate opposing pathways to regulate a process.

Subsection Four: Intracellular trafficking in platelets

Intracellular trafficking of vesicles carrying proteins, lipids and various cargo is critical for the growth, function, and survival of cells. This process is crucial for cells to sense (through endocytosis) and react (through exocytosis) to their environment. Platelets contain clathrin-coated vesicles (King and Reed 2002) and endocytose IgG, albumin and fibrinogen (Handagama, Shuman et al. 1989) mainly through integrin $\alpha\text{IIb}\beta\text{3}$ receptors. In fact, Klinger et al., showed that fibrinogen, vWF and fibronectin co-localize with clathrin-coated vesicles (Klinger and Kluter 1995). Platelets also endocytose and recycle purinergic receptors P2Y1 and P2Y12 (Cunningham, Nisar et al. 2013). Behnke showed that fibrinogen (alpha granule marker) is endocytosed through a clathrin-dependent pathway while acid phosphatase (lysosomal marker) is endocytosed through clathrin-independent pathway indicating that human platelets can implement both of these pathways (Behnke 1992).

Clathrin-dependent endocytosis-

Platelets contain dynamin, disabled, and dynamin-related protein which are components of the clathrin-dependent endocytosis machinery. Dynamin 2 is the predominant form present in murine platelets and mutations in dynamin 2 are associated with Charcot- Marie- Tooth disease (Zuchner, Nouredine et al. 2005). Bender et al., used a platelet-specific KO mouse model to study its role in hematopoiesis. These animals have severe thrombocytopenia, macrothrombocytopenia, and increased abundance of clathrin-coated vesicles (Bender, Giannini et al. 2015). Koseoglu et al., showed that dynamin-related protein-1 regulates fusion pore stability during platelet granule secretion (Koseoglu, Dilks et al. 2013).

Clathrin-independent endocytosis-

This mode of endocytosis is further divided into dynamin-dependent (caveolae and/or Rho families of GTPases) and dynamin-independent (Cdc42 and ADP-ribosylation factor (Arf) families of small GTPases). Using a megakaryocyte-specific RhoA deficient mouse model, Pleines et al., showed that RhoA deficiency leads to macrothrombocytopenia and granule secretion defects. RhoA is crucial for integrin-mediated clot retraction but not for platelet spreading on fibrinogen. These animals had a severe hemostatic deficiency but also were protected from arterial thrombosis indicating that RhoA is an essential component of platelet function (Pleines, Hagedorn et al. 2012).

Platelets contain multiple ADP ribosylation factor/Arf isoforms-Arf1, Arf3, and Arf6 (Burkhart, Schumbrutzki et al. 2012). Using a platelet-specific Arf6 KO mouse model Huang et al., showed that fibrinogen uptake was significantly decreased in these platelets in both *in vitro* and *in vivo* analysis. Arf6 deficiency led to increased spreading and faster clot retraction suggesting its role in endocytic trafficking. Arf6 deficiency does not affect platelet secretion, tail bleeding time or thrombosis indicating that it is dispensable for platelet exocytosis. This feature makes these mice an important tool to study endocytosis without affecting exocytosis (Huang, Joshi et al. 2016).

SNAREs in intracellular trafficking in platelets-

The role of SNAREs in vesicular trafficking was first uncovered in yeast studies (Nichols and Pelham 1998). Since then, monumental work has shown that multiple members of SNARE family facilitate almost every stage of endocytosis, trafficking and homotypic and heterotypic fusion events inside diverse types of cells including plants (Fujiwara, Uemura et al. 2014), *Drosophila* (Morelli, Ginefra et al. 2014), and macrophages (Murray, Kay et al. 2005, Murray and Stow 2014).

In platelets, however, the picture is not yet clear. As discussed above, VAMP-8 has been shown to play a role in homotypic early or late endosome fusion events while VAMP-7 is vital for heterotypic endosome to lysosome fusion events. However, animals

deficient in VAMP-8 (Ren, Barber et al. 2007), VAMP-7, (Koseoglu, Peters et al. 2015), syntaxin-8 (Golebiewska, Harper et al. 2015) or munc 13-4 (Ren, Wimmer et al. 2010) did not exhibit any accumulation of transport vesicles or cargo deficiency. Similarly, extensive studies that investigated the roles of t- SNAREs and SNARE regulators did not find any significant cargo level defects (Al Hawas, Ren et al. 2012, Ye, Karim et al. 2012, Karim, Zhang et al. 2013). This suggests that either these proteins may play a role in trafficking but are compensated for by other isoforms. Recently, our lab showed that VAMP-3 plays a major role in endocytosis of fibrinogen and this is crucial for an immunogenic response from platelets (Banerjee, Joshi et al. 2017).

Table 1.3 Summary of various molecules crucial for platelet secretion

Protein/ strain	Dense (%)	ATP Release (%)	Aggregation (%)	Alpha (%)	Lysosome (%)	Bleeding	Thrombosis	Reference
WT	100	100	100	100	100	no	No	
V-3	100	100	100	100	100	no	No	(Schraw, Rutledge et al. 2003)
V-7	70	50	30-40	55	NA	no	No	(Koseoglu, Peters et al. 2015)
V-8 Het	100	100	100	100	100	no	No	(Ren, Barber et al. 2007)
V-8	70	50	80?	30	35	no	Delayed	(Ren, Barber et al. 2007)
V-2/3	100	90	100	100	90	no	No	Joshi <i>et al.</i> (unpublished)
V-8 het2/3	100	90	100	100	90	no	No	Joshi <i>et al.</i> (unpublished)
V-2/3/8	40	30	80	15	20	+++	+++	Joshi <i>et al.</i> (unpublished)
Syntaxin 2/4	100	NA	100	100	100	no	no	(Ye, Karim et al. 2012)
Syntaxin 11	0	0	10-60	0	40	+++	NA	(Ye, Karim et al. 2012)
Syntaxin 8	50	50	70	100	100	no	Embolism increased	(Golebiewska, Harper et al. 2015)

Protein/ strain	Dense (%)	ATP Release (%)	Aggregation (%)	Alpha (%)	Lysosome (%)	Bleeding	Thrombosis	Reference
SNAP 29	NA	100	100	90	NA	NA	NA	(Williams, Savage et al. 2016)
Munc 18b	0	0	NA	0	50	variable	NA	(Al Hawas, Ren et al. 2012)
Munc 13-4	0	0	50	25	25	+++	NA	(Ren, Wimmer et al. 2010)
Tomosyn	90	60	50	50	60	+++	+++	(Ye, Huang et al. 2014)
serglycin	25	NA	50	100	NA	NA	+++	(Woulfe, Lilliendahl et al. 2008)
Alpha-synuclein	90	NA	NA	80	90	No	NA	Whiteheart, unpublished
Rab 27A	100	NA	NA	100	NA	No	NA	(Tolmachova, Abrink et al. 2007)
Rab 27B	76	NA	60	100	NA	+++	NA	(Tolmachova, Abrink et al. 2007)
Rab 27A/B	46	NA	60	100	NA	+++	NA	(Tolmachova, Abrink et al. 2007)
IKK- β	25	NA	NA	30	30	+++	defect	(Karim, Zhang et al. 2013)
Cal DAG	20	NA	10	20	NA	+++	+++	(Piatt, Paul et al. 2016)

Protein/ strain	Dense (%)	ATP Release (%)	Aggregation (%)	Alpha (%)	Lysosome (%)	Bleeding	Thrombosis	Reference
ROCK2	NA	NA	30	60-90	NA	+++	+++	(Sladojevic, Oh et al. 2017)
HPS (pearl, pallid and light ear)	0	0	0	25-30	20	NA	+++	(Meng, Wu et al. 2015)
NBEAL2	100	100	50	50	NA	+++	+++	(Deppermann, Cherpokova et al. 2013)
NBEAL2	NA	NA	70-80	30	NA	NA	NA	(Kahr, Hinckley et al. 2011)
VPS33A	NA	20	30	NA	NA	NA	NA	Suzuki <i>et al</i> , 2003
VPS33B	NA	50	50	NA	NA	+++	+++	(Xiang, Zhang et al. 2015)
VPS33B	NA	70	100	60	NA	+++	+++	(Bem, Smith et al. 2015)
SERT	NA	50	50	NA	NA	NA	NA	(Carneiro, Cook et al. 2008)
SGK-1	50	50	70	NA	NA	normal	+++	(Walker, Schmid et al. 2015)

Subsection Five: SNARE regulatory proteins

SNAREs are essential for membrane fusion, but their associations to form membrane-fusing complexes are controlled temporally and spatially by several types of regulatory proteins and by post-translational modifications of the SNAREs themselves (Figure 1.4 and Table 1.2). Some SNARE regulators are chaperones (*e.g.*, the Sec/Munc proteins), while others promote the apposition of fusing membranes, indirectly (or directly) affecting SNARE association (*e.g.*, Munc13, Rabs, STXBP5/Tomosyn 1, SLP4/granuphilin, *etc.*). These so-called docking factors also affect where fusion occurs.

Munc18b

The Sec1/Munc18 (SM) family of cytosolic proteins is crucial for membrane trafficking and exocytosis. In mammals, the seven SM proteins act as syntaxin chaperones, targeting and directing the t-SNAREs to form specific SNARE complexes (Archbold, Whitten et al. 2014, Rehman, Archbold et al. 2014). Platelets contain VPS33a and 33b, which are involved in dense and alpha-granule biogenesis, respectively (Huizing, Didier et al. 2001, Bem, Smith et al. 2015). Recently, Xiang et al., reported that VPS33b binds with the integrin β subunit and this can potentially regulate α IIb β 3 mediated fibrinogen endocytosis, platelet activation, spreading, clot retraction, platelet aggregation and *in vivo* hemostasis and thrombosis (Xiang, Zhang et al. 2015). Platelets also contain Munc18a, Munc18b, and Munc18c (STXBP1, 2, 3); however, Munc18b is significantly more abundant (Table 1.2 and (Al Hawas, Ren et al. 2012). Previous studies suggested that Munc18c contributes to platelet secretion by interacting with syntaxin-4 (Houng, Polgar et al. 2003); however, platelets from Munc18c^{+/-} mice have normal secretion indicating that partial loss (~30%) of this isoform is insufficient to depress secretion (Schraw, Crawford et al. 2004). Recent studies of FHL type 5 patients, which have defects in the gene encoding Munc18b, show that Munc18b is critical for platelet secretion from all three granule types (Al Hawas, Ren et al. 2012) (Table 1.3). Consistent with Munc18b's role as a chaperone, deficient platelets showed a decrease in syntaxin-11; no other syntaxins were affected. Platelets from biallelic patients have robust secretion defects and heterozygous patients have intermediate deficits. Such

haploinsufficiency suggests that Munc18b is limiting for the secretion process in platelets. Consistently, semi-quantitative western blotting data suggest that syntaxin-11 and SNAP23 are in molar excess over Munc18b in human platelets (Al Hawas, Ren et al. 2012, Ye, Karim et al. 2012). Several reports suggest that the Munc18s are phosphorylated in platelets and that phosphorylation affects Munc18/syntaxin interactions (Reed, Houg et al. 1999, Houg, Polgar et al. 2003, Schraw, Lemons et al. 2003). However, many of the studies focused on Munc18c, whose role in secretion is uncertain. Detailed studies of Munc18b phosphorylation in platelets have not been done.

STXBP5/Tomosyn 1

Another t-SNARE regulator is Syntaxin Binding Protein 5 (STXBP5), also known as Tomosyn 1, meaning friend (tomo) of syntaxin (Fujita, Shirataki et al. 1998). In neurons, STXBP5 is a negative regulator of exocytosis. STXBP5 contains WD-40 repeats, which are thought to interact with the cytoskeleton (Ashery, Bielopolski et al. 2009). C-terminal of these repeats is a variable linker region with multiple predicted phosphorylation sites. Adjacent to the linker is a v-SNARE-like domain that interacts with t-SNARE heterodimers (*e.g.*, syntaxin-11/SNAP23) and is thought to be a regulatory “place-holder”. Consistently in platelets, STXBP5 antibody only precipitated t-SNAREs but no VAMPs (Ye, Huang et al. 2014). Surprisingly, platelet secretion is significantly diminished in platelets lacking STXBP5 (Table 1.3). Mice lacking STXBP5 showed a robust bleeding diathesis that exceeded expectations based on their secretion defect. The bleeding was due to defective platelet secretion based on bone marrow transplantation studies.

A recent GWAS linked polymorphisms in the STXBP5 gene to increased plasma vWF and thus to an increased risk of cardiovascular diseases (Smith, Rice et al. 2011) (van Loon, Leebeek et al. 2010), which conflicts with the phenotype of the KO mice. Lowenstein and colleagues resolved this inconsistency by showing that STXBP5 negatively regulates Weibel Palade Body release from endothelial cells. Consistently, the KO mice had increased plasma vWF (Zhu, Yamakuchi et al. 2014). Interestingly, rs1039084, a non-synonymous single nucleotide polymorphism (SNP) in the STXBP5

gene, correlated with increased bleeding (van Loon, Sanders et al. 2012). Taken together, these data suggest that STXBP5 is a critical regulator of vascular health and plays distinct roles in both endothelial cells and platelets.

Tethering and docking factors

The factors discussed above directly control SNAREs; however, there are other factors that control secretion by affecting docking/tethering of granules to exocytosis sites. Many of these regulators are recruited from the cytosol through interactions with lipids and/or membrane proteins to promote SNARE engagement and enhance membrane fusion. The relevance of granule docking in platelets is understudied; however, dense granules do appear “docked” in resting platelets, which may account for their rapid release (Chicka, Ren et al. 2016).

Rab27

Rab27a/b are small GTPases that direct granule docking and tethering in many secretory systems (Fukuda 2013). In a seminal study using Rab27a^{-/-} (*ashen*), Rab27b^{-/-} and Rab27a/b^{-/-} mice, Seabra and colleagues (Tolmachova, Abrink et al. 2007) showed that only mice homozygous for Rab27b loss had a significant bleeding diathesis. Loss of Rab27a alone affected pigmentation but not bleeding. Detailed studies showed that Rab27b was important for dense granule release, but its loss had no effect on P-selectin exposure (alpha-granule release) (Table 1.3). Rab27b did appear to contribute to dense granule biogenesis since endogenous serotonin levels were lower in Rab27b^{-/-} platelets, irrespective of Rab27a's presence. Since platelets contain both Rab27a and b, it appears that some level of compensation does occur, but it is not complete. Given the interactions between Rab27 and other elements discussed below (*i.e.* SLPs and Munc13-4), this small GTPase is a key to dense granule release in platelets.

Munc13-4

Munc13 proteins are a family of multi-domain proteins, not to be confused with the Munc18 proteins. Munc13s contain a characteristic Munc Homology Domain (MUN) domain and, at least, two calcium/ lipid-binding C2 domains. Some also contain

calmodulin and diacylglycerol (DAG) binding sites (Rizo and Xu 2015). Munc13s have been described as “priming factors” which promote trans-SNARE complex formation (James and Martin 2013, Rizo and Xu 2015). We classify Munc13-4 together with tethering factors due to its effects on platelets and *in vitro* assays, discussed below. Munc13-4 is a known Rab27 effector (Fukuda 2013). *In vitro* studies with liposomes show that Munc13-4 bridges, but does not fuse, membranes in a calcium-dependent manner (Chicka, Ren et al. 2016). This clustering requires both C2 domains. Secretion is dramatically abolished in Munc13-4^{-/-} platelets. Consistently, Munc13-4^{-/-} mice have a significant bleeding diathesis (Ren, Wimmer et al. 2010). Quantitative analysis suggests that Munc13-4 may be limiting (Table I.2), which was confirmed by titration experiments using permeabilized Munc13-4^{-/-} platelets and in Munc13-4^{+/-} mice which had an intermediate phenotype. Loss of Munc13-4 also increased the *in-situ* mobility of dense granules, which is consistent with its role as a docking/tethering factor (Ren, Wimmer et al. 2010). Parenthetically, these results imply that dense granules are pre-docked in resting platelets, perhaps accounting for their rapid release rates.

Although Munc13-4 clearly plays a role in dense granule release, its role in α -granule and lysosome release is less certain. Ren et al., (Ren, Wimmer et al. 2010) showed that the alpha granule and lysosome release defects in Munc13-4^{-/-} platelets are less apparent at higher secretagogue concentrations. Poole and colleagues showed that ADP addition overrides much of the α -granule secretion defect (Harper, van den Bosch et al. 2015). These data underline the fact that dense granule secretion deficiency precipitates defects in α -granule and lysosome release; a point that has been noted by others (Meng, Wu et al. 2015, Sharda, Kim et al. 2015). Thus, the autocrine signaling from released ADP plays a critical role in modulating α -granule and lysosome release.

As in platelets, Munc 13-4 also plays a role in mast cell degranulation (Rodarte, Ramos et al. 2017). Using global and mast cell-specific KO murine models, Rodarte et al showed that Munc 13-4 is a crucial component of mast cell exocytosis that facilitates compound fusion. Although another isoform Munc 13-2 is present and in some degree could compensate for Munc 13-4, it cannot compensate for all its functions.

Synaptotagmin-like proteins

Rab effectors such as synaptotagmin-like-proteins (SLPs) are present in platelets and appear to have both stimulatory and inhibitory roles (Fukuda 2013). Using a yeast two-hybrid assay, Smolenski and colleagues showed that SLP1 forms a trimeric complex with Rap1, a Ras-like GTPase, and the Rap GTPase activating factor RAP1GAP2. SLP1 had a negative effect on dense granule release from permeabilized platelets while RAP1GAP2 addition increased release (Neumuller, Hoffmeister et al. 2009). The same group showed that SLP4/granuphilin interacts with Rab8 in human platelets and its addition to permeabilized platelets enhanced dense granule release (Hampson, O'Connor et al. 2013). This enhancement required SLP4 binding to Rab8. SLP4 is also a Rab27 effector (Fukuda 2013) and interacts with Munc18/Syntaxin complexes in platelets (Ye, Huang et al. 2014). SLP proteins contain calcium/lipid-binding, C2 domains and thus could serve as calcium sensors. Despite these data, a clear mechanistic understanding of their function is still lacking.

Sorting complexes

Tethering/Sorting complexes appear to be important for granule biogenesis (Masliah-Planchon, Darnige et al. 2013); however, it is unclear how such complexes influence exocytosis. One potential example, the Exocyst, is thought to be important for polarized secretion (Liu and Guo 2012). Exocyst is a conserved octameric complex that directly interacts with SNAREs and SM proteins (Wu and Guo 2015). Platelet proteomics studies show that the Exocyst subunits are expressed in stoichiometric amounts (Table 1.2; (Burkhart, Schumbrutzki et al. 2012)). The Exocyst is targeted to mammalian cell membranes *via* an interaction with a prenylated, ras-like GTPase called Ral (Gentry, Martin et al. 2014). Ral is expressed in platelets and is activated to its GTP-bound state following thrombin-treatment (Wolthuis, Franke et al. 1998). There are two Ral isoforms, A and B; both are present in platelets. Horiuchi and colleagues showed that by blocking Ral- GTP binding to the Sec5/Exoc2 subunit, they could inhibit GppNHp-induced dense granule release from permeabilized platelets (Kawato, Shirakawa et al. 2008). Consistently Ral and Exocyst interact in thrombin-stimulated platelets. Despite these

insights, there are many unanswered questions about the roles of Ral and Exocyst. Does the presence of Exocyst imply some polarization of platelet secretion? Ral A and B have distinct functions in other systems (Shirakawa and Horiuchi 2015). Will that be true in platelets as well? Given the development of Ral inhibitors as anti-cancer drugs (Yan, Liu et al. 2014), it seems important that the roles of this protein and the Exocyst be determined to appreciate how these drugs might affect platelet function.

NSF and SNAPs

The first membrane trafficking proteins purified were the AAA+ ATPase, N-ethylmaleimide Sensitive Factor (NSF) and its adaptors, the Soluble NSF Attachment Proteins (SNAPs) (Block, Glick et al. 1988, Clary, Griff et al. 1990). These proteins disassemble spent SNARE complexes, post-fusion, and thus are critical for SNARE recycling (Zhao, Smith et al. 2012). Lemons et al., (Lemons, Chen et al. 1997) first reported their presence in platelets and Polgar et al., (Polgar and Reed 1999) showed that inhibitory peptides that blocked NSF activity also blocked release from permeabilized platelets. Consistently, nitrosylation reversibly inhibits platelet NSF, accounting for a portion of the inhibitory effect of NO on platelet exocytosis (Morrell, Matsushita et al. 2005). Initially, NSF's role was contentious since platelet exocytosis was thought to be a "one-shot" event; thus, there was no need to recycle spent SNAREs. Subsequent studies suggest that platelets have multiple, membrane fusion/ trafficking processes (*i.e.* endocytosis, autophagy, *etc.* (Ouseph, Huang et al. 2015, Huang, Joshi et al. 2016) explaining the presence of NSF and SNAPs. However, their role in recycling SNAREs for exocytosis is still puzzling.

Actin cytoskeleton in platelet secretion

Upon agonist stimulation, platelets change shape before undergoing secretion. These instigated investigations into the role of the platelet cytoskeleton in secretion. Resting platelets contain about 40% filamentous actin/F actin which dramatically increases up to 80% upon stimulation. Multiple reports suggested that the inhibition of actin polymerization by latrunculin A or cytochalasins (Cox 1988, Flaumenhaft, Dilks et al. 2005) or cleavage of F-actin by scinderin leads to increased dense granule (Marcu,

Zhang et al. 1996) and alpha-granule release (Flaumenhaft, Dilks et al. 2005) suggesting inhibitory role of actin polymerization in platelet granule secretion. Contrary to these findings, Woronowicz et al., showed that inhibition of actin polymerization prevents alpha-granule release (Woronowicz, Dilks et al. 2010). t- SNARE SNAP23 associates with the actin cytoskeleton in resting and activated states, however, it is not clear how this association plays a role in secretion.

Post-translational modifications of the secretory machinery

There have been several reports of phosphorylation of platelet secretory machinery components (*e.g.*, syntaxin-4, Munc18, STXBP5, *etc.*), although few have definitively linked modifications to exocytosis control (Houng, Polgar et al. 2003, Polgar, Lane et al. 2003, Schraw, Lemons et al. 2003). One example where phosphorylation clearly affects function is the phosphorylation of SNAP23 by I κ B kinase (IKK). Originally seen in mast cells, IKK phosphorylates specific serines (Ser95) on SNAP23, which affects SNARE complex dynamics (Cabaniols, Ravichandran et al. 1999, Hepp, Puri et al. 2005, Puri and Roche 2008, Suzuki and Verma 2008, Karim, Zhang et al. 2013).

Consistently, platelet-specific deletion of IKK β or treatment with IKK inhibitors blocks platelet secretion from all three granules and leads to a bleeding diathesis (Karim, Zhang et al. 2013). *In vitro* studies with SNARE-containing proteoliposomes suggest that SNAP23 phosphorylation enhances membrane fusion rates, though it is not required for fusion. Immunoprecipitation studies show that phospho-SNAP23 preferentially incorporates into SNARE complexes in activated platelets.

Another noteworthy post-translational modification is acylation. Proteomics studies had identified a number of acylated proteins in platelets that have thioester-linked fatty acids attached to cysteines (Dowal, Yang et al. 2011). This is particularly important to syntaxin-11 and SNAP23, which lack classical transmembrane domains (TMD) but behave as membrane proteins. Both t-SNAREs contain cysteine-rich domains (at the C-terminus for syntaxin-11 and between the two SNARE domains in SNAP23) that are thought to be acylated. Consistently, treatment of platelet membranes with a

thioesterase releases SNAP-23 and affects secretion in permeabilized platelets (Sim, Dilks et al. 2007). The significance of t-SNARE acylation is yet to be understood; however, since neither SNARE has a classical TMD, acylation must affect membrane fusogenicity. Studies using reconstituted proteoliposome fusion assays show that t-SNARE TMDs can be functionally replaced by lipid moieties so long as they are hydrophobic enough. C15 prenyl groups are not sufficiently hydrophobic but C55 groups are (McNew, Weber et al. 2000). Both SNAP23 and syntaxin-11 have multiple potential acylation sites; however, it remains to be determined the extent to which t-SNARE acylation occurs and is controlled in platelets. In other cell types, removal or modification of the t-SNARE cysteine-rich domains does affect secretion (Valdez, Cabaniols et al. 1999, Salaun, Gould et al. 2005).

A recent study by Malmersjo and coworkers suggest that in mast cells, VAMP-8 is phosphorylated by PKC (Malmersjo, Di Palma et al. 2016). This phosphorylation occurs at multiple residues in a SNARE domain of VAMP-8 and is inhibitory to *in vitro* and *in vivo* fusion events. Interestingly, the authors also report that these phosphorylation motifs are absent in all eukaryotic neuronal VAMPs but present in all non-neuronal ones indicating a special secretion regulatory mechanism in non-neuronal cells.

Subsection Six: Importance of platelet secretion

As discussed in earlier sections, platelet granule secretion is a critical central event that shapes thrombus composition downstream. The importance of this event is highlighted by following facts:

1. Granulopathies- As discussed in detail in previous sections, dense (CHS and HPS) granulopathies present a severe bleeding defect while alpha (GPS and ARC) granulopathies display a wide array of bleeding diathesis ranging from mild to severe defects. This demonstrates that the lack of granule cargo or the failure to release these cargoes does have a significant impact on hemostatic balance.
2. SNPs in VAMPs – Single nucleotide polymorphisms in the VAMP-8 gene may potentially affect its expression and are associated with hyperreactive platelets. These

events are associated with high susceptibility to acute myocardial infarction. This suggests that increased secretion by hyperreactive platelets could skew the hemostatic balance leading to hyper-thrombotic events.

3. Immunological response- Multiple reports indicate that platelets act as immune cells (Semple, Italiano et al. 2011, Manne, Xiang et al. 2017) by sensing their micro-environment by endocytosis and take up viral particles (Simon, Sutherland et al. 2015) and bacteria (Gaertner, Ahmad et al. 2017). Consequently, the release of cytokines and chemokines from alpha granules and P-selectin exposure help recruit professional phagocytic cells to clear up the infection (Etulain, Martinod et al. 2015). Evidently, secretion-deficient platelets fail to generate this immune response underlining the significance of platelet secretion in immunity and infection (Hwaiz, Rahman et al. 2015). In fact, antiplatelet therapies are being tapped to modulate platelet-leukocyte immune responses (Evangelista, Manarini et al. 2005).

4. B cell sialylation- Increasing evidence suggests that the impact of platelet secretion may not be limited to only hemostasis and thrombosis. Platelet secretion does play a role in facilitating B cell-independent sialylation of IgG by providing necessary substrate (CMP-sialic acid) (Jones, Oswald et al. 2016).

Subsection Seven: Future directions

Though the relevance of SNAREs is established, the protein-protein interactions leading to membrane fusion are not completely defined in platelets. Examples are dynamin and the dynamin-related proteins, which affect fusion pore opening and thus cargo release (Koseoglu, Dilks et al. 2013). Clearly, there are more secretory machine elements yet to be identified, especially given that we have not filled the gaps between known signaling cascades and secretory machinery.

As implied by the phenotypes of the platelets lacking specific SNAREs (V-7^{-/-}, V-8^{-/-}, syntaxin-8^{-/-}, and syntaxin-11/ FHL4^{-/-}), it seems possible that more than one SNARE complex may mediate granule release. Future studies should focus on determining if different SNARE combinations convey distinct properties to specific membrane fusion

events. It seems possible that some complexes will be more fusogenic than others and thus could fine-tune release kinetics by facilitating single or compound fusion event, preferentially and perhaps alter platelet releasate composition.

A major question is, “How is secretion controlled by calcium?”. Calcium is critical to most regulated secretory processes; yet in platelets, there are no clear calcium sensors that affect secretion. Synaptotagmins, which are calcium sensors in neurons have not been characterized in platelets (Rizo and Xu 2015). Platelets contain several proteins which contain calcium-binding domains (C2 domains like synaptotagmins) or can be phosphorylated by Protein Kinase C. However, direct mechanistic data are lacking.

A second consideration is whether platelet exocytosis is polarized. While at first glance, it may seem unlikely, there is clear polarity once platelets are adherent. Given the core and shell architecture of a growing thrombus (Welsh, Stalker et al. 2014), it's plausible that cargo release is oriented: either toward the vascular wall to affect wound healing or toward the vessel lumen to recruit more platelets. Sakurai et al., showed that platelet secretion orients to the boundary of a fibrinogen-coated micro-dot to facilitate spreading beyond the dot's edge (Sakurai, Fitch-Tewfik et al. 2015). Additionally, in an elegant study, Gaertner and colleagues showed that platelets actively migrate at the site of vascular injury and this ability is crucial for their ‘immune cell’ like behavior to collect bacteria and aid neutrophil activation (Gaertner, Ahmad et al. 2017). In the context of VAMPs, the work of Peters et al., (Peters, Michelson et al. 2012) clearly shows that VAMP-8- and VAMP-7-positive granules spatially segregate; is that to mediate different release events? Given VAMP-7's role in spreading and the importance of Ral and Exocyst, perhaps the answer is yes.

Finally, are granules the only things secreted from platelets? Cargoes that are generally thought to be markers of cellular organelles (*i.e.*, sugar nucleotides from Golgi; Protein Disulfide Isomerase from ER; *etc.*) are detected in platelet releasates and are functionally relevant (Wandall, Rumjantseva et al. 2012, Lee, Nasirikenari et al. 2014,

Crescente, Pluthero et al. 2016). Do these release events use the same machinery as granules? These are exciting questions that get to the very heart of platelet cell biology and its importance. Their answers are destined to change our views of platelet function and hemostasis.

The focus of the dissertation

Platelets are crucial for a multitude of pathophysiological processes including hemostasis, thrombosis, wound healing, angiogenesis, microbial defenses, and immunity, among others. Platelets participate in these processes by releasing their granule cargo to modulate the vascular microenvironment. Though a plethora of molecules has been identified and credited for platelet secretion (Table 1.3), it remains to be seen how secretion takes place on a molecular level and how much secretion is sufficient to achieve an ideal hemostatic balance. The work presented in this dissertation addresses these questions. The first part of the dissertation discusses generation and characterization of platelet-specific VAMP KO mouse strains and how defective platelet secretion affects hemostasis. By titrating amounts and types of VAMPs in platelets we report for the first time how much secretion is necessary to prevent occlusive thrombosis without causing unwanted bleeding. This is the first quantitative study to present a therapeutic range that will help identify an optimal hemostatic balance. Moreover, these reagents are valuable for future studies to determine the contribution of platelets in various physiological and pathological processes.

In the second part of the study, we examined in detail how the rates of alpha-granule cargo are regulated. These rates decide the fate of thrombus composition and therefore should be controlled dynamically. Here using high-pressure freeze technology and 3D SIM, we show that progressive dissolution of cargo in time and agonist concentration-dependent manner affects platelet alpha-granule morphology during the fusion process. We also demonstrate that the degranulation is VAMP-dependent and follows granule fusion.

This is a first comprehensive study investigating the molecular nature of platelet granule fusion and the regulation of granule cargo release along with physiological implications of this process on maintaining optimal hemostatic balance. Insights gleaned from this study are not only valuable to develop anti-platelet therapeutics but are also

crucial to determine the efficacy of these therapies on platelets and to determine the optimal dosage of medicine to maintain hemostatic balance.

Chapter 2 Materials and Methods

Materials

Reagents

Apyrase, human fibrinogen, hirudin, and proteinase K were from Sigma (St. Louis, Mo). Thrombin, ADP, and CHRONO-LUME reagents were from Chronolog (Havertown, PA). U46619 and prostaglandin I₂ (PGI₂) was from Cayman (Ann Arbor, MI). Paraformaldehyde (P6148, Sigma-Aldrich), 70% glutaraldehyde was from Electron Microscopy Sciences (Hatfield, PA). Other reagents used were at least laboratory grade.

Antibodies

Mouse monoclonal antibodies against FITC-anti-CD62P and PE-anti-LAMP1 antibody were from BD Biosciences (San Jose, CA). Rabbit anti-VMAT2 antibody, was from Abcam (Cambridge, MA). FITC-anti-CD41/CD61 antibody and PE-Jon/A antibody were from Emfret Analytics (Eibelstadt, Germany). Rabbit anti-fibrinogen antibody was from Dako (Glostrup, Denmark). Rabbit anti-RabGDI polyclonal antibody was generated by Dr. Tara Rutledge in our laboratory using recombinant RabGDI α as antigen (Rutledge and Whiteheart 2002). The anti-SNAP23 antibody, (Schraw, Rutledge et al. 2003) anti-Arf 6 (Choi, Karim et al. 2006), and polyclonal anti-VAMP-8 (Ren, Barber et al. 2007) were as described previously. Monoclonal anti-VAMP-2 antibody (SP10) (catalog no NBP1-19332), Polyclonal anti-VAMP7 antibody (catalog no NBP2-41183), Polyclonal anti-VAMP-3 antibody (catalog no NBP 300-510) were from Novusbio (Littleton, CO).

Methods

Murine Strains and Genotyping

The following mice were used for the experiments described in this thesis.

WT mice (C57BL/6 background), initially purchased from Jackson Laboratory and bred in our animal vivarium.

Global VAMP-3^{-/-} mice

These mice were bred and genotyped as described (Schraw, Rutledge et al. 2003).

Forward primer, (3F) 5'-CACAGGCACTCTGTTGCATT-3',

Reverse primer, (6R) 5'-CCACACAGGCTCCTGATCTT-3',

Neo primer, (mutant allele) 5'-GAGCAGCCGATTGTCTGTTG-3'.

The PCR reactions were run for WT (with 3F WT and 6R reverse) and for KO (with Neo and 6R reverse). The PCR yields a 500 bp DNA product for WT allele and a 750 bp one for the mutant allele.

The PCR conditions for VAMP-3 reactions were: 94°C for 7 min for 1 cycle, followed by 30 cycles of 94°C for 1 min, 60°C for 1 min and 72°C for 90 sec, and lastly 72°C for 10 min for 1 cycle.

Global VAMP8 -/- mice

These mice were a generous gift from Dr. Wanjin Hong (Institute of Molecular and Cellular Biology, Proteas, Singapore) and were bred and genotyped as described in (Wang, Ng et al. 2004). These mice were on a mixed background of 129 SvJ and C57/BL6.

Primers and genotyping conditions –

Forward primer- 5' -GATCCTCTGCTGCCCGCCTAAT-3' and

Reverse primer (WT allele)- 5' -CCGTGGCTTCCAAGTCCTCTGTC-3')

Reverse primer (mutant allele) (5' -GCCTGCAAAGGGTCGCTACAGAC-3').

The PCR reactions were 95°C for 7:00 min for 1 cycle, 95°C for 0:30 min, 65°C for 0:30 min, 72°C for 1:00 min for 28 cycles, finally 72°C for 10:00 min for 1 cycle

The PCR products for wild-type allele and mutant allele were 204 bp and 323 bp, respectively.

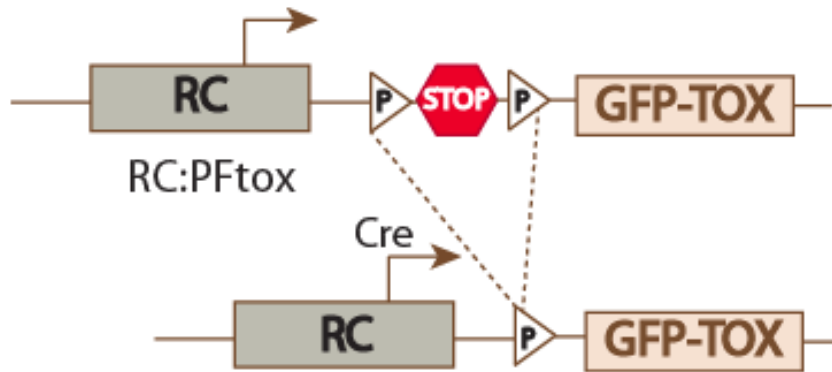


Figure 2.1. Conditional expression of tetanus toxin light chain

Conditional expression of Cre recombinase under control of megakaryocyte-specific PF4 promoter facilitated removal of floxed stop cassette upstream of GFP- tetanus toxin light chain.

(Schematic based on (Kim, Cook et al. 2009) RC- enhancer sequences used R26 and CAG. PF-Parallel fibers)

Platelet-specific VAMP-2/3^{-/-} strain

To specifically delete VAMP-2 and -3, we crossed a RC::PFtoX strain from Dr. S. Dymecki (Harvard Medical School) with a PF4-Cre strain (from Dr. R. Skoda, Basel, (Tiedt, Schomber et al. 2007)) The RC::PFtoX strain has a genomic insertion encoding the catalytic subunit of the tetanus toxin endopeptidase, which can be expressed upon Cre-mediated excision of a STOP cassette. The STOP cassette consists of the 3' portion of the yeast His3 gene, an SV40 polyadenylation sequence, and a false translation initiation codon followed by a 5' splice donor site. The floxed STOP cassette is inserted between the promoter and tetanus toxin coding sequences of a transgene, ensuring that few, if any, transcripts containing the coding region are generated (Figure 2.1). Tetanus toxin specifically cleaves only VAMP-2 (Schiavo, Benfenati et al. 1992) and -3 (McMahon, Ushkaryov et al. 1993) This strategy was needed because VAMP-2^{-/-} mice are embryonic lethal (Schoch, Deak et al. 2001) as are VAMP-3/8^{-/-} mice (Whiteheart unpublished). These RC::PFtoX/PF4Cre mice (referred to as V-2/3^{-/-} in this manuscript) were further crossed with a VAMP-8^{-/-} strain (referred to as V-8^{-/-}) to create a RC::PFtoX/PF4Cre/VAMP-8^{-/-} strain (referred to as V-2/3/8^{-/-}).

Tetanus toxin harboring strain

Platelet-specific conditional KO was made according to the strategy described by (Kim, Cook et al. 2009) (Figure 2.1). These mice were a generous gift from Dr. Susan M. Dymecki (Dept. of Genetics, Harvard Medical school).

Primers and genotyping conditions-

Forward primer -5' GCCGATCACCATCAACAACTTC 3'

Reverse primer-5' GCAGAGCTTCACCAGCAACG 3'

The PCR reactions were 94°C for 4:00 min for 1 cycle, 94°C for 0:30 min, 58°C for 0:30 min, 72°C for 1:00 min for 35 cycles, finally 72°C for 5:00 min for 1 cycle

PCR reaction yielding about 300 bp DNA production indicates the presence of tetanus toxin light chain.

Platelet factor 4 (PF4)/cre recombinase harboring strain

These mice were a kind gift from Dr. Radek Skoda (University Hospital Basel, Switzerland) (Tiedt, Schomber et al. 2007).

Primers and genotyping conditions-

Forward primer -5' CCCATACAGCACACCTTTTG 3'

Reverse primer -5' TGCACAGTCAGCAGGTT 3'

PCR reaction conditions were- 94°C for 7:00 min for 1 cycle, followed by 30 cycles of 94°C for 1:00 min, 50° C for 1:00 min, 72°C for 1:30 min, and finally 72°C for 10:00 min for 1 cycle.

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

V-2/3/8^{-/-} mice

These mice were generated by a cross between platelet-specific V-2/3^{-/-} and V-8^{-/-} mice.

Genomic DNA isolation from mouse tail tip

Mouse tail tips (3-5 mm) from 3-4 weeks old weanlings were cut and digested overnight (O/N) in 400 µl of tail lysis buffer (50 µM Tris/HCl, pH 7.5, 100 µM EDTA, 100 µM NaCl, and 1% SDS) containing 30 µl of 10 mg/mL proteinase K at 55° C.

After digestion was completed, the solution was mixed with 200 µl saturated 6 M NaCl and centrifuged at 13,000 x g for 30 min at room temperature (RT). The supernatant containing DNA was collected and mixed with equal volume of 100% ethanol. After incubation for 10 min at RT, DNA was pelleted down by centrifugation at 13,000 x g for 10 min. DNA pellets were washed once with 1 mL 70% ethanol and then dried in a vacuum centrifuge for around 10 min. Genomic DNA was finally dissolved in 50 µl autoclaved ddH₂O.

Platelet preparation from mouse blood

Mice were sacrificed by CO₂ inhalation. About 120 µl of 3.8% sodium citrate in a 1 mL syringe attached to 26 G needle was used to collect blood from the right atrium (final concentration 0.38%). Collected blood samples were diluted with 1:1 1X filtered PBS. Apyrase (0.2 U/mL) and PGI₂ (10 ng/mL) was added and blood was incubated for 5 min at RT. The samples were then centrifuged at 237 x g at RT. Platelet Rich Plasma (PRP) was collected, apyrase and PGI₂ were supplemented and centrifuged at 657 x g to get platelet pellet. For secretion assays, PRP was incubated with 1uCi [³H] serotonin for 30 min. Platelet pellet was resuspended in HEPES Tyrode buffer pH 6.5 (10 mM HEPES/NaOH, 5.56 mM glucose, 137 mM NaCl, 12 mM NaHCO₃, 2.7 mM KCl, 0.36 mM KH₂PO₄, 1 mM MgCl₂) with 0.2U/mL apyrase, 10 ng/mL PGI₂, 1mM EGTA and centrifuged again at 657 x g to recover platelets. The platelet pellet was resuspended in HEPES Tyrode buffer pH 7.4. Platelet concentrations were measured using a Z2 Counter (Beckman Coulter, Inc., Miami, FL).

Whole blood count to study hematological parameters

Blood was drawn via cardiac puncture from KO and control WT littermates in 3.8 % sodium citrate as described above and whole blood counts were performed using a Hemavet (Drew Scientific, Dallas, TX). Statistical analyses were done using the statistical software program in SigmaPlot (v13.0).

Platelet counting

Washed platelets prepared as described above were diluted 1: 20,000 in HEPES Tyrode's buffer (pH 7.4). The platelet counts were measured in triplicates using a Z2 Coulter particle counter and size analyzer (Beckman Coulter, Brea, CA). The final count was an average of triplicates. The concentration of platelets was adjusted as indicated in each experiment.

Secretion measurement from mouse platelets

To measure secretion, mouse platelets were collected and isolated as described above with incubation with [³H] serotonin (Perkin-Elmer Cetus Life Sciences, Boston,

MA). Platelet stock was made at a concentration of 2.5×10^8 /mL and 0.7 mM CaCl₂ was supplemented. After at least 5 min after adding CaCl₂, 50 µl of this stock was aliquoted in 1.5 mL Eppendorf tubes. Platelets were stimulated with either different concentrations of thrombin (0.01, 0.05, 0.1, 0.5 U/mL) or different time points (15, 30, 45, 60, 90, 120, and 300 sec). Hirudin (2X the concentration of thrombin) was used to stop the reaction. Samples were centrifuged at 16.2 x g for 2 min on tabletop centrifuge machine. The supernatant was removed to another tube and the pellet was incubated with 60 µL of lysis buffer (1% Triton X 100% in 1X PBS) for 45 min on ice.

Dense granule secretion measurement-

To measure secretion from dense granules, 25 µL of supernatant or pellet samples were added to 3 mL of scintillation cocktail (Econo-safe™, Research Products International Corp, Mt. Prospect, IL) in vials. Protocol 16 was used to measure secretion from all the samples in a Tri-Carb 2100TR liquid scintillation analyzer (Beckman, Fullerton, CA).

Alpha granule secretion measurement-

To measure secretion from alpha granules, a commercial sandwich ELISA assay was performed as per the manufacturer's instructions (R&D). Briefly, clear ELISA plates (Costar, #3369, Fisher Scientific) were coated with capture antibody against mouse PF4 (concentration of antibody) overnight at RT. The next day, plates were washed and blocked with blocking buffer (1x PBS + 0.05% Tween twenty+ 1% BSA+5% sucrose) for an hour. After washes, diluted supernatant and pellet samples (reagent diluent recipe) were added to the plate and incubated at RT for two hours. Recombinant mouse PF4 was plated in serial dilution to make a standard curve, with a maximum concentration of 2 ng/mL. After washes, the plate was incubated with biotinylated detection antibody at RT for two hours. After washes, streptavidin-conjugated horseradish peroxidase was used to detect signal. Release was measured as percent release= [(amount in supernatant or pellet)/ (amount in supernatant + amount in pellet)] *100.

Lysosomal secretion measurement

A colorimetric assay was used to measure the activity of β -hexosaminidase which is a soluble cargo in lysosomes on PNP gluconic substrate. Briefly, 6 μ L of supernatant or 3 μ L of pellet sample was mixed with 100 μ L of citrate phosphate buffer (53.4 mM citric acid, 93.2 mM Na_2HPO_4 , pH 4.5) containing 10 mM p-nitrophenyl-N-acetyl- β -D-glucosaminide. These samples were incubated at 37°C in a parafilm sealed, 96 well plate for 18 hrs. These reactions were then stopped with 100 μ L of 2 M NaOH. Using a Biotek Elx808 plate reader (BioTek Instruments Inc., Winooski, VT), the optical density of each well was measured at 405 nm. Release was measured as percent release = [(amount in supernatant or pellet)/ (amount in supernatant + amount in pellet)] *100.

Platelet aggregometry

Aggregation of washed platelets prepared as described above was measured using a Chrono-Log Model 460VS Lumi-aggregometer (Havertown, PA). Mice platelets (250 μ L of 4×10^8 /mL) were placed in siliconized glass cuvette (Chrono-log) with a metal stirring bar (Chrono-log), spinning at 800 rpm for 2-3 min at 37°C. Agonists were added to initiate platelet activation as indicated. The aggregation traces were monitored using Model 810 Aggro/Link computer interface and Aggro/Link software (Chrono-log).

Platelet ATP release

Agonist-stimulated ATP release from platelet dense-core granules was monitored by the luciferin/ATP-mediated luminescence using a Chrono-Log Model 460VS Lumi-aggregometer (Havertown, PA). Washed murine platelets (250 μ L, 4×10^8 /mL) were preincubated with 12.5 μ L of Chrono-Lume reagent (Chrono-log) containing luciferin and luciferase for 2 min at 37°C. Addition of thrombin, collagen (Chrono-Log), or A2317 (Calbiochem, La Jolla, CA), initiated ATP release from platelets, ATP-driven activation of luciferase, followed by production of luciferin which was monitored by absorption at 560 nm. Luminescence traces along with aggregation were using the same interface and software.

Flow cytometry Analysis

Washed murine platelets ($20 \mu\text{L}$ of $1 \times 10^8 / \text{mL}$) were either in a resting state (no agonist) or were stimulated with thrombin (0.1 U/mL) for 2 min at room temperature/RT. The reaction was stopped with the addition of a 2-fold excess of hirudin. Platelets were incubated with $2.5 \mu\text{L}$ FITC-conjugated or PE-conjugated antibodies for 15-20 min at RT. The platelets were 10-fold diluted by adding HEPES-Tyrode's buffer (pH 6.5) and transferred to a polystyrene Falcon™ tube (BD Biosciences, San Jose, CA). Fluorescent intensity was measured using FACScan™ flow cytometer (BD Biosciences). The platelet population was detected by adjusting the voltages for forward light scattering (FSC; E-01/linear) and side light scattering (SSC; 450/linear). Fluorescent intensity was optimized by adjusting the voltages for excitation of blue (FITC) and red (PE) channels. Platelet fluorescent intensities were monitored using an acquisition and analysis application, CellQuest™ (BD Biosciences). A total of 10,000 events were analyzed and fluorescent intensities were plotted as a histogram with statistical values.

Western blotting and quantification

Mouse platelets were prepared from the indicated strains as described above. After adjusting to $1 \times 10^9 / \text{mL}$, an equal volume of 2X SDS was added and the samples were boiled at 95°C for 5 min. The appropriate volume of this stock was loaded to achieve 5×10^7 platelets per well onto a 1.5 mm 10% or 12.5% SDS-PAGE gel and the separated proteins were transferred to Immobilon- P polyvinylidene fluoride (PVDF) membranes (Millipore Corp., Bedford, MA) for 1 hr at 100 V. The PVDF membrane containing the transferred proteins was first incubated with the blocking buffer (5% nonfat milk in TBST (25 mM Tris, 150 mM NaCl, 0.1% Tween-20, and pH 7.4)) for 1 hr. at RT and then in the indicated dilution of primary antibody (in Blocking Buffer) for overnight at 4°C . The membrane was washed with TBS-T - 10 min for 3 times and incubated with the 1:20,000 dilution of alkaline phosphatase -conjugated secondary antibody for 1 hr. at RT. After washing the membrane with TBS-T, Vista- ECF substrate (Amersham Biosciences) was incubated with the membrane for 5 min at RT. The proteins were visualized with a Typhoon 9400 scanner using excitation at 488 nm and

measuring emission at 532 nm and quantified using ImageQuantTL software (v 7.0, GE Healthcare).

Antibody Arrays

Proteome Profiler Mouse Angiogenesis Antibody Array (ARY 015; R&D Systems) was used according to the manufacturer's instructions. Briefly, the nitrocellulose membranes were first incubated in blocking buffer for 1 hr. Platelet extracts (from 10^8 platelets) were prepared in 1 ml lysis buffer (1% NP-40; 20 mM Tris-HCl, pH 8.0; 137 mM NaCl; 10% glycerol; 2 mM EDTA; and 10× protease inhibitor cocktail) and mixed with a cocktail of biotin-labeled detection antibodies against different individual angiogenesis-related proteins. After incubation for 1 hr., the mixture of the platelet sample and detection antibodies were then incubated with nitrocellulose membranes O/N at 4°C. The membranes were washed 3 times with wash buffer and incubated with HRP-conjugated streptavidin for 30 minutes. After washing the membranes 3 times, the signals for each array spot were detected with Supersignal ELISA Femto Maximum Sensitivity Substrate (Thermo-Scientific) using a ChemiDoc MP System (BioRad). The array experiments were run in duplicate. Fluorescence intensities for each spot were measured, the background was subtracted, and the KO/WT ratio was calculated for each strain and plotted using SigmaPlot (version 13.0; Systat Software Inc.).

Spreading Assays

Human fibrinogen-coated slides were prepared by incubating human fibrinogen (50 µg/mL, Enzyme Research Laboratory) in 1X filtered PBS onto Nunc Lab - Tek II 16 well chamber slide (Thermo Scientific) for overnight at 4°C. The slide was incubated with denatured 2% FBS (boiled for 10 min at 95°C) for at least one hr. Washed mouse platelets (2×10^7 /mL) were prepared as explained earlier and resuspended in HEPES Tyrode buffer (pH 7.4) supplemented with 1 mM CaCl₂. Platelet-poor plasma (1:100) was added in washed platelets before seeding the platelet suspension on the fibrinogen-coated slide. The plate was incubated at 37° C. After each time point (as indicated in figures- 30, 60, 90, 120 min), unbound platelets were removed by washing twice with HT 7.4 and spread platelets were fixed using 4% paraformaldehyde (in 1 X

PBS) at 4°C for overnight. The next day, after two washes with HT 7.4, plastic sealing from the slide is removed with forceps. VectaShield preservative (Vector Laboratories) was mounted on a slide and Lab- Tek cover glass is placed. The cover glass was sealed with nail polish and samples were visualized using Nikon Eclipse E600 microscope (Nikon, Melville, NY) with a 100 X /1.40 Numeric aperture DIC H oil objective lens (Nikon). ImageJ (v1.48 NIH) software is used to measure the area of spread platelets.

Tail Bleeding Assay

Tail bleeding time was determined as described by (Ye, Huang et al. 2014). Briefly, 6-8-week-old mice were anesthetized using ketamine 75 mg/kg *i.p.* Anesthetized animals were kept in a 50 mL conical tube which was perforated for ventilation. A transverse incision was made with a scalpel at a position around 3 mm from the tip of the murine tail. To avoid operational error, the same person transected tails from all the animals. After incision, the tail was immediately immersed into a 15 mL tube filled with 37°C normal saline. The time from incision to bleeding cessation was recorded as the bleeding time. After initial cessation of bleeding, mice were observed for additional 1 min to exclude re-bleeding. Tails of animals with bleeding times greater than 10 min were stopped manually by applying pressure.

FeCl₃-induced arterial thrombosis assay

FeCl₃-induced arterial thrombosis is a method to analyze platelet functional defects *in vivo*. This model is believed to be one of the better models to mimic endothelial denudation because it generates a controlled and localized vascular damage that the platelets respond to (Hechler, Nonne et al. 2010). Briefly, 8-12 weeks old mice were anesthetized with Avertin (0.2 g/kg, *i.p.*) in a supine position on a 37°C heating pad. The toe-pinch reflex was used to monitor the anesthesia state. The left carotid artery was exposed using blunt dissection under a dissecting microscope. A miniature Doppler flow probe (0.5VB, Transonic system Inc., Ithaca, NY, USA) was placed on the carotid artery to monitor blood flow. The position of the probe was adjusted to record maximum flow. After baseline readings, the area was dried to prevent dispersion of the FeCl₃ solution from filter paper to neighboring tissue. Thrombus formation was induced

by placing a round filter paper (1 mm diameter) saturated with 5% FeCl₃ solution (1 μL) in 1X PBS on the top of the vessel for 3 min. After 3 min, the paper was removed, and saline was added to facilitate flow. The time from the application of ferric chloride to the cessation of blood flow was recorded. The recording was stopped after 30 min of application of ferric chloride. To monitor the stability of thrombus, fluctuations in blood flow were monitored. A progressive decrease in blood flow followed by a sudden increase in flow was characterized as a potential indicator of unstable thrombus at the site of injury.

Coagulation assays

Mice platelets were harvested from whole blood as described above. Platelet-poor plasma was collected and preserved at -80°C. This platelet poor plasma was used to measure Prothrombin time (catalog number 5248) and activated Partial Thromboplastin Time (catalog number 5389) as per manufacturer's instructions (Helena Laboratories, Beaumont, Texas).

Electron microscopy studies of mice platelets

For a resting sample, blood was drawn in a syringe prefilled with Acid Citrate Dextrose/ACD (12.5 gm trisodium citrate dehydrate+ 7 gm citric acid+ 10 gm anhydrous D (+) glucose in 500 mL of distilled dH₂O) and an equal volume of 2X fixative (6% PFA and 0.2% glutaraldehyde in 1x PBS). The blood was fixed at RT for 20 min before collecting Platelet Rich Plasma (PRP) by centrifugation at 237 x g for 8 min in Sorvall legend RT centrifuge (Thermo Scientific). PRP was then centrifuged at 695 x g for 10 min to collect platelets and the second fixation was performed with 2.5% glutaraldehyde for 20 min at RT.

For the WT stimulation series, washed platelets were supplemented with 0.7 mM CaCl₂. Resting samples were fixed with 2X fixative (2% PFA and 5% glutaraldehyde). The activation samples were stimulated with 1U/mL thrombin to achieve a final concentration of 0.1 U/mL. The reaction was stopped at various time points (10, 20, 30, 60, 90, 120 and 300 sec) by adding an equal volume of 2X fixative to attain a final

concentration of 1% PFA and 2.5% glutaraldehyde. The samples were fixed for 30 min at RT. After fixation, platelets were centrifuged at 695 x g and the platelet pellet was resuspended in HEPES Tyrode's buffer (pH 7.4).

For morphology study of VAMP KO mutant platelets, experimental sample platelets were stimulated with 1 U/mL thrombin to achieve 0.1 U/mL final concentrations. The reactions were stopped at either 90 or 300 sec by adding 2X fixative. Platelets were fixed at RT for 30 min and were recovered by spinning at 695 x g for 10 min. Fixed platelets were resuspended in a small volume of HEPES Tyrode's buffer (pH 7.4) and shipped on ice to the Storrie lab for EM analysis.

The following procedures were performed by Irina Pokrovskaya at University of Arkansas Medical Sciences, Little Rock, AR.

High-pressure freezing (HPF)/Freeze Substitution (FS)

Following fixation platelet preparations were washed 3 x 5 min with PBS and resuspended in PBS containing 2% ultra-low gel agarose (Sigma, St. Louis, MO). Platelets were then frozen using a Leica EM PACT2 high-pressure freezer with rapid transfer system at high pressure (2,100 bar) and further processed by FS.

Freeze substitution dehydration

Samples were transferred under liquid nitrogen to cryovials containing 2% osmium tetroxide/0.1% glutaraldehyde/1% H₂O in acetone. Samples were freeze substituted in a Leica EM AFS2 freeze substitution and low-temperature embedding system under the following schedule: -90° C for 22 hrs., warm 3°C/hr. to -60°C, -60°C for 8 hr., warm 3°C/hr. to -30°C, -30°C for 8 hrs., warm 3°C/hr. to 0°C. Following freeze substitution, samples washed 3 x 10 min in acetone followed by tannic acid at 4°C (1% tannic acid with 1% H₂O in acetone) for 1 hr. Samples were washed 3 x 10 min in acetone followed by 1 hr. osmium wash (1% osmium tetroxide/1% H₂O solution in acetone) at 4°C. Samples were washed 3 x 10 min in acetone and dehydrated over a series of ethanol gradations (25%, 50%, 75%, 100%) using automatic resin infiltration protocol for PELCO Bio-Wave Pro laboratory microwave system. Specimens final

polymerization was done at 60°C for 48 hr. in a regular oven.

Thin section TEM

Thin sections were cut at a thickness of 50 nm and post-stained with uranyl acetate and lead citrate (EMS).

Electron microscopy and image handling

Samples were imaged using a FEI Tecnai TF20 intermediate-voltage electron microscope operated at 80 keV (FEI Co.). Images were acquired with a FEI Eagle 4k digital camera controlled with FEI software.

Semi-thick section transmission electron tomography

Samples were sectioned at 300 nm and post-stained with uranyl acetate and lead citrate, followed by carbon coating. 15 nm gold (EMS) was applied to each side of the grids. Samples were imaged using a FEI Tecnai TF20, 200 keV field-emission gun, using SerialEM software (University of Colorado). Dual axis tilt series were collected $\pm 60^\circ$ with a tilt increment of 1° . Images were taken at 7800x, pixel size 1.42 nm.

Tomogram reconstructions

Reconstructions were computed using weighted back projection (IMOD software, University of Colorado). STEM tomograms were binned by 2 and post-processed with an anisotropic diffusion filter.

Statistical Analysis

All statistical analysis was performed using Student's t-test using Sigma Plot (version 13.0; Systat Software Inc.). P value less than 0.05 was considered significant

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

Study Approval

All animal work was approved by the Institutional Animal Care and Use Committee at the University of Kentucky.

Chapter 3 Fine-tuning hemostasis and thrombosis *by altering* platelet secretion

Introduction

In addition to hemostasis, blood platelets also play a central role in a myriad of other conditions, including angiogenesis, wound healing, infection, inflammation and immunity. These functions are mediated by platelet granule exocytosis/secretion in which three types of platelet granules, each containing specialized cargo fuse with the plasma membrane and release their contents. The importance of these cargoes is underlined by the fact that various platelet storage pool deficiencies show a wide array of bleeding diathesis ranging from mild to severe, sometimes fatal bleeding defects. This suggests that there should be an optimal level of granule cargo that needs to be available to attain hemostatic balance, preventing occlusive thrombosis without causing spurious bleeding.

The overarching question we ask is how much platelet granule secretion is needed for hemostatic balance? Platelet granule exocytosis is a complex process facilitated by a family of membrane proteins termed Soluble N-ethylmaleimide Attachment Protein Receptors (SNAREs). Based on their location in platelets, and a residue at the center of conserved SNARE domain, these proteins are classified as t- target SNAREs/ Q (Glu) SNAREs and v- vesicle SNAREs/ R (Arg) SNAREs, also known as Vesicle-Associated Membrane Proteins (VAMPs). Past studies have found that t- SNAREs such as SNAP23 (Karim, Zhang et al. 2013), syntaxin-11 (Ye, Karim et al. 2012) and syntaxin-8 (Golebiewska, Harper et al. 2015) are required for platelet secretion. Proteomic studies have found the presence of 6 VAMP isoforms in platelets- (2, 3, 4, 5, 7 and 8); however, only 2, 3, 7, and 8 are abundant in both mouse and human platelets. VAMP-8 (Ren, Barber et al. 2007) and VAMP-7 (Koseoglu, Peters et al. 2015) have been shown to play critical roles in platelet exocytosis while VAMP-2 and VAMP-3 have been considered as secondary v- SNAREs. The importance of VAMP-8 in platelet exocytosis is underscored by genetic studies suggesting that Single Nucleotide Polymorphisms in

VAMP-8 are associated with early onset myocardial infarction (Shiffman, Rowland et al. 2006) and a microRNA that regulates VAMP-8 expression potentially contributes to hyperreactive platelets (Kondkar, Bray et al. 2010). Thus, SNAREs potentially control platelet secretion and ultimately platelet function.

In platelets, VAMP-7 (Koseoglu, Peters et al. 2015) and VAMP-8 are primary isoforms crucial for granule secretion while VAMP-2 and VAMP-3 are ancillary (Ren, Barber et al. 2007). Why is this redundancy in v- SNARE usage valuable? V- SNARE usage in few other secretory systems such as neurons (Borisovska, Zhao et al. 2005), mast cells (Puri and Roche 2008), and neutrophils (Mollinedo, Calafat et al. 2006) indicate that heterogeneous usage of VAMPs influences regulated secretion by altering the rates and the extent of secretion or preferentially releasing specific cargo.

Based on these studies, we hypothesized that by regulating VAMP types and amounts in platelets, we could determine the optimal level of platelet exocytosis required for an ideal hemostasis. To study this, we generated platelet-specific $V-2/3^{-/-}$ and $V-2/3/8^{-/-}$ animals and studied the characteristics of granule secretion. We found that the loss of VAMP-2 and VAMP-3 do not have any significant effect on the kinetics and the magnitude of the secretion from platelet granules. However, $V-2/3/8^{-/-}$ platelets showed significantly diminished secretion from all three granules. Additionally, $V-8^{-/-}$ and $V-2/3/8^{-/-}$ platelets spread remarkably slowly on fibrinogen indicating that secretion is necessary for spreading. *In vivo* assays showed that $V-2/3/8^{-/-}$ animals bled profusely and failed to form occlusive thrombus indicating that secretion defects led to a robust hemostatic deficiency. Surprisingly, phosphatidylserine exposure was minimally affected in $V-2/3^{-/-}$ platelets but significantly depleted in $V-2/3/8^{-/-}$ platelets suggesting that the deletion of these VAMP isoforms does have at least some effect on the coagulation cascade and this potentially could have exacerbated hemostatic deficiencies.

The release of platelet granule cargo shapes the thrombus composition. Hence, anti-platelet molecules are appealing antithrombotics. However, it is challenging to equilibrate platelet function with no clear understanding of the threshold of platelet secretion necessary to prevent uncontrolled thrombosis without compromising

hemostasis. This is a first systematic quantitative study that addresses this question. Additionally, by generating these platelet-specific VAMP KO strains, we have created a key reagent system to study the role of platelet secretion in a multitude of other processes where platelets play major roles.

Results

Generation of platelet-specific V-2/3^{-/-} and V-2/3/8^{-/-} mouse model

To overcome the challenges of perinatal lethality of V-2^{-/-} (Schoch, Deak et al. 2001) and embryonic lethality of V-3/8^{-/-} animals (Whiteheart unpublished), we used broadly accepted megakaryocyte/platelet-specific PF4 promoter-driven Cre recombinase system (Tiedt, Schomber et al. 2007). Tetanus toxin light chain cleaves VAMP-2 (Schoch, Deak et al. 2001) and VAMP-3 (McMahon, Ushkaryov et al. 1993, Ren, Barber et al. 2007) while VAMP-8 and -7 are insensitive to this toxin and are not affected (Figure 3.1). The mice generated, V-2/3^{-/-} were later crossed with V-8^{-/-} animals (Wang, Ng et al. 2004, Ren, Barber et al. 2007) to get V-2/3/8^{-/-} animals.

Animals from both novel strains – V-2/3^{-/-} and V-2/3/8^{-/-} followed Mendelian ratios and were healthy and fertile. They did not have any gross developmental or anatomical abnormalities and had a normal lifespan. The average weight of littermate control at 12 weeks (24.41 ± 2.96 gm) was comparable with V-2/3^{-/-} (23.48 ± 2.97 gm) and V-2/3/8^{-/-} (23.58 ± 1.9 gm) suggesting the absence of growth defects in these animals. Hematological parameters including RBC count, platelet number and mean platelet volume were comparable to littermate controls (Figure 3.1). Additionally, electron micrographs show that the shape and size of platelets were normal in V-2/3^{-/-}, V-8^{-/-} and V-2/3/8^{-/-} indicating normal hematopoiesis (Figure 3.2) in absence of VAMP isoforms. Detailed morphological features will be discussed in Chapter 4.

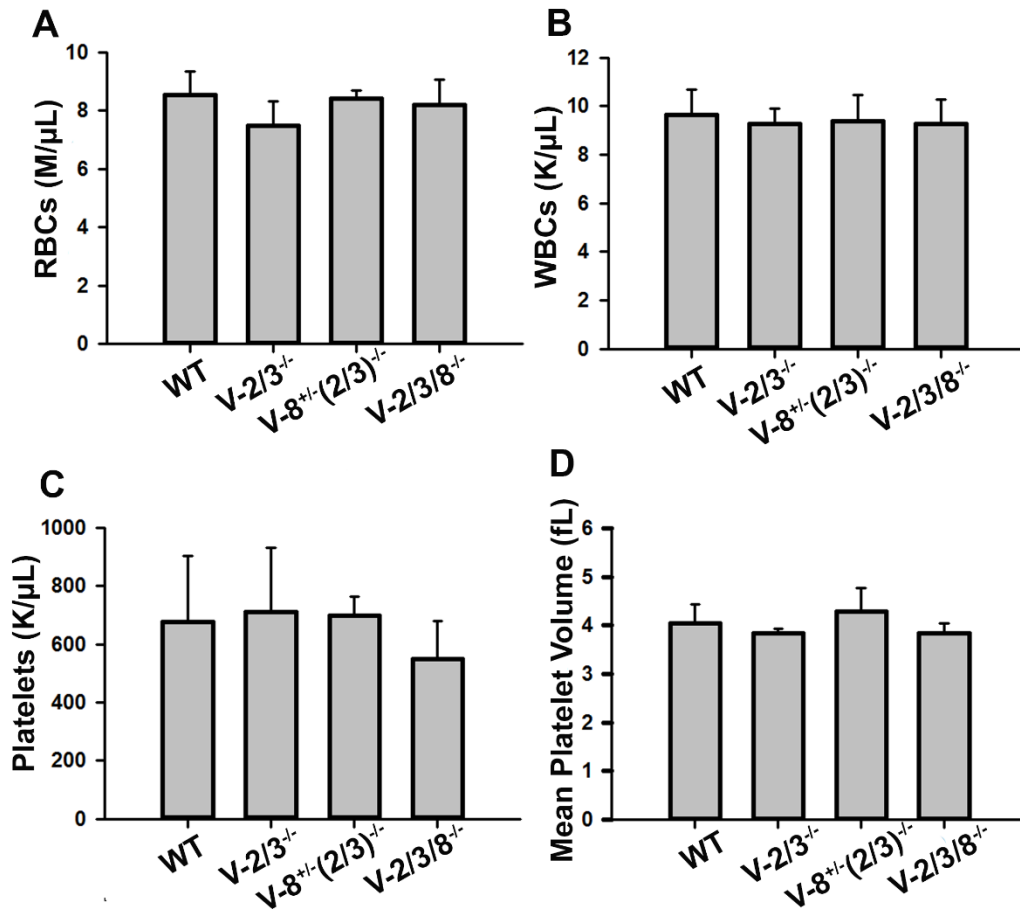


Figure 3.1. Characteristics of blood cells from WT and VAMP deficient animals

Whole blood was collected from WT (n=11), VAMP-2/3^{-/-} (n=6), V-8^{+/(2/3)}^{-/-} (n=6), and V-2/3/8^{-/-} (n=8) animals and a Hemavet instrument was used to measure RBCs (A), WBC (B), platelet concentration (C) and mean platelet volume (D).

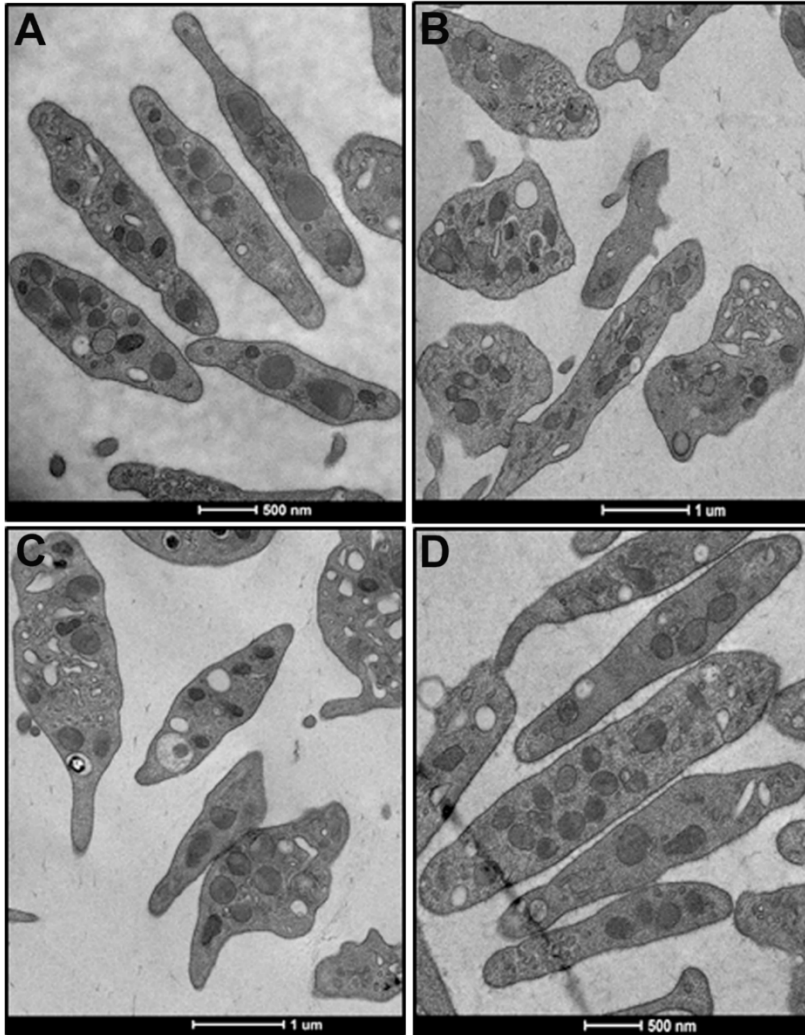


Figure 3.2. Ultrastructure of resting VAMP-deficient platelets

Mouse platelets from WT (A), $V-2/3^{-/-}$ (B), $V-8^{-/-}$ (C), $V-2/3/8^{-/-}$ (D) mice were fixed directly while collecting blood by cardiac puncture. High-pressure freeze technique was used to process samples. Thin sections (50 nm) were cut and post-stained with uranyl acetate and lead citrate. Samples were imaged using an FEI Tecnai TF20 intermediate-voltage electron microscope. (Samples prepared by Smita Joshi, imaging by Irina Pokrovskaya)

SNAREs in V-2/3^{-/-}, V-8^{+/-}(2/3)^{-/-} and V-2/3/8^{-/-} platelets

To validate the generation of VAMP-deficient strains, western blotting was used along with tail DNA PCR. Western blot analysis confirmed the deletion of VAMP-2, and VAMP-3, in V-2/3^{-/-} and VAMP-2, VAMP-3, and VAMP-8 in V-8^{+/-}(2/3)^{-/-} and V-2/3/8^{-/-}, strains (Figure 3.1). The levels of VAMP-8 in V-2/3^{-/-} platelets were increased by about 10% while they were decreased by about 40% in V-8^{+/-}(2/3)^{-/-} platelets (Figure 3.3 A and B). Previously it was noted that global V-2^{+/-}/3^{-/-} platelets had about 30% increase in VAMP-8 levels though VAMP-7 levels were not altered (Ren, Barber et al. 2007); however, there was no change in expression of VAMP-7 and VAMP-8 in platelet-specific V-2/3^{-/-} platelets. Additionally, the loss of VAMP-8 did not change VAMP-7 levels (Ren, Barber et al. 2007). Similarly, the loss of VAMP-7 did not result in increased in VAMP-8 levels (Koseoglu, Peters et al. 2015). Surprisingly, VAMP-7 expression was significantly increased (about 2X) in V-8^{+/-}(2/3)^{-/-} platelets while it diminished by about 50% in V-2/3/8^{-/-}. Since it is well established that VAMP-7 is tetanus toxin insensitive (Galli, Zahraoui et al. 1998), it is unlikely that our transgenic strategy affected VAMP-7 expression. One possibility is that the early loss of multiple VAMP isoforms may have interrupted VAMP-7 trafficking from megakaryocytes to proplatelets to ultimately platelets resulting in the observed reduction in VAMP-7 levels. VAMP-8 has been implicated in endosomal trafficking in other secretory systems and VAMP-3 is crucial for platelet endocytosis (Antonin, Holroyd et al. 2000, Banerjee, Joshi et al. 2017). Since V-3^{-/-} and V-8^{-/-} platelets do not show any defect in VAMP-7 levels, these two VAMP isoforms could function in the same pathway and possibly compensate for each other. Therefore, complete loss of both isoforms may have contributed to this trafficking defect. However, the more detailed analysis is necessary to determine the mechanism of VAMP-7 trafficking in platelets.

The expression of t-SNAREs -syntaxin-11 and SNAP23 were not affected. As expected, fibrinogen levels were decreased in V-2/3^{-/-} and V-2/3/8^{-/-} animals by about 20% and 50% respectively mainly due to the deficiency of VAMP-3 which plays an

essential role in endocytosis (Banerjee, Joshi et al. 2017). Cytoplasmic protein Rab- GDI was used as a loading control for the analysis.

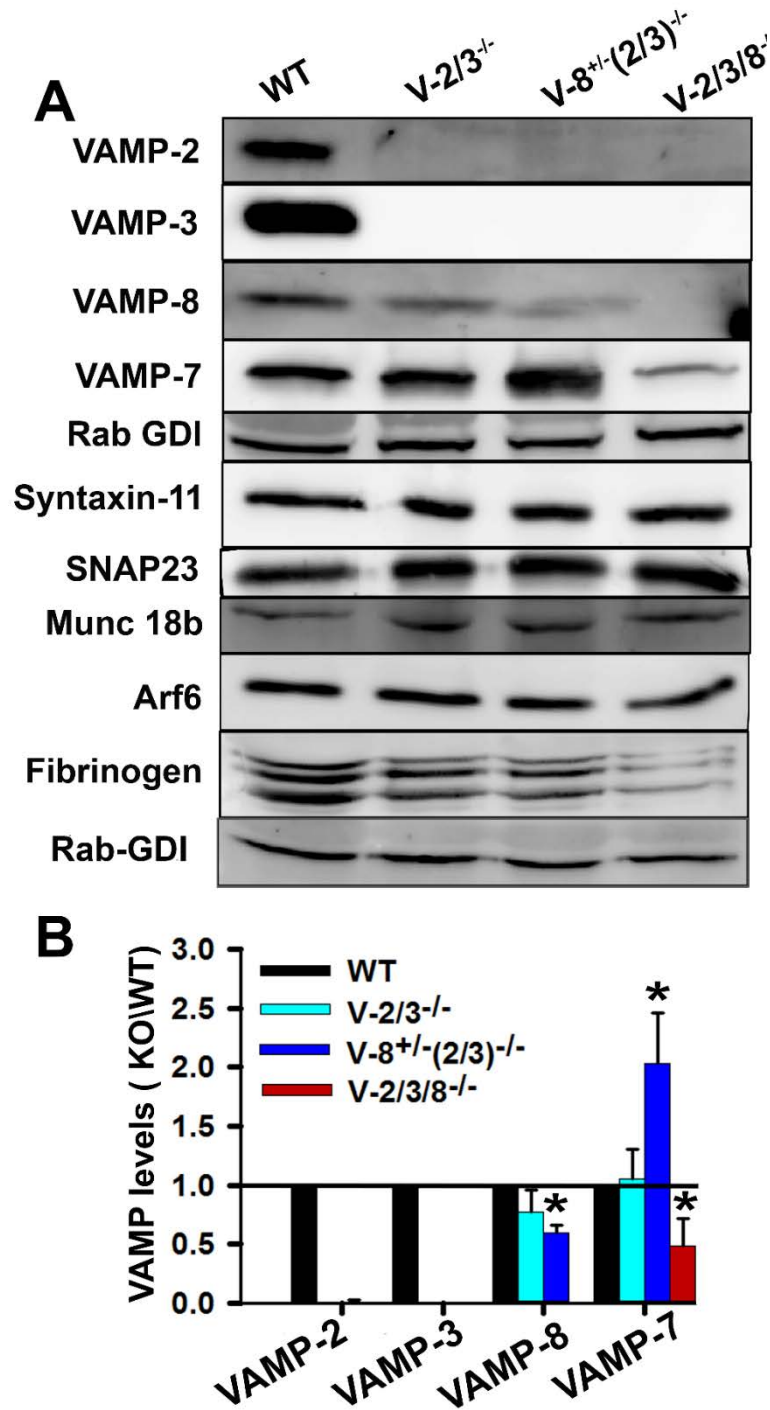


Figure.3.3. Generation of V-2/3^{-/-} and V-2/3/8^{-/-} animals

(A) Comparisons of protein levels among V-2/3^{-/-}, V-8^{+/-}(2/3)^{-/-} and V-2/3/8^{-/-} platelets. Washed platelet extracts (5×10^7 platelets/lane) were prepared from WT, V-2/3^{-/-}, V-8^{+/-}(2/3)^{-/-} and V-2/3/8^{-/-} mice, and the indicated proteins were analyzed by western

blotting. **(B)** Quantification of protein levels in $V-2/3^{-/-}$, $V-8^{+/-}(2/3)^{-/-}$ and $V-2/3/8^{-/-}$ platelets was performed using Rab GDI as a loading control and ImageQuantTL software was used for image analysis. The data were plotted as the ratio of KO over WT. The horizontal solid line indicates the ratio of 1. Data are representative of platelets pooled from two mice and at least 2 independent experiments.

Role of VAMPs in platelet secretion

To understand how VAMP-2, -3 and -8 influence the kinetics and the extent of granule secretion, we examined the stimulation-dependent and time-dependent release of cargo from all three platelet granules: dense, alpha, and lysosomes. Platelet granule secretion upon agonist titration indicates the extent of cargo release upon various strengths of stimulation while the time-dependent release of cargo aids in the understanding of the kinetics of this process. Washed mouse platelets were incubated with [³H] serotonin and then stimulated with an agonist for a certain time before stopping the reaction with hirudin. Releasate (supernatant) was separated from the pellet and tritium was measured in both the fractions to calculate percent release from dense granules. A sandwich ELISA assay was used to measure PF4 secretion from alpha granules and PNP glucnac was used as a substrate to measure the enzymatic activity of a lysosomal enzyme β -hexosaminidase as explained previously (Ye, Karim et al. 2012).

Role of VAMP-8 in platelets granule secretion

Increased concentrations of thrombin facilitated release from V-8^{-/-} platelets that was delayed and reduced at low concentrations of thrombin but reached comparable levels with WT as thrombin concentration increased (Figure 3.4). This shows that VAMP-8 is probably more important for early stages/burst phase of secretion than in sustained release once secretion reaches the maximal extent. VAMP-8 seems to have more influence on alpha granule (Figure 3.4 B and E) and lysosomal release (Figure 3.4 C and F) than on dense granule release (Figure 3.4 A and D). Evidently, defective dense granule secretion that is crucial for availability of secondary agonists such as ADP, ATP *etc.* may have impact on downstream alpha and lysosomal secretion making these events less efficient in the context of both the rate and the degree of release (Graham, Ren et al. 2009, Harper, van den Bosch et al. 2015). Consistent with our previous report, though the secretion was diminished, it was not completely abolished in V-8^{-/-} platelets. suggesting that the loss of VAMP-8 could be compensated for by residual VAMP isoforms at higher agonist concentrations (Ren Q et al, 2007).

Role of VAMP-2 and VAMP-3 in platelets granule secretion

Previously, Ren et al., showed that global V-2^{+/-} animals and global V-2^{+/-}/3^{-/-} animals did not have secretion deficiency (Ren, Barber et al. 2007). Similarly, an earlier investigation with global V-3^{-/-} animals indicated that lack of VAMP-3 does not affect secretion (Schraw, Rutledge et al. 2003). In fact, some reports indicated that the loss of VAMP-3 shows a small but significant increase in secretion suggesting that it might be an inhibitory v-SNARE (Varlamov, Volchuk et al. 2004). Since global V-2^{-/-} are perinatal lethal, we made platelet-specific V-2/3^{-/-} animals to study how it influences platelet secretion and function (Figure 2.1).

Secretion of granule cargo from V-2/3^{-/-} platelets displayed little change compared to WT platelets in response to increased thrombin concentrations (Figure 3.4). Dense and alpha-granule secretion was not altered however, there was a minimal but non-significant decrease in lysosomal secretion (Figure 3.4 C and F). Flow cytometry data show that P-selectin (alpha granule membrane marker) exposure is about 10-15% reduced in V-2/3^{-/-} platelets whereas LAMP-1 (Lysosomal membrane marker) expression is also decreased by about 20% (Figure 3.6 A and B).

Secretion deficiency in V-2/3/8^{-/-} platelets

The lack of VAMP-2, -3, and -8 had a significant impact on granule secretion. V-2/3/8^{-/-} platelets displayed delayed and decreased dense granule secretion, the most rapid among all three granule release events (Figure 3.4 A and D). Dense granules are docked and therefore the first and fastest ones to release their cargo upon stimulation (Jonnalagadda, Izu et al. 2012, Chicka, Ren et al. 2016). This may be the reason why the loss of three major VAMP isoforms has a comparatively lower effect on dense granule release. Upon thrombin stimulation at the concentration of 0.05 U/mL, WT platelets reached 50% of their maximal release level while V-2/3/8^{-/-} platelets hardly showed any release. The maximal extent of V-2/3/8^{-/-} release was about 50% lower than that of WT. Consistent with VAMP-8 deficient phenotype, the deletion of VAMP-2, -3, and -8 severely affected the alpha and lysosomal release than the dense granule release. There was about 80% reduction in the extent of the alpha-granule release (Figure 3.4 B and E)

and about 75% decrease in lysosomal release compared to WT (Figure 3.4 C and F). This pattern also indicates the role of dense granule cargo, ADP as a secondary agonist to stimulate release from downstream/late granule fusion (alpha and lysosome) events (Harper, van den Bosch et al. 2015).

The only major residual VAMP isoform in V-2/3/8^{-/-} platelets is VAMP-7 and the data suggest that it may need higher stimulation to reach WT levels. This pattern was evident in both alpha and lysosomal release. It should be noted that since VAMP-7 levels were significantly decreased in these platelets, it may have compounded the secretion deficiency in all three granules, at least to some extent.

The rates of cargo release from V-8^{-/-} and V-2/3/8^{-/-} platelets were also notably reduced upon activation with 0.05 U/mL thrombin (Figure 3.4 D, E, and F). This thrombin concentration was chosen to study kinetics because it was optimal to observe the release kinetics at about half-maximal rate without reaching a plateaued pattern which could obscure the subtle differences among strains.

In the early phase of secretion, burst phase (first 30 sec on secretion time scale), serotonin release from V-8^{-/-} platelets was remarkably slower (Figure 3.4 D). The additive loss of VAMP-2 and VAMP-3 significantly delayed this phase further. This pattern of delayed release was consistent with all three granule secretion events. Additionally, the extent of secretion was also significantly affected. While the loss of VAMP-8 resulted into about 50% deficit in alpha-granule secretion (Figure 3.4 E) and about 30% decrease in lysosomal secretion (Figure 3.4 F), its effect on dense granule secretion extent was minimal, mainly affecting rates in early burst phase than the extent. Deficiency of VAMP-2, VAMP-3, and VAMP-8 led to about 40% deficit in dense granule, 80% deficit in alpha granule and 50% deficit in a lysosomal release (Figure 3.4 D, E, and F).

To probe if a partial loss of VAMP-8 affects secretion kinetics and extent of V-2/3^{-/-} platelets, we performed secretion assays. The data show that these platelets did not display any secretion deficiency in any of the granules (Figure 3.5). The secretion

patterns are like those observed in V-2/3^{-/-} platelets in context of both the extent (Figure 3.5 A, B, C) and the kinetics (Figure 3.5 D, E, and F).

To further confirm that primary, as well as secondary VAMPs, are required for α granule and lysosome release, exposure of the respective markers P-selectin and LAMP-1 was analyzed by flow cytometry. Consistently, activation-dependent exposure of P-selectin was decreased by 90% and LAMP-1 exposure was reduced by about 70% in V-2/3/8^{-/-} platelets (Figure 3.6 A and B). Additionally, FITC conjugated total integrin staining showed that the total integrin levels were significantly reduced in V-2/3^{-/-} and in V-2/3/8^{-/-} platelets. Since translocation of integrin to plasma membrane requires membrane fusion which is affected in absence of VAMPs, these levels may have been affected. The activation of integrins was not affected as probed by PE-conjugated Jon A staining (Figure 3.6 C and D). This defect is more pronounced in absence of VAMP-2, -3, and -8.

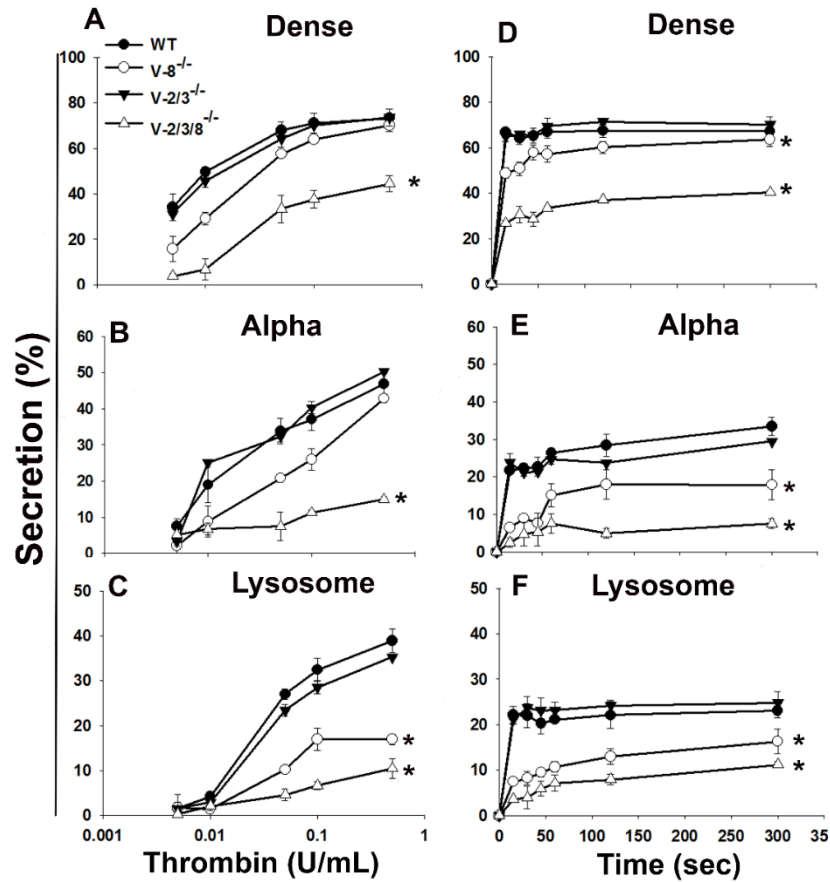


Figure 3.4. The loss of VAMP-2, -3, and -8 affects the kinetics and the extent of platelet secretion

[³H]- Serotonin–labeled platelets from WT, V-2/3^{-/-}, and V-2/3/8^{-/-} mouse were prepared as described in Methods. The release of [³H]-serotonin from dense (A and D) granules, PF4 from α-granules (B and E), and β-hexosaminidase from lysosomes (C and F) was measured, and percent secretion was calculated as described in methods. For thrombin dose-response experiment; platelets were stimulated for 2 min with indicated concentrations of thrombin (A, B, and C). For time-course experiment; platelets were stimulated with 0.05 U/mL thrombin for indicated times (D, E, and F). Data are mean ± SEM of triplicate measurements. Asterisk represents significant *P* values (*P*<0.05, Student’s t-test) as indicated.

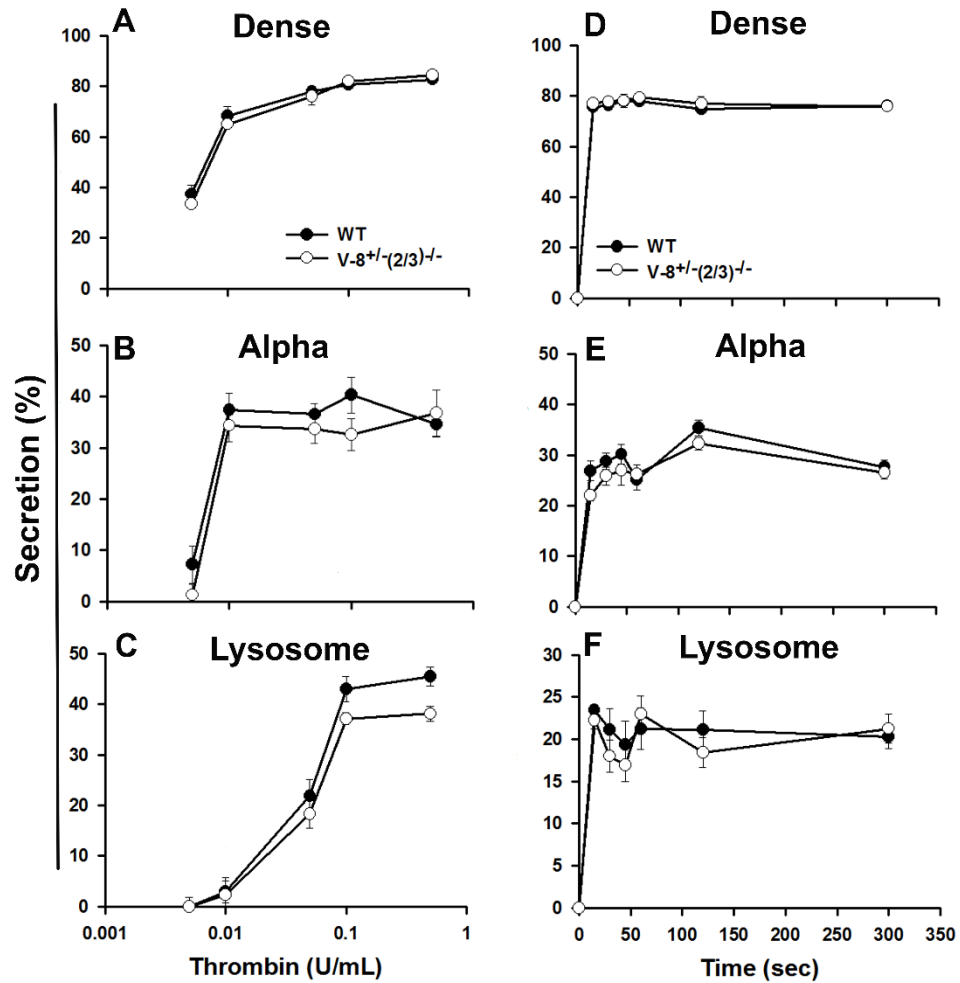


Figure 3.5. Granule secretion is not affected in $V-8^{+/-}(2/3)^{-/-}$ platelets

$[^3\text{H}]$ - Serotonin-labeled platelets from WT and $V-8^{+/-}(2/3)^{-/-}$ mice were prepared as described in Methods. The release of $[^3\text{H}]$ -serotonin from dense granules (A and D) using scintillation counter, PF4 from alpha granules (B and E) using commercial ELIZA assays, and β -hexosaminidase from lysosomes (C and F) using enzymatic activity of β -hexosaminidase was measured, and percent secretion was calculated as described in methods. For thrombin dose-response experiment; platelets were stimulated for 2 min. (A, B and C). For time-course experiment; platelets were stimulated with 0.05 U/ml thrombin (D, E, and F). Data are representative of at least two experiments.

V-2/3^{-/-} and V-2/3/8^{-/-} have diminished integrin α IIb β 3 levels but not integrin activation

To exclude the possibility that defective platelet activation may impact platelet granule secretion, we measured integrin α IIb β 3 activation in V-2/3^{-/-} and V-2/3/8^{-/-} platelets. A flow cytometry assay using FITC-conjugated JonA antibody that recognizes an active form of α IIb β 3 was used to probe platelet activation. The data indicate that integrin activation is normal in both V-2/3^{-/-} and V-2/3/8^{-/-} strains (Figure 3.6 C).

Since fibrinogen levels were reduced in V-2/3^{-/-} and V-2/3/8^{-/-} we wanted to test if fibrinogen receptor integrin α IIb β 3 levels were affected in these animals. Total surface staining for α IIb β 3 integrin showed about a 10-15% decrease in V-2/3^{-/-} platelets and about 25% reduction in V-2/3/8^{-/-} platelets may be due to a trafficking defect to the plasma membrane. However, these levels did not affect platelet activation. (Figure 3.6 D).

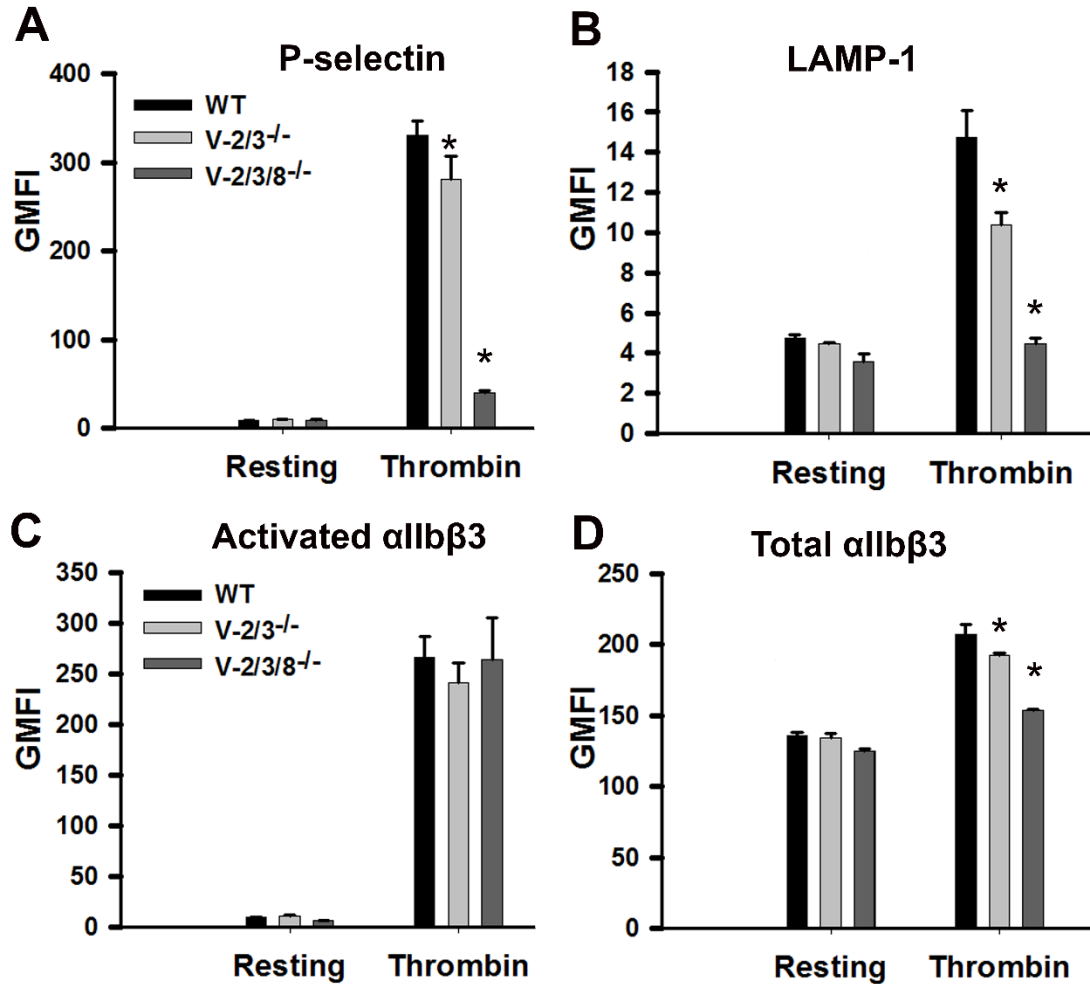


Figure 3.6. Platelets from $V-2/3/8^{-/-}$ mice have lower P-selectin and LAMP-1 exposure but a little defect in integrin $\alpha_{IIb}\beta_3$ activation.

Washed platelets (5×10^7) from WT, $V-2/3^{-/-}$, and $V-2/3/8^{-/-}$ mice were kept resting or stimulated with thrombin (0.05 U/mL) for 2 min and then incubated with FITC-conjugated anti-P-selectin (A), PE-conjugated LAMP-1(B), PE-conjugated JonA (C), or FITC-conjugated CD41/61 (D) Abs for 20 min at RT. Fluorescence intensities were measured by flow cytometry. Shown are representative data (WT- black bars, $V-2/3^{-/-}$ - light gray bars, $V-2/3/8^{-/-}$ - dark gray bars) and geometric MFI (mean \pm SEM) of at least 2 independent experiments. Significant P values ($p < 0.05$, Student's t-test) are indicated.

Integrin activation and aggregation are not altered in V-2/3/8^{-/-} platelets

To support our findings from secretion assays, we also used Lumi-aggregometry to analyze ATP/ADP release from dense granules. While ATP/ADP release in response to 0.05, 0.1, and 0.5 U/mL thrombin was minimally affected in V-2/3^{-/-} platelets, it was substantially inhibited in V-2/3/8^{-/-} platelets, whereas aggregation was not (Figure 3.7). At stimulation with 0.05 U/mL thrombin, there was virtually no ATP secretion and therefore no aggregation from V-2/3/8^{-/-} platelets (Figure 3.7 A and D). Minimal ATP release at 0.1 U/mL and 0.5 U/mL thrombin might be sufficient to support normal aggregation (Figure 3.7 B, E, C, and F).

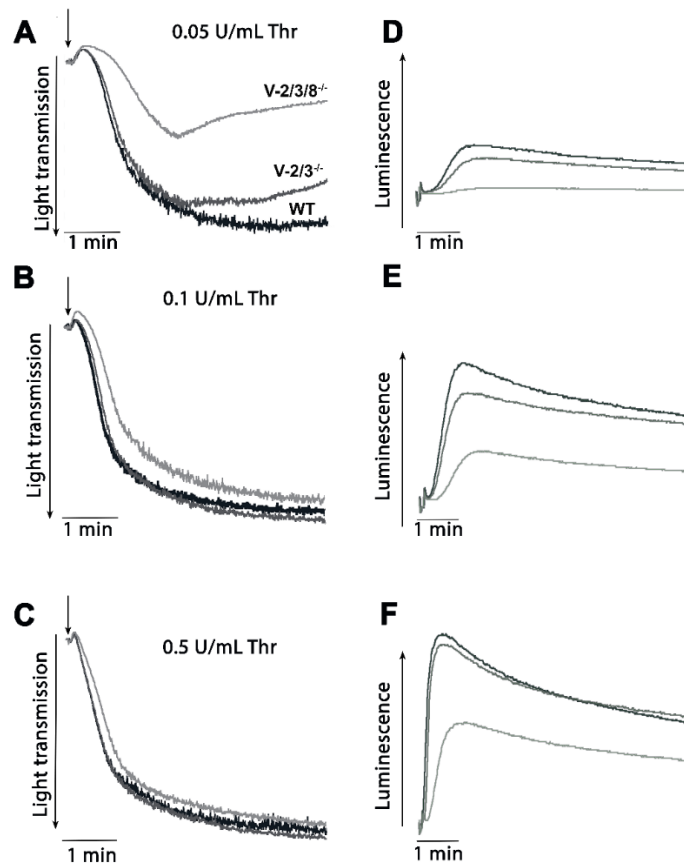


Figure 3.7. Depletion of VAMP-2, -3, and -8 in platelets affects ATP/ADP release, but not aggregation

Aggregation (A-C) and ATP/ADP release (D-F) were monitored simultaneously in a Lumi-aggregometer. Washed platelets ($4 \times 10^8/\text{mL}$) from WT (black traces), V-2/3^{-/-} (dark gray traces) and V-2/3/8^{-/-} (light gray traces) platelets were stimulated with thrombin (0.05 U/mL; Thrombin) (A), or 0.1 U/mL (B), or 0.5 U/mL (C) and ATP release and aggregation were measured for 5 min.

V-8^{-/-} and V-2/3/8^{-/-} platelets show defective spreading

Upon stimulation, platelets spread to increase the surface area that enhances interaction with other platelets and aids in thrombus formation. Previous studies have reported that granule secretion is important for platelet spreading (Peters, Michelson et al. 2012). Loss of VAMP-7 affects spreading on fibrinogen-coated surfaces (Koseoglu, Peters et al. 2015). To determine if secretion deficiency in VAMP-deficient strains affects the rate and the extent of platelet spreading, we studied their spreading on fibrinogen. Washed platelets were supplemented with calcium and plated on fibrinogen-coated coverslips and fixed with paraformaldehyde at 30, 60, 90, and 120 min at 37°C.

Image analysis using ImageJ showed that the platelet spreading was heterogeneous. The data show that V-8^{-/-} platelets spread slowly compared to WT platelets (Figure 3.8 C), but there was no significant difference between the number of platelets spread at each time point in both the WT and V-8^{-/-} platelets (Figure 3.8 D). This suggested that granule secretion is important for spreading. Surprisingly the V-2/3^{-/-} group had more spread platelets than the control group at the 30 min time point, although the area covered by spread platelets did not change (Figure 3.8 A and B). This is most likely due to the loss of VAMP-3, which we previously showed causes platelets to spread more rapidly (Banerjee, Joshi et al. 2017).

V-2/3/8^{-/-} platelets spread remarkably slower than WT platelets (Figure 3.8 A). At each time point, fewer platelets spread than the platelets in WT samples (Figure 3.8 B). The area covered by spread platelets was also significantly reduced in V-2/3/8^{-/-} platelets. This supports previous reports that granule secretion deficiency affects platelet spreading (Peters, Michelson et al. 2012). Peters et al., showed that human HPS platelets that have dense granules show normal spreading, however, human GPS platelets with defective alpha granules had impaired spreading. This suggests that though dense granules are dispensable, the alpha-granule release is crucial for normal platelet spreading. The spreading defect observed in V-2/3/8^{-/-} platelets is the result of significantly diminished alpha-granule release in these platelets (Figure 3.4). VAMP-7 has been suggested to have associations with cytoskeletal elements. Loss of VAMP-7

severely affects platelet spreading (Koseoglu, Peters et al. 2015). In the light of this role of VAMP-7, it should be noted that reduction in VAMP-7 levels in these platelets may have also impacted this spreading, at least to some degree (Figure 3.3).

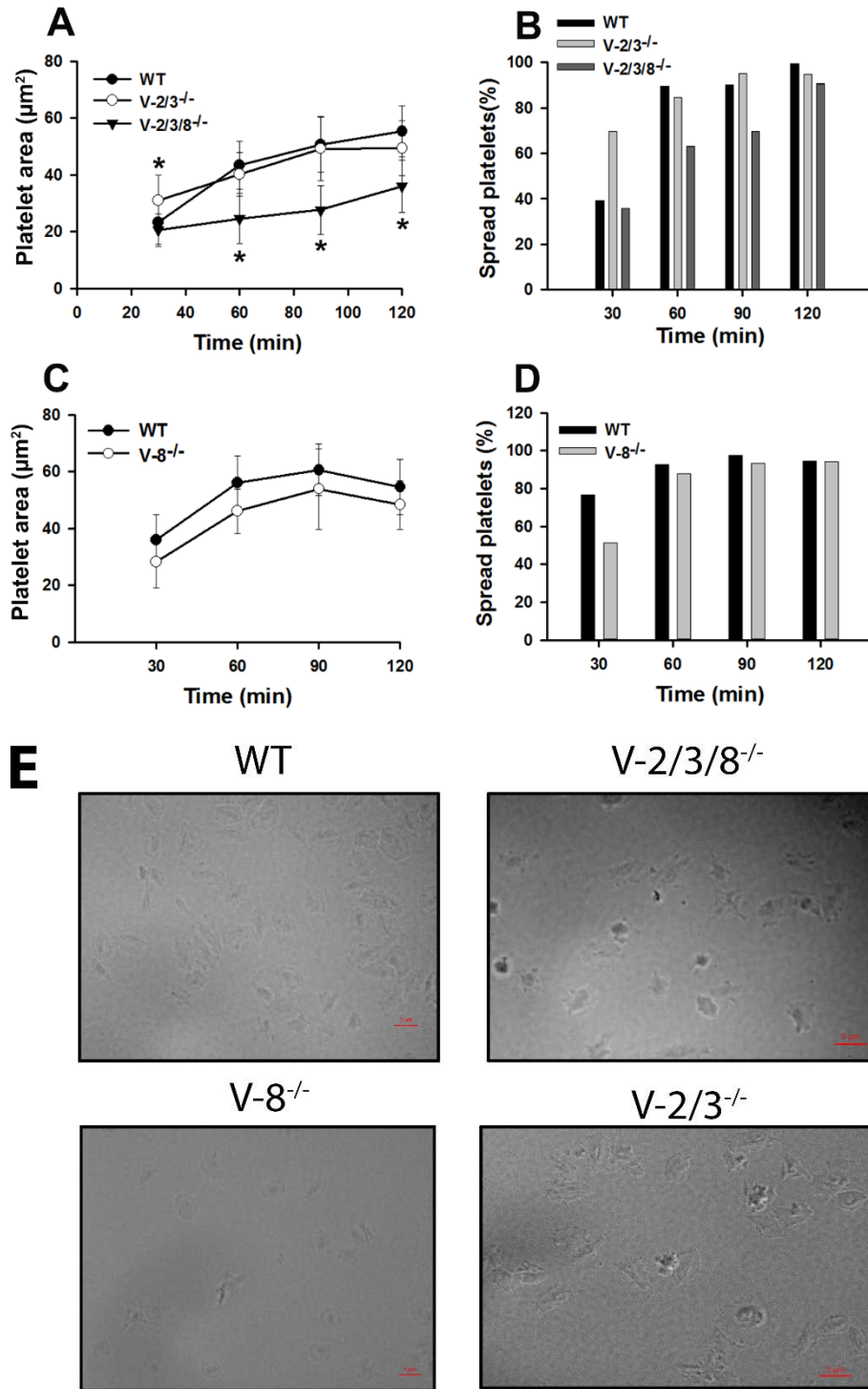


Figure 3.8. *V-8^{-/-}* and *V-2/3/8^{-/-}* platelets have a spreading defect

Platelets from the indicated mouse strains were allowed to adhere and spread on fibrinogen-coated coverslips. At the indicated times, the samples were fixed and imaged

for analysis. (A). Quantification of the area spread by platelets from WT, V-2/3^{-/-} and V-2/3/8^{-/-} mice. (B). Distribution of spread platelets over time for WT, V-2/3^{-/-} and V-2/3/8^{-/-} platelets as a percent of platelets imaged. (C). Quantification of the area spread by platelets from WT and V-8^{-/-} strains. (D). Distribution of spread platelets over time for WT and V-8^{-/-} platelets as a percent of platelets imaged. For each time point, >100 platelets were counted for the quantification. The data are representative of two independent assays. Examples of spread platelets from various strains at 120 min are shown in (E). Scale bar is 5 μm.

V-2/3/8^{-/-} animals have a severe hemostatic deficiency

To study how secretion deficiencies affect *in vivo* hemostasis, we used two assays that represent different types of vascular injuries. Tail bleeding time measurement mimics transection injury and FeCl₃ injury model that mirrors endothelial denudation. While tail bleeding time was comparable among WT, V-3^{-/-}, V-2/3^{-/-} and V-8^{-/-} models, it was significantly increased in V-2/3/8^{-/-} (mean time = 571.74 ± 72.61 sec) compared to WT animals (mean time = 250.07 ± 86.82 sec) (Figure 3.9 A). The measurement of time to form occlusive thrombus post- FeCl₃ application indicates that in WT animals, thrombus formed in the meantime of around 6.5 min (Figure 3.9 B). This time was comparable among other strains tested- V-8^{+/-} (~ 7 min), V-2/3^{-/-} (~ 7 min), V-8^{+/-}(2/3)^{-/-} (~ 6.5 min). However, it was notably increased in V-8^{-/-} (~ 21 min) animals, confirming our previous findings from the laser injury model data (Graham, Ren et al. 2009). As expected, most V-2/3/8^{-/-} (~ 28 min) animals failed to form occlusive thrombus during our observation window suggesting that the loss of these three VAMP isoforms responsible for platelet secretion deficiency also leads to *in vivo* thrombosis defect. Additionally, we did not find any sexual dimorphism in these processes (Figure 3.10).

To test if defective secondary hemostasis impacted coagulation processes and contributed to increased bleeding time and decreased thrombosis, we measured clotting times using two broadly used tests, PT (prothrombin time) and aPTT (activated partial thromboplastin time). PT is mainly used to analyze the role of extrinsic pathway while aPTT is more focused on the intrinsic and common pathway. The data show that both PT and aPTT times were comparable among all the strains tested (Figure 3.9 C). This argues that the coagulation pathways were not affected in the absence of platelet secretion.

Expression of phosphatidylserine/PS upon platelet activation provides a platform for thrombin generation. To exclude the possibility that secretion deficiency affected PS exposure and ultimately thrombin generation leading to insufficient hemostasis, we performed flow cytometry-based assays using FITC-conjugated anti-PS antibody. The data show that though mildly reduced, PS exposure was not significantly depleted in V-

2/3^{-/-} platelets (Figure 3.9 D). In V-2/3/8^{-/-} platelets, however, it was significantly diminished by about 50%. This suggests that platelet granule secretion is probably necessary for either the release of flippases/floppase/translocases from a vesicle/granule is crucial for PS exposure or the trafficking and/or targeting of these enzymes to the plasma membrane of these platelets is defective. It should be noted that about 25-30% reduction in PS exposure does not cause hemostatic deficiencies in V-2/3^{-/-} animals.

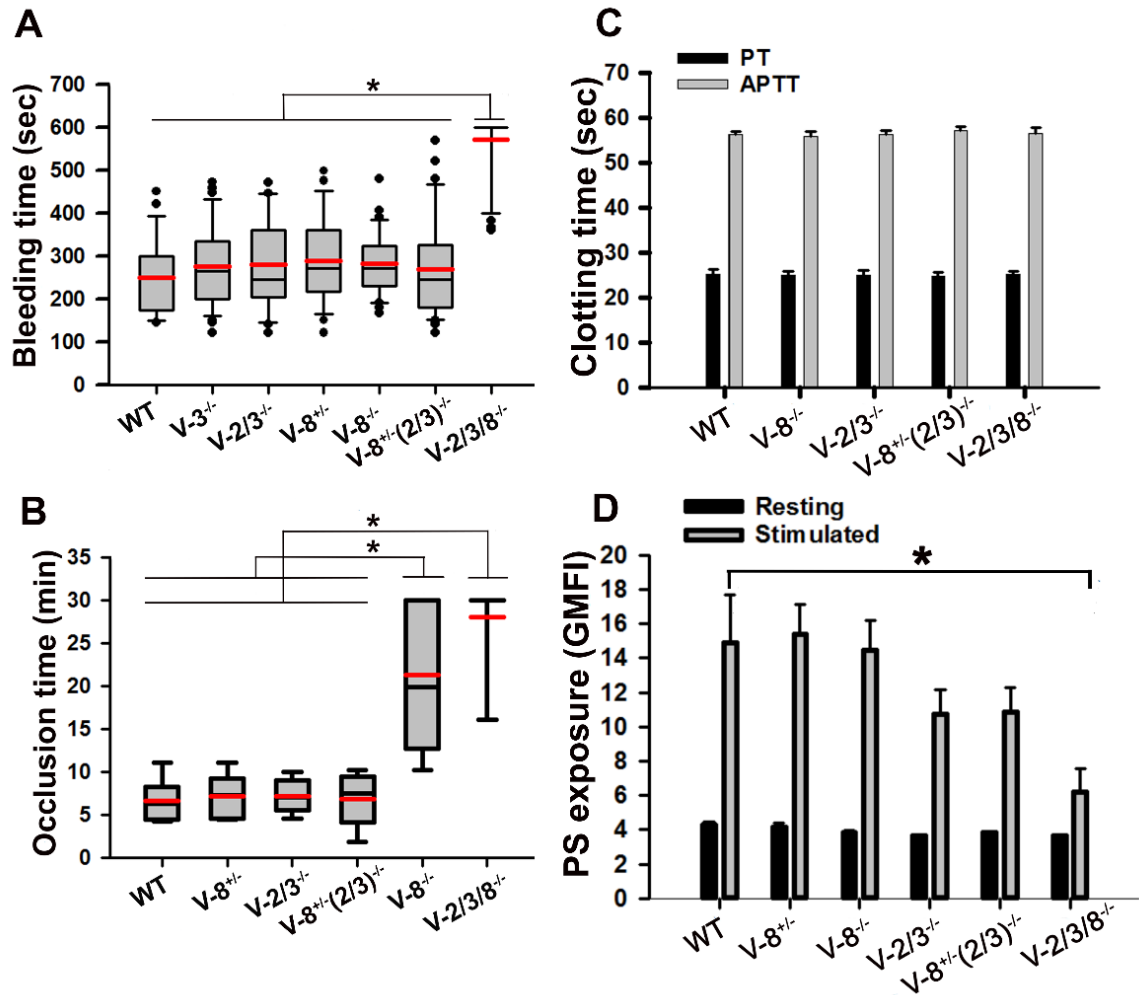


Figure 3.9. Hemostasis and thrombus formation are impaired in V-2/3/8^{-/-} animals

(A) Tail bleeding times were measured after 3 mm tail transection of WT (n=28), V-3^{-/-} (n=38), V-2/3^{-/-} (n= 20), V-8^{+/-} (n=26), V-8^{-/-} (n=31), V-8^{+/-}(2/3)^{-/-} (n=41), and V-2/3/8^{-/-} (n= 34) mice (B) Thrombus formation in the carotid artery was induced by topical application of 5% FeCl₃, and blood flow was monitored in WT (n=6), V-8^{+/-} (n=6), V-2/3^{-/-} (n=7), V-8^{+/-}(2/3)^{-/-} (n=9), V-8^{-/-} (n=7), and V-2/3/8^{-/-} (n=7) mice. Mean ± SEM and P values (log-rank test) are indicated. Red line indicates the mean and the black line indicates the median. The box represents 25th-75th percentile. While whiskers/error bars above and below the box indicate the 90th and 10th percentiles. Outliers are as shown in tail bleeding figure. (C) Activated partial Thromboplastin Times (aPTT) and Prothrombin Times (PT) were measured in platelet-poor plasma prepared from WT (n=7), V-8^{-/-} (n=5),

V-2/3^{-/-} (n=8), V-8^{+/-}(2/3)^{-/-} (n=3) and V-2/3/8^{-/-} (n=5) mice. **(D)** Phosphatidylserine exposure on the surface of thrombin-stimulated platelets from WT, V-8^{+/-}, V-8^{-/-}, V-2/3^{-/-}, V-8^{+/-}(2/3)^{-/-} and V-2/3/8^{-/-} mice were measured by FITC-conjugated lactadherin binding and flow cytometry. The data are representative of two independent assays. Asterisk represents *p*-value (paired Student's t-test, *p*<0.05)

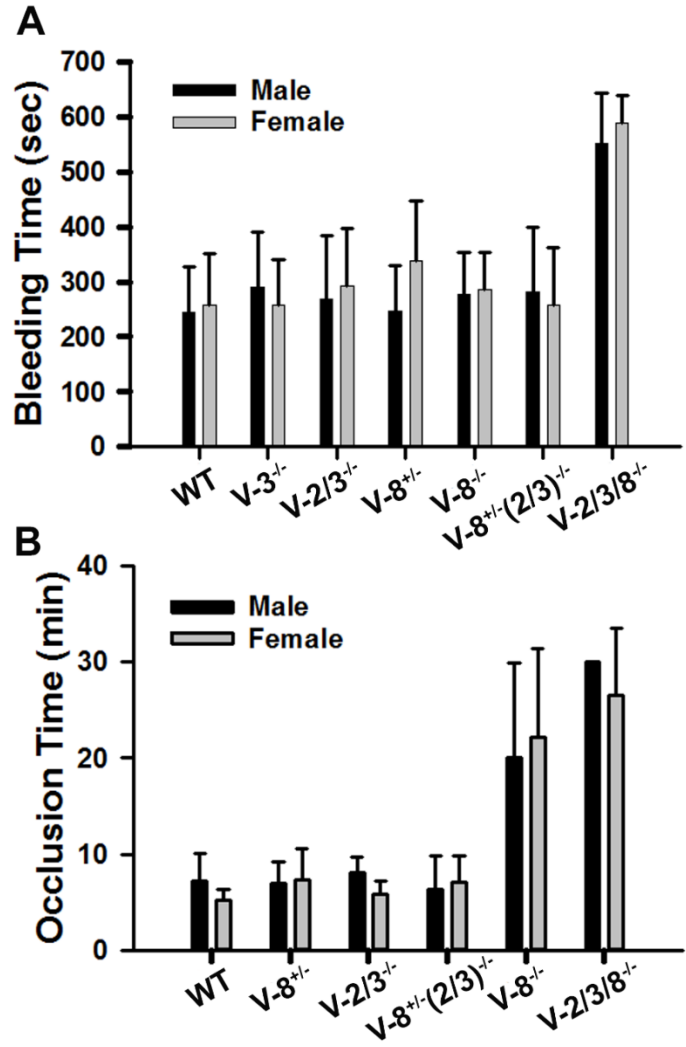


Figure 3.10. Hemostatic deficiencies indicate no sexual dimorphism in VAMP-deficient strains

(A) Tail bleeding times were measured after tail transection of WT (n=28, 13 females, 15 males), V-3^{-/-} (n=38, 18 females, 20 males), V-2/3^{-/-} (n= 20, 9 females, 11 males), V-8^{+/-} (n=26, 12 females, 14 males), V-8^{-/-} (n=31, 15 females, 16 males), V-8^{+/-}(2/3)^{-/-} (n=41, 22 females, 19 males), and V-2/3/8^{-/-} (n=34, 19 females, 15 males) mice (B) Thrombus formation in the carotid artery was induced by topical application of 5% FeCl₃, and blood flow was monitored in WT (n=6, 2 females, 4 males), V-8^{+/-} (n=6, 3 males, 3 females), V-2/3^{-/-} (n=7, 3 females, 4 males), V-8^{+/-}(2/3)^{-/-} (n=9, 5 females, 4 males), V-8^{-/-} (n=7, 4

females, 3 males), and V-2/3/8^{-/-} (n=7, 4 females, 3 males) mice. Mean \pm SEM and p values (log-rank test) are non-significant ($p>0.05$).

Conclusions

Platelet granule secretion is central to the platelet function. Lack of granules or secretion deficiencies that impact platelet granule cargo release affect the hemostatic balance. On the other hand, hyperactive platelets increase the likelihood of thrombotic events underlining the importance of the platelet secretion in maintaining hemostatic balance. Though our understanding of molecules that play a role in platelet secretion has expanded, it is still unclear how much secretion is needed to maintain this hemostatic balance to prevent occlusive thrombosis without triggering unwanted bleeding.

In this Chapter, we address this question by using v-SNAREs to regulate the rate and the magnitude of platelet secretion. By generating platelet-specific $V-2/3^{-/-}$ and $V-2/3/8^{-/-}$ mouse models, we show that each VAMP isoform plays a differential role in determining the rate and the degree of platelet granule secretion. VAMP-8 is a primary VAMP that is crucial for determining the rate of secretion in the early phase of secretion. Loss of VAMP-8 leads to defective granule secretion. Residual VAMPs- VAMP-2, VAMP-3, and VAMP-7 compensate for VAMP-8's loss indicating at least partial functional overlap. The deficiency of VAMP-2 and VAMP-3 in platelets minimally affects secretion, however, the additive loss of VAMP-2, VAMP-3, and VAMP-8 significantly diminishes secretion. This decrease is more evident in alpha and lysosomal secretion than in dense granule release.

Using tail bleeding and arterial thrombosis assays, we show that the secretion deficiency in $V-2/3^{-/-}$ did not cause any hemostatic deficiency. While $V-8^{-/-}$ animals did not bleed, they did seem to have at least some protection from arterial thrombosis since the thrombus formation was delayed and diminished in these animals. $V-2/3/8^{-/-}$ animals bled profusely and failed to form a thrombus. Further investigation showed that though coagulation times were not changed, PS exposure was significantly reduced in $V-2/3/8^{-/-}$ animals potentially affecting thrombin formation that led to a hemostatic defect.

Chapter 4 Alpha-granule cargo decondensation is VAMP-dependent

(This Chapter of the dissertation is a work done as a collaboration with Dr. Brian Storrie and Irina Pokrovskaya from the University of Arkansas for Medical Sciences. Credits for imaging and analysis has been given under each figure)

Introduction

Platelet granule exocytosis is a central event for platelet function in a wide array of physiological and pathological conditions including hemostasis and thrombosis. Platelets contain three types of granules: dense, alpha and lysosomes. Dense granules contain small molecules like ATP, ADP, and calcium that work as secondary agonists. Alpha granules contain more than >200 proteins that play roles in angiogenesis, infection, immunity and wound healing. Lysosomal contents include β -hexosaminidase and cathepsins, important for clot remodeling. These granule cargoes are crucial for hemostasis since granulopathies that affect these granules lead to a wide range of bleeding disorders (Joshi and Whiteheart 2017).

Alpha-granule cargoes are the most diverse in their functions and critical for a myriad of conditions, hence their composition has been a subject of intense investigation. While some reports suggest that each granule contains subpopulations of cargo residing at specific zones inside granules, others indicate that cargo is packed in subpopulations of alpha granules themselves and their release is agonist-dependent (Chatterjee, Huang et al. 2011). Though an appealing mechanism, multiple studies failed to find a correlation between the composition of cargo and agonist stimulation or function-dependent spatial segregation of cargo (Kamykowski, Carlton et al. 2011). In fact, a study by Jonnalagadda et al., showed that there is hardly any difference in cargo composition released after stimulation from four different agonists. The stronger the agonists, the more cargo molecules come out and the only constant feature is that release comes in three waves (Jonnalagadda, Izu et al. 2012).

Recent advances in electron microscopy have provided necessary tools to prevent artifacts during sample preparation, fixation, and the imaging. Using these new technologies, the study of alpha granules suggests that they have a dense, condensed appearance perhaps due to the accumulation of dehydrated proteins that need to be dissolved to be released from granules. Alpha granules form small necks or longer pipes to fuse with the plasma membrane which play a role as routes for cargo release or conduits to allow an influx of extracellular plasma or water (Eckly, Rinckel et al. 2016, Pokrovskaya, Aronova et al. 2016). This influx of a solvent has been credited for the progressive increase in granule volume, solubilization of cargo leading to alterations in electron density of granules, and subsequent exodus of cargo from a fused granule. A granule decondensation has been observed in secretory vesicles (trichocysts) of Paramecium cells (Bilinski, Plattner et al. 1981), and mucin granules of Goblet cells (Perez-Vilar, Mabolle et al. 2006, Perez-Vilar 2007). However, though expected, the decondensing granules have not been yet identified in platelets.

Here using secretion-deficient mouse strains, we show that the decondensation of alpha granules is a time-dependent and agonist concentration-dependent process and decondensation constitutes a real intermediate leading to cargo release. We also show that this process follows a granule fusion, and therefore VAMP deficiency leads to accumulation of these intermediates mainly in $V-2/3/8^{-/-}$ platelets. Secretion deficiency in $V-8^{-/-}$ platelets translates into delayed decondensation of alpha granules, while $V-2/3^{-/-}$ platelets do not show any differences. $V-2/3/8^{-/-}$ platelets have smaller alpha granules suggesting their role in vesicular trafficking.

Results

Condensation and decondensation of alpha granules

Electron micrographs of resting and activated mouse platelets showed a heterogeneous population of alpha granules. Alpha granules in resting platelets displayed uniform density of contents- with darker nucleoid and lighter material packed in a granule (Figure 3.2 A). On the other hand, in agonist-stimulated platelets, various types of fusion events- heterotypic (granule to the plasma membrane/simple) (Figure

4.1 black arrow), and homotypic (granule to granule fusion/compound) were noted (Figure 4.1 red and purple arrows). These granules also differed from each other in the density of cargo suggesting an ongoing process of solubilization of cargo that may facilitate its release from the fusing granule. To see if these various stages of decondensation are really intermediates of granule exocytosis, we decided to conduct a detailed morphological study of stimulated platelets.

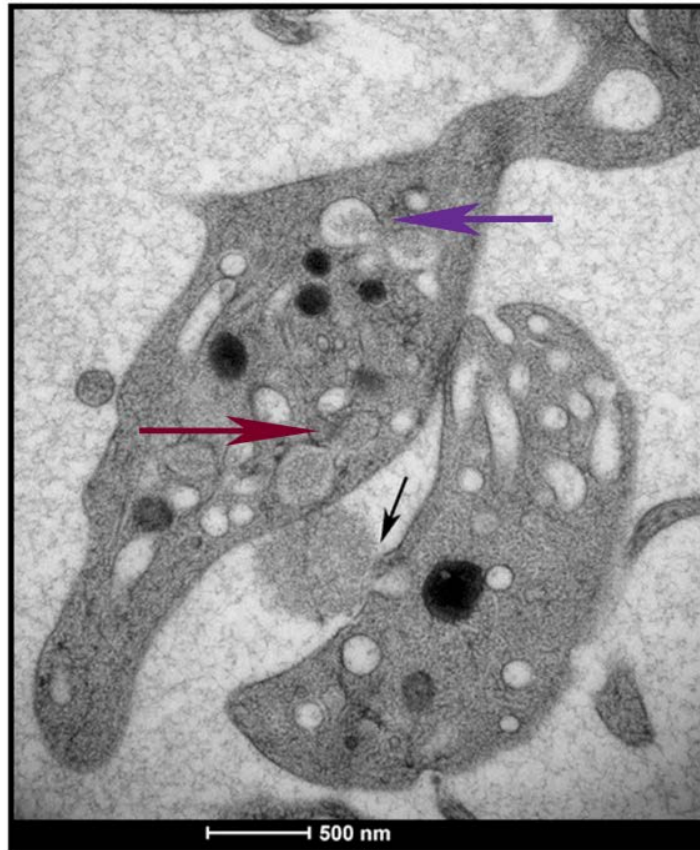


Figure 4.1. Condensed and decondensed alpha granules in activated mouse platelets

An electron micrograph of a thin section (50 nm) of stimulated mouse platelets (0.1 U/mL thrombin for 5 min) depicting simple fusion (black arrow) and various stages of decondensing compound fusion (red and purple arrows) events.

(Samples generated by Smita Joshi and processing and imaging by Irina Pokrovskaya)

Alpha-granule decondensation is time-dependent

Our previous experiments showed that the extent of granule secretion increases as the time increases, indicating that it is a time-dependent process (Figure 3.4 E). We hypothesized that since platelet secretion is a time-dependent process, the form of secreting/decondensing granules will alter in a time-dependent manner. To determine how the appearance of secreting granules alters over time, we performed a time course analysis by stimulating mouse platelets for various time points and fixing them for microscopic studies. We measured the frequency of condensed and decondensed granules at each time point. Condensed granules have a well-defined dense nucleoid like structure and an intact membrane (Figure 4.3 B, white star) while decondensed granules are fused to other granules, the plasma membrane or the open canalicular system (Figure 4.3 B, white arrowhead). Decondensed granules also vary in the appearance of granule cargo since the density of these cargoes is usually lighter than the condensed granules. Our data show that as stimulation time increased, the frequency of condensed granules decreased while decondensed granules increased. This is a classic example of a precursor-product relationship whereas the quantity of a precursor decreases, the quantity of a product increases. The data show that the frequency of condensed alpha granules decreased progressively with time, going from about 5 per platelet profile to less than 1 over 300 sec. Correspondently, the frequency of decondensed alpha granules increased for approximately the first 90 sec following stimulation. At 90 sec stimulation, the numbers for condensed and decondensed alpha granules added together were ~5, the initial value. At later time points, however, the sum was less as the frequency of condensed granules continued to decrease while that of decondensed granules failed to increase and, in fact, decreased slightly. This could be attributed to compound/granule-granule fusion events, which were already apparent at 90 sec (Figure 4.3 B, arrowheads). Moreover, the gradual change in the appearance of decondensed granules may be challenging to detect at the maximal extent of release when the cargo become remarkably less dense. These data confirmed that the decondensation of granule cargo represents progressive intermediates in the secretion

process and the degree of decondensation may represent the extent of the granule cargo release.

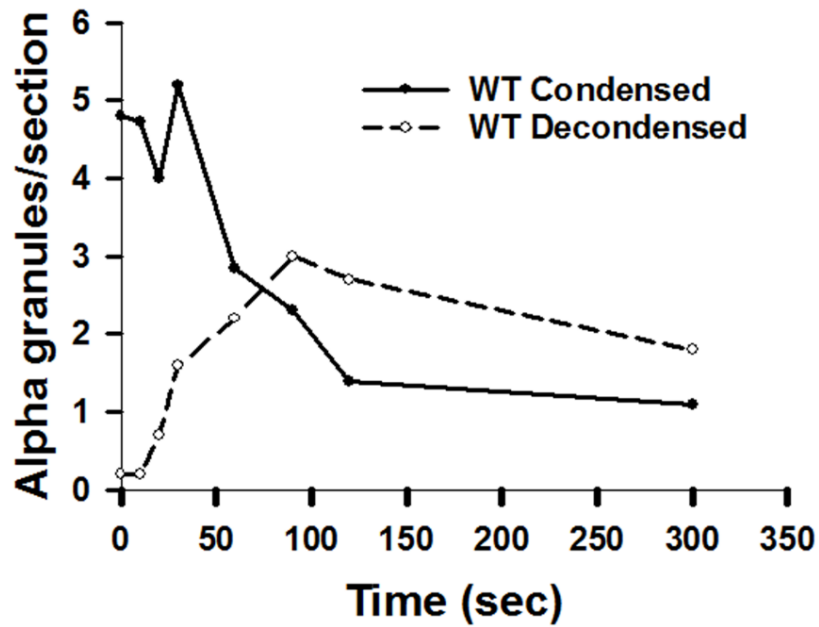


Figure 4.2. A precursor-product relationship between condensed and decondensed granules

The graph shows quantification of condensed and decondensed granules for eight-time points upon stimulation of mouse platelets with 0.1 U/mL thrombin. The average number of platelet profiles scored per data point was 45, range 19 to 50 and the average number of alpha granule profiles scored per time point was 213, range 96 to 295.

(Samples generated by Smita Joshi and processing and imaging by Irina Pokrovskaya)

Arresting secretion intermediates

To investigate if and how the appearance of alpha granules changes as secretion proceeds, we selected a few time points for a detailed study of the morphology of decondensing granules based on earlier profiles (Figure 4.2). Washed mouse platelets were either kept resting or stimulated for 90 sec to monitor an intermediate/partial release step and for 300 sec to observe a maximal release from granules. These platelets were fixed, and microscopy was performed.

Resting platelets maintained a characteristic discoid shape and contained condensed alpha granules with electron-dense matrix (Figure 4.3). Upon stimulus with either 0.1 U/mL or 0.5 U/mL thrombin, the platelets became rounded and extended pseudopods (Figure 4.3 B-D) indicating activation. Post 90 sec stimulation, alpha-granule cargoes appeared more fibrous and less electron dense (Figure 4.3 B, 90 sec stimulation, white arrowheads), indicating decondensation. Additionally, these decondensed granules also appeared slightly dilated with an increased diameter compared to condensed ones.

The agonist dose affected granule decondensation process by influencing both the extent and the frequency of decondensation. First, alpha-granule cargo matrix displayed a greater extent of decondensation upon stimulation with 0.5 U/mL compared to 0.1 U/mL dose (Figure 4.3 B and D). Moreover, the frequency of decondensed vs condensed alpha granules was greater at the higher thrombin dose (data not shown). These data show that the stronger stimulation progressively affects granule release events and a similar effect could be achieved by a lower dose for a longer duration of stimulation.

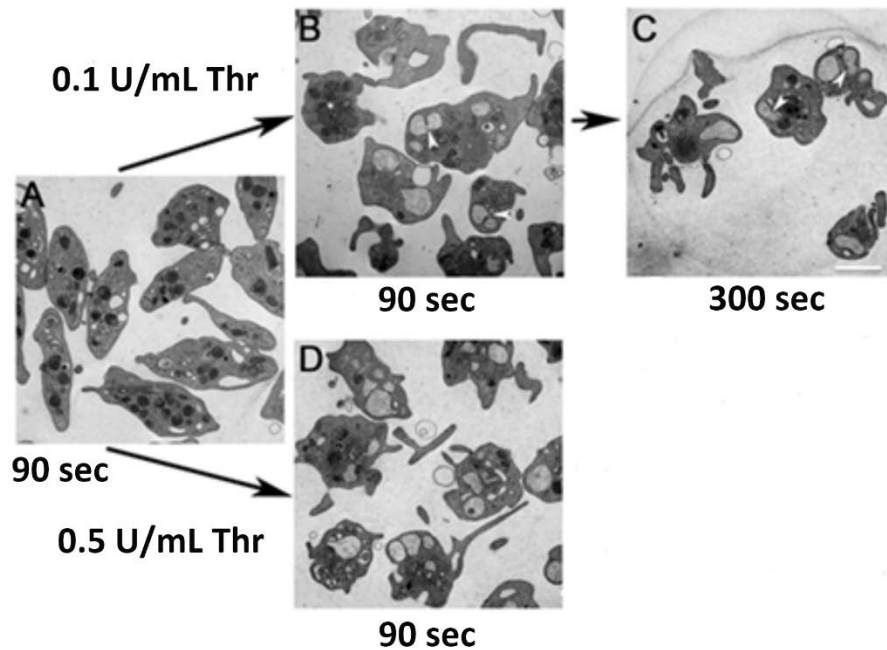


Figure 4.3. A precursor to product relationship between condensed and decondensed alpha granules upon thrombin stimulation of wild-type mouse platelets

A) In electron micrographs of thin sections of resting mouse platelets (0 sec), alpha granules appeared as round to ovoid structures marked by an electron-dense matrix. Discoid shape indicates a resting state. B) Upon thrombin stimulation (0.1 U/mL for 90 sec), platelets show filopodia and lamellipodia formation with decondensed alpha granules that are marked by the residual fibrous matrix. White arrows show compound fusion event and the white asterisk indicates condensed granules C) Upon longer stimulation (0.1 U/mL thrombin for 300 sec), progressive decondensation noted with decondensed granules and compound fusion events are indicated with white arrows. D) Higher concentration of agonist (0.5 U/mL thrombin for 90 sec) stimulation, showed faster decondensation of granules. Scale bar-1 μm . The average number of platelet profiles scored per data point was 45, range 19 to 50 and the average number of alpha-granule profiles scored per time point was 213, range 96 to 295.

(Samples generated by Smita Joshi and processing and imaging by Irina Pokrovskaya)

Granule decondensation is VAMP-dependent

Since the decondensation extent is dependent on the stimulation strength and the time, we next asked if granule fusion is required for decondensation to occur. To test this, we employed VAMP KO mouse models where multiple VAMP isoforms were deleted that resulted in various degrees of granule secretion deficiencies in these platelets. Briefly, isolated mouse platelets were radiolabeled with tritiated serotonin and were stimulated with 0.1 U/mL thrombin. This concentration was chosen to optimally stimulate platelets to monitor morphological differences (Figure 3.4 A, B and C). The data show that the deficiency of VAMP-8 leads to decreased dense granule secretion suggesting that VAMP-8 is important for dense granule release. The loss of VAMP-8 has a greater impact on alpha-granule and lysosomal release compared to dense granule release implying that the defective release of secondary agonists from dense granules has downstream consequences. The loss of VAMP-2 and VAMP-3 in V-2/3^{-/-} platelets did not affect dense and alpha-granule release but had a minor defect on a lysosomal release. The accumulative loss of all three isoforms in V-2/3/8^{-/-} platelets led to a significant decrease in cargo release from all three granules (Figure 4.4). In these platelets, dense granule release was affected by 50% and there was about only 25% release from alpha-granules and lysosomes indicating severe secretion deficiency.

To study the morphology of the platelets and platelet alpha granules at each of these time points, we stopped the reaction with a fixative and performed an electron microscopy. We decided to select a few time points that would yield a good representation of degranulation intermediates in this process, so we chose resting, 90 sec stimulation (early intermediate), and 300 sec (maximal release) points for further structural studies based on our earlier analysis with WT mouse platelets (highlighted with arrows in Figure 4.4).

To study if decondensation is VAMP-dependent, we used secretion defective VAMP-deficient strains explained in Chapter 3. V-8^{-/-} platelets which have been shown to be significantly deficient in secretion showed delayed alpha-granule decondensation (Figure 4.5 and Figure 4.6 A). At 90 sec stimulation, decondensed granules were fewer

than the condensed ones. However, the loss of VAMP-8 could be overcome by ancillary VAMP isoforms at a higher or longer stimulation. This is evident at the 300 sec time points where decondensed granules were more prevalent than the condensed ones. VAMP-2, -3, and -7 act as ancillary VAMPs in absence of VAMP-8 and their loss had a minimal impact on alpha-granule secretion (Figure 4.5 and Figure 4.6 B). These platelets showed remarkable decondensation even at the 90 sec time point. In contrast, the loss of all these three VAMP isoforms inhibited granule secretion in V-2/3/8^{-/-} platelets (Figure 4.5 and Figure 4.6 C).

In sum, these data strongly indicate that alpha-granule decondensation follows VAMP-mediated granule fusion events prior to cargo release to the external microenvironment. This cargo decondensation may depend on extracellular solvent influx inside the granule.

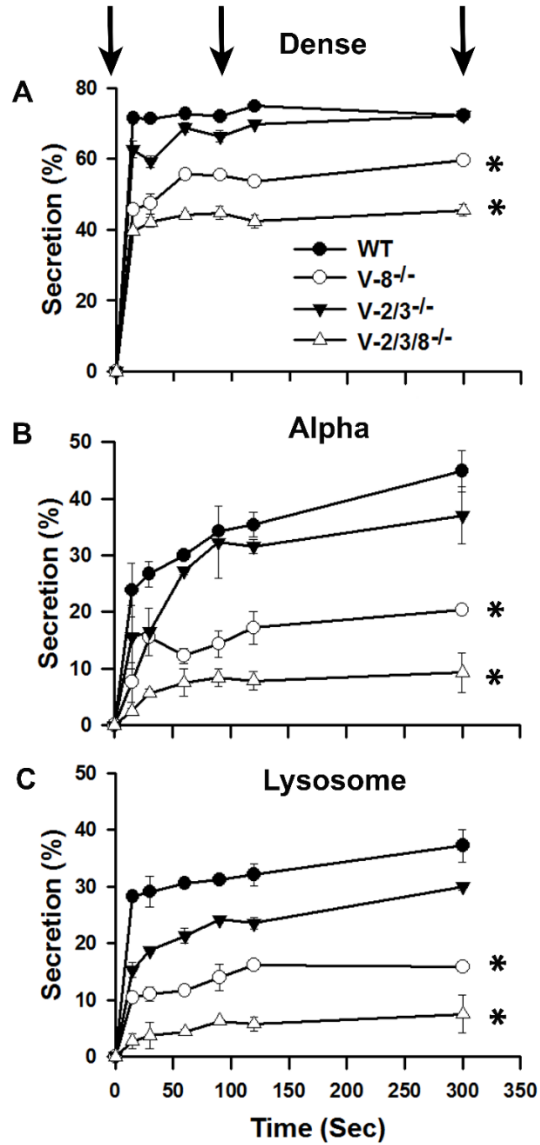


Figure 4.4. Granule secretion is differentially affected in V-8^{-/-}, V-2/3^{-/-} and V-2/3/8^{-/-} platelets

Mouse platelets from indicated VAMP strains were stimulated with 0.1 U/mL thrombin for indicated time points and secretion from each type of granule was measured. A- Dense, B- Alpha, C- Lysosome. Arrows point out resting, 90 sec and 300 sec time points used for further analysis. Statistical analysis done by one-way ANOVA and asterisks represent p value < 0.05.

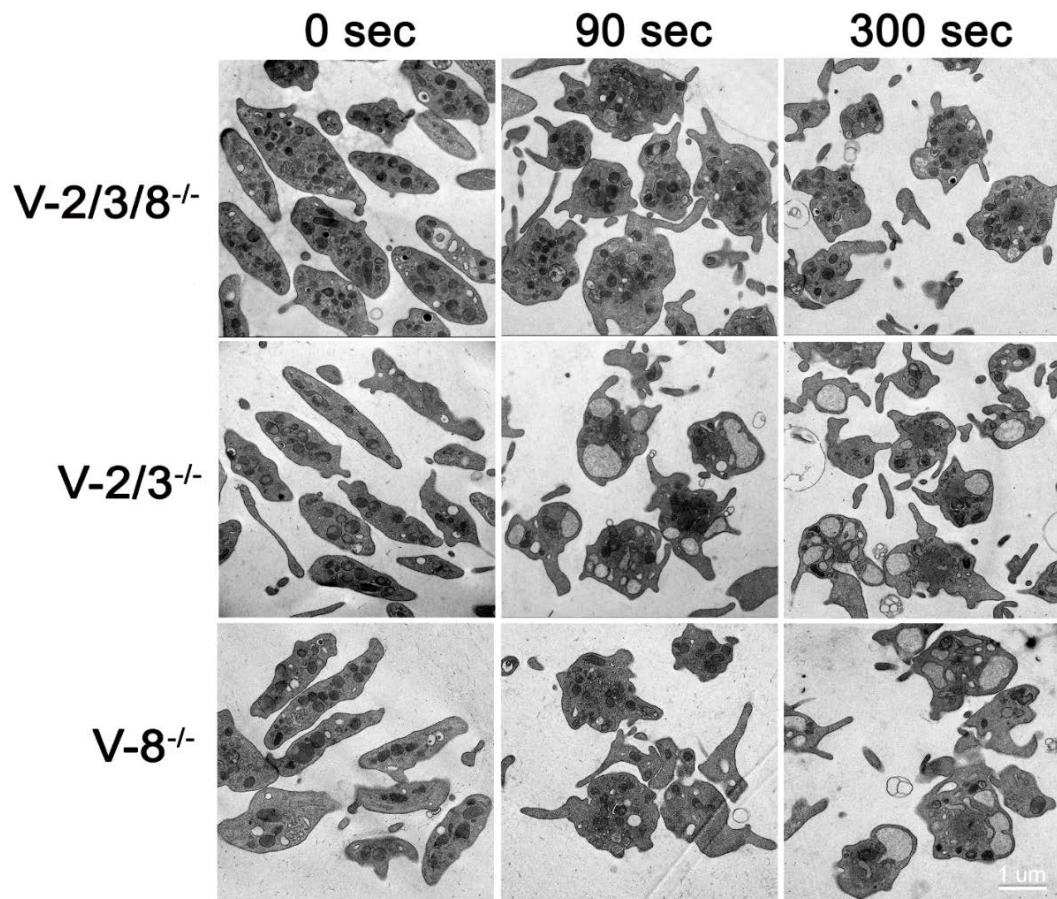


Figure 4.5. V-8^{-/-} platelets exhibit delayed and diminished alpha-granule secretion while it is essentially blocked in V-2/3/8^{-/-} platelets

(A). Thin section electron microscopy of V-8^{-/-} platelets: 0, 90, and 300 sec stimulation with 0.1 U/mL thrombin. (B). Thin section electron microscopy of V-2/3^{-/-} platelets: 0, 90, and 300 sec 0.1 U/mL thrombin stimulation. (C). Thin section electron microscopy of V-2/3/8^{-/-} platelets: 0, 90, and 300 sec 0.1 U/mL thrombin stimulation. The number of platelet profiles scored for each time point ranged from 41 to 50 and the total number of alpha-granule profiles ranged from 403 to 118, the number decreases with compound fusion. Means and standard error of the mean are presented.

Scale bar equals 1 μm.

(Samples generated by Smita Joshi and processing and imaging by Irina Pokrovskaya)

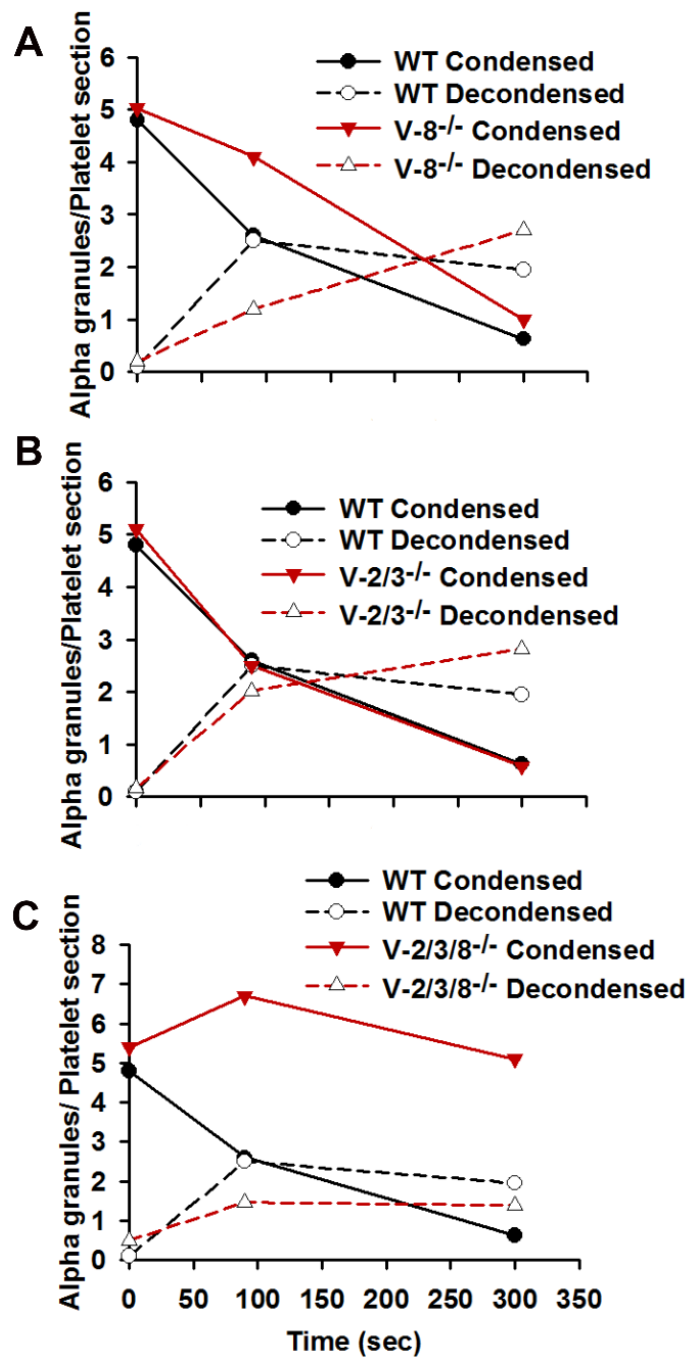


Figure 4.6. Quantification of condensed and decondensed granules from V-8^{-/-}, V-2/3^{-/-} and V-2/3/8^{-/-} platelets.

Quantification of the effect of VAMP-8 deletion (A), VAMP-2 and VAMP-3 deletion (B) or VAMP-2, -3 and -8 deletions (C) on the decondensation kinetics of mouse platelet alpha granules. The number of platelet profiles scored for each time point ranged from 41 to

50 and the total number of alpha-granule profiles ranged from 403 to 118, the number decreases with compound fusion. Means and standard error of the mean are presented.

(Samples generated by Smita Joshi and processing and imaging by Irina Pokrovskaya)

Alpha-granule decondensation is linked to granule-plasma membrane and granule-granule fusion

The data from the time course analysis indicated that decondensation is VAMP-dependent and granule fusion-dependent. This means that granule fusion may provide entry to an extracellular solvent that is necessary for cargo solubilization facilitating the release of these cargoes. To find evidence of this mechanism, we wanted to analyze fusion events and see how they correlate with decondensation. In thin sections (50 nm thickness), granule fusion events were rare. It was not surprising since the sampling volume was comparatively small relative to alpha-granule volume (200-500 nm) and platelet size (2-3 μm diameter). To overcome this challenge, we used thick sections (300 nm) that significantly increase sampling volume for electron tomography.

As shown in Figure 4.7, various possible routes of granule membrane fusion and potential routes of water/solvent influx were noted. These routes include small, short necks (Figure 4.7 A, D white arrows), longer pipes (Figure 4.7 B right arrow), and notably wider openings (Figure 4.7 B- C). All these routes were marked by residual fibers indicating the exodus of the fibrous material from granules. Additionally, multiple compound fusion events (granule to granule fusion) were observed where multiple granules fused to each other either before or after fusing to the plasma membrane (Figure 4.7 C, right arrow). Even in these 300 nm thick sections of platelets, the sample volume was insufficient to study the full granule volume and hence a direct linkage between membrane fusions and granule decondensation could be established for only a small portion of the alpha-granule population. We did not observe any fused granule substantially merge with the plasma membrane. In fact, a granule marked with a dilute luminal matrix remained present in all instances. This suggests that the granule matrix may adhere or associate in some form to the platelet exterior and may also contribute to the release of cargo long after the cargo is dispelled from granules.

Next, we studied the incidence of simple (granule to plasma membrane) or compound (granule to granule) fusion events. Three-dimensional structure of activated platelets was reconstructed based on z tilt series. This technique facilitated analysis of

different modes of fusion, however, though the samples size was bigger (300 nm) compared to thin section (50 nm) microscopy, it was sufficient to provide the full volume of only a few platelets for fusion measurements.

Our data show that the incidence of simple fusion events was dominant in all WT, V-2/3^{-/-} and V-8^{-/-} platelets. These events were lower at early time points but rapidly increased as stimulation time increased (Figure 4.7 E). However, V-2/3/8^{-/-} platelets showed minimal simple fusion events supporting their severe secretion deficiency. Our data show that the incidence of compound fusion events was about three times higher than that of simple fusion events (Figure 4.7 F), perhaps due to some technical challenges that affect detection of simple fusion events. V-2/3^{-/-} platelets showed an increase in compound fusion events compared to WT platelets while the loss of VAMP-8 affected the compound fusion events supporting previous reports suggesting the role of VAMP-8 in compound fusion events (Eckly, Rinckel et al. 2016).

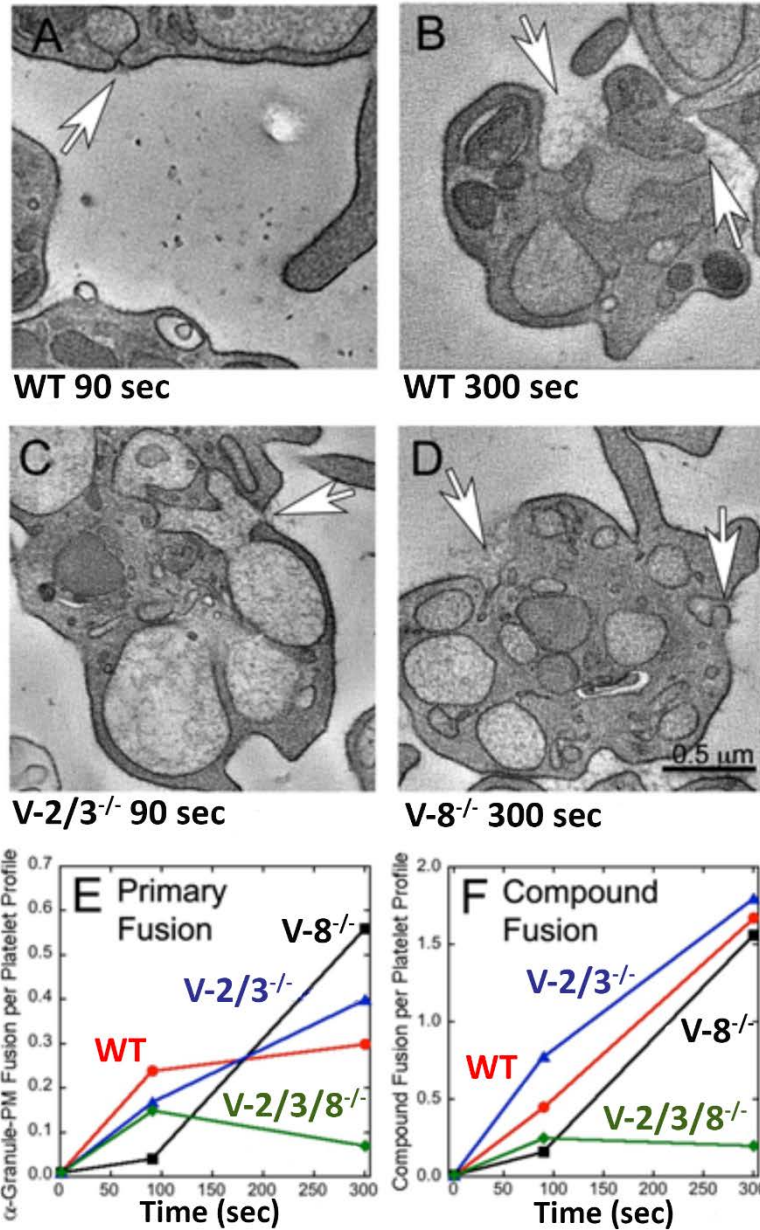


Figure 4.7. Alpha-granule fusion with the plasma membrane strongly correlates with granule decondensation

Examples of simple and compound fusion events are shown with decondensing granules containing fibrous material. An alpha-granule fusion with plasma membrane with a short neck marked by a white arrow (A), and (C). Decondensing granules also showed a wider neck (B). The right white arrow shows a short neck and left arrow shows residual

fibrous material near fusion pore (D). Incidence plot for simple (granule to plasma membrane) fusion events vs time. The average number of platelet profiles scored was 16, range 9 to 28 and the average number of fusion events scored per time point was 3.3, range 0 to 11. Incidence plot for compound (granule-granule) fusion vs time. The average number of platelet profiles scored was 16, range 9 to 28 and the average number of alpha-granule profiles scored per time point was 9.3, range 0 to 28 (F).

(Samples generated by Smita Joshi and processing and imaging by Irina Pokrovskaya)

VAMP-8 depletion is associated with decreased fusion pore diameter

To understand if fusion pore size affects the decondensation and secretion process, we stimulated platelets with 0.1 U/mL thrombin for either 90 sec or for 300 sec and fixed for electron microscopic analysis. We measured the diameters of fusion pores from z stack images (Figure 4.8 I-K) and based on the measurements, classified these pores in three groups: 0-50 nm, 51-100 nm, and more than 100 nm diameter. The data show that fusion pore diameter increases in a time-dependent manner. In WT platelets, upon 90 sec stimulation, few pores have a diameter more than 100 nm while the number increases notably upon 300 sec stimulation time (Figure 4.8 A and E). In absence of VAMP-2 and -3, few fusion pores with less than 100 nm diameter are observed at 90 sec stimulation (Figure 4.8 B), the number increases upon 300 sec stimulation time (Figure 4.8 F). The loss of VAMP-8 however, severely affects fusion pore sizes and which are recovered upon longer stimulation time (Figure 4.8 C and G). The cumulative loss of VAMP-2, -3, and -8 drastically reduces fusion pore size at both shorter and longer stimulation times (Figure 4.8 D and H).

These data suggest that VAMPs may regulate fusion pore size. The loss of VAMPs reduced the pore size at 90 sec stimulation but not at longer (300 sec) stimulation time suggesting that the absence of VAMP-8 is compensated by other residual VAMPs. In contrast, the loss of VAMP-2 and -3 did not have any negative influence at 90 sec stimulation however at 300 sec stimulation time, the incidence of the pores wider than 100 nm slightly decreased. As expected the incidence of fusion events was lower in V-2/3/8^{-/-} platelets.

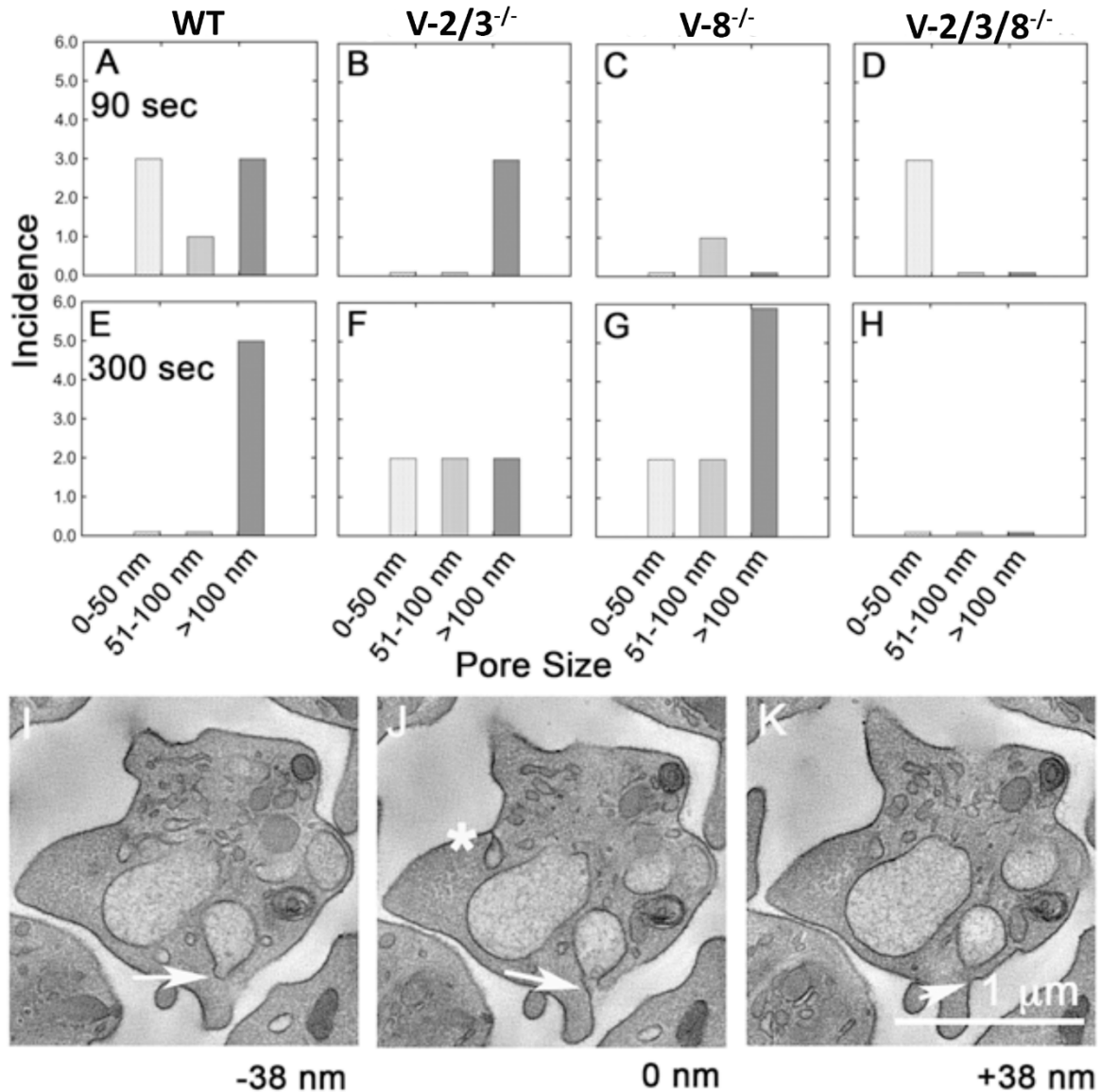


Figure 4.8. VAMP-8 depletion affected fusion pore size.

Platelets from the indicated mouse strains were stimulated for 90 (A-D) or 300 (E-H) sec with 0.1 U/mL of thrombin and fixed for electron microscopy analysis. Maximum pore sizes (diameter) were measured from the appropriate slices of tomograms with a Z-step size of 1.8 nm. Pores of given sizes were grouped into three bins, and the total number of pores counted was used to calculate an incidence value (# pores in a bin/total pores). At the indicated time points, 15-29 platelet profiles were scored for each mouse variant. I-J) Example illustrations showing the information gained by tracing the pore formation

process at different depths, VAMP-2/3^{-/-} platelets. (I). Arrow points to what appeared at -38 nm as a tubular granule extension, in (J) as the maximum diameter membrane fusion pore, and an asterisk marks a very small pore of unknown stability and in (K) as a small indentation of the plasma membrane.

Conclusions

The rate of alpha-granule cargo release is diverse with three different rates that vary from slow to fast release (Jonnalagadda, Izu et al. 2012). Various theories suggested that either there might be different subpopulations of alpha-granules that contain specific cargoes which are released upon specific agonist stimulation or there might be spatial segregation of cargo within the same granule population. A systematic study showed that cargo release is dependent on an agonist potency and kinetically, the rates at which alpha-granule cargoes come out could be divided into three groups: fastest, medium and slow release. Though the kinetic heterogeneity could explain the nature of the release, it was not clear how does this heterogeneity arise.

Here we address this question by using VAMP-deficient mouse strains that show secretion defects of various ranges and modern microscopic techniques- high-pressure freeze for sample preparation and scanning electron microscopy and three-dimensional tomography for imaging. The data indicate that the decondensation of granule cargo is an agonist-dependent and stimulation time-dependent process. A granule fusion is necessary to occur for cargo decondensation to take place. The rate of solubilization may dictate the rate of cargo release even after the complete exodus of cargo from the fused granule.

The insight from this study provides a mechanistic understanding of how the rates of release vary depending on the strength of an agonist or the duration of stimulation. These findings are crucial for understanding the rates and extents of platelet alpha-granule secretion since platelet secretion regulates the thrombus composition. This study is unique because it synchronizes the biochemical analysis of platelet secretion to granule morphology. Moreover, it also presents platelet secretion as a model system to study mechanistic details.

Chapter 5 Role of VAMPs in intracellular trafficking

Introduction

Intracellular trafficking in platelets is a crucial process for granule biogenesis and cargo packaging. Studies focused on dense and alpha granulopathies have been instrumental in shaping our understanding of these processes. Lack of granule cargo in these SPDs leads to multiple anomalies including lack of pigmentation, significant immunodeficiencies, and most importantly, severe bleeding diathesis. Though our understanding of these cellular events has expanded in recent years, a clear picture is yet to emerge.

Platelets contain translation machinery, and can *de novo* synthesize at least some part of their proteome such as PF4. Additionally, they also endocytose proteins from plasma (e.g., fibrinogen) to survey their microenvironment. Endocytosed cargo travels through early and late endosomes and multivesicular bodies, before being trafficked to the alpha granules while *de novo* synthesized cargo could be trafficked to the endosomal system or directly to granules (Figure 6.6). Recent reports suggest that Nbeal2, VPS 33B, and VPS 16B play roles in these trafficking events, though mechanistic details are still unknown (Gissen, Johnson et al. 2004, Lo, Li et al. 2005, Cullinane, Straatman-Iwanowska et al. 2010, Albers, Cvejic et al. 2011, Gunay-Aygun, Falik-Zaccai et al. 2011, Kahr, Hinckley et al. 2011, Bem, Smith et al. 2015).

VAMP isoforms are known to play roles in membrane trafficking in other systems as discussed earlier in Chapter 1. In platelets, VAMP-3 is crucial for endocytosis. Roles of other VAMP isoforms are not clear since V-7^{-/-} and V-8^{-/-} platelets do not show any changes in cargo levels suggesting compensation by other VAMPs. We hypothesized that deletion of multiple VAMP isoforms will affect intracellular trafficking in both endocytic as well as synthetic pathways influencing cargo levels in these platelets. To address this, we studied the morphology of VAMP deficient platelets and using arrays we probed for the extent of deficits in cargo levels. We found that alpha-granules are smaller and more numerous in V-2/3/8^{-/-} platelets and cargo levels are notably

decreased. This indicates that the loss of multiple VAMP isoforms affects platelet intracellular trafficking.

Results

Fibrinogen levels are decreased in V-2/3^{-/-} and V-2/3/8^{-/-} platelets

Western blot analysis of extracts from V-2/3^{-/-}, V-8^{+/-}(2/3)^{-/-} and V-2/3/8^{-/-} platelets show that fibrinogen levels were diminished by about 20%, 20% and 50% in these strains, respectively (Figure 3.3). VAMP-3 plays a crucial role in endocytosis and the absence of VAMP-3 leads to a defect in fibrinogen uptake. These data show that progressive loss of VAMP-8 in addition to the absence of VAMP-2 and VAMP-3 further depresses fibrinogen intake in these platelets causing a significant decrease in fibrinogen levels in V-2/3/8^{-/-} platelets. A deficiency of VAMP-3 in the global KO mouse strain leads to about a 50% decrease in fibrinogen uptake while it is only about 20% diminished in platelet-specific V-2/3^{-/-} platelets suggesting that the expression of the PF4 promoter-driven Cre recombinase at early stages may contribute to this phenotype.

Selected dense granule, alpha-granule, and lysosomal cargoes were unaltered in V-8^{-/-}, V-2/3^{-/-} and V-2/3/8^{-/-} platelets

To study if the loss of VAMP-2, -3 and -8 causes any defects in cargo uploading in dense granules, we incubated washed platelets with [³H] serotonin for 45 min at 37°C. After washing to remove excess serotonin, we stimulated platelets with either various concentrations of agonists or at different time points to measure the serotonin release from platelets. We calculated total serotonin uptake levels in the supernatant (releasate) and pellet. The data showed that there was no significant difference among the levels of serotonin uptake in WT, V-8^{-/-}, V-2/3^{-/-} and V-2/3/8^{-/-} platelets suggesting that cargo uptake in dense granule is not affected in these strains (Figure 5.1 A). PF4 is a *de novo* synthesized protein in platelets. Using commercial PF4 sandwich ELISA assays, we measured the total amount of PF4 in these platelets (releasate +pellet) and found no difference among strains indicating that PF4 synthesis and/or trafficking is not altered in these strains (Figure 5.1 B). Similarly, we also measured the soluble lysosomal cargo β-hexosaminidase levels among these strains and found no difference (Figure 5.1 B).

These data show that soluble cargo levels in dense, alpha and lysosomes were not affected in these strains.

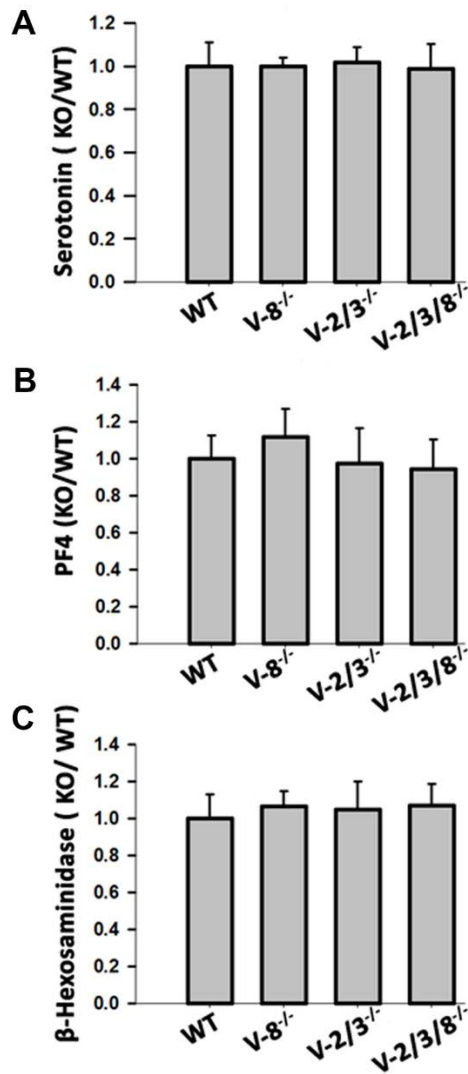


Figure 5.1. Dense, alpha, and lysosomal cargo levels are normal in V-8^{-/-}, V-2/3^{-/-} and V-2/3/8^{-/-} platelets

Cargo levels were calculated from *in vitro* platelet secretion assays. Total serotonin levels indicate tritiated serotonin uptake in platelets PF4 levels were calculated from sandwich ELISA and β-hexosaminidase levels were calculated based on the colorimetric assay (see methods for details). Cargo levels in KO strains were normalized by levels in WT platelets. Data are representative of three independent experiments Student's t-test indicates no significant difference.

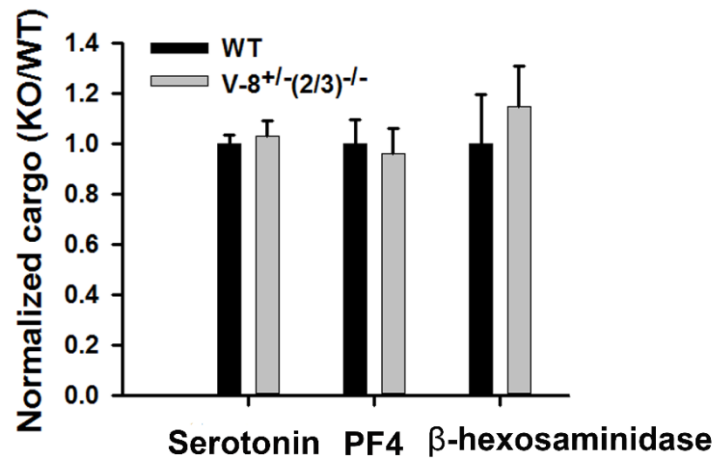


Figure 5.2. Dense, alpha and lysosomal cargo levels are normal in V-8^{+/-}(2/3)^{-/-} platelets

Cargo levels were calculated from *in vitro* platelet secretion assays. Total serotonin levels indicate tritiated serotonin uptake in platelets PF4 levels were calculated from sandwich ELISA and β-hexosaminidase levels were calculated based on the colorimetric assay. (see methods for details). Cargo levels in KO strain were normalized by levels in WT platelets. Data are a representative of two independent experiments. Student's t-test indicates no significant difference.

V-2/3/8^{-/-} platelets have smaller alpha-granules

To study if the loss of multiple VAMP proteins affects platelet morphology, we used a novel approach. It has been shown that even minimal handling and washing could stimulate platelets (Pokrovskaya, Aronova et al. 2016). To prevent that, platelets were immediately fixed while drawing blood through cardiac puncture and prepared for microscopic studies.

SEM images show that the gross morphology of platelets was not affected in these VAMP KO strains. Platelet granules, mitochondria, and microtubules were evident in micrographs. Though the size of alpha-granules was not affected in V-8^{-/-} and V-2/3^{-/-} platelets, alpha-granules in V-2/3/8^{-/-} platelets were about 30% smaller than controls suggesting that the granule genesis might be affected in these platelets. Though smaller in size, the number of alpha-granules was not increased in these platelets. The loss of VAMP-8 or the loss of VAMP-2 and -3 did not influence the granule size or number suggesting that the loss of one or two isoforms does not influence granule formation.

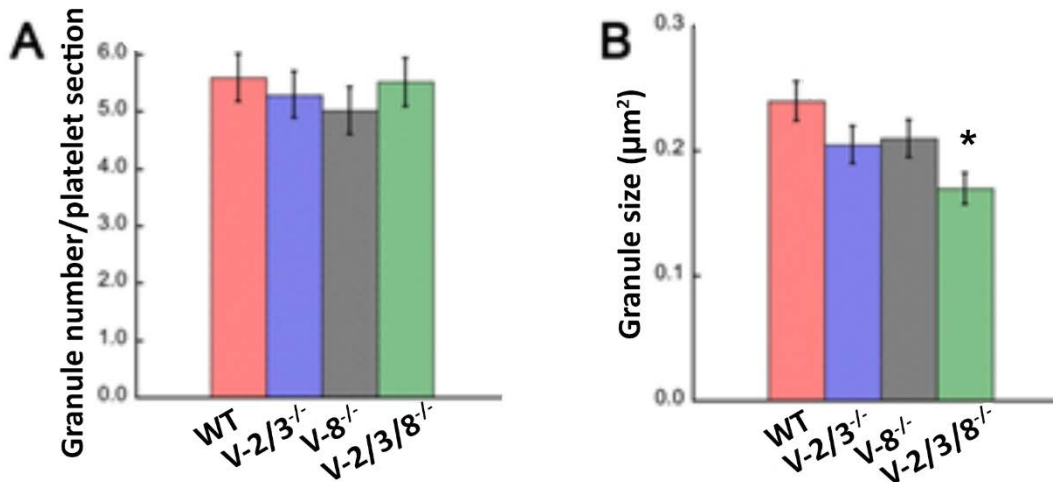


Figure 5.3. *The loss of VAMP-2, -3 and -8 affects alpha granule size but not granule numbers per platelet.*

Thin section, electron microscopy images of platelets from the indicated mouse strains were analyzed for granule number per platelet profile (A). and cross-sectional granule area (B). Fifty platelet profiles were scored for each mouse strain and the averages and standard deviations are presented. Student's t-test was used to establish statistical significance as indicated.

V-2/3^{-/-} and V-2/3/8^{-/-} platelet granules have a trafficking defect

We used antibody arrays to determine if these platelets have any cargo defects. We measured alpha-granule cargo platelet factor 4\PF4 using ELISA assays which showed no significant difference among these strains (Figure 5.1 B). PF4 levels were used as an internal control to interpret the results from antibody arrays. Array data showed that PF4 levels were not altered significantly in V-8^{-/-} (Figure 5.4), V-2/3^{-/-} (Figure 5.5), or in V-2/3/8^{-/-} (Figure 5.6) validating our findings from ELISA assays and indicating that the cargo deficits noted using these antibody arrays could be reproducible. The results show that there is a global deficit in protein levels in these platelets. VAMP-8 deficient platelets show a milder defect than V-2/3^{-/-} platelets. This defect progressively gets worse in V-2/3/8^{-/-} platelets. It should be noted that nothing is completely missing from V-2/3/8^{-/-} platelets. All proteins tested are present, but the efficacy of biogenesis/trafficking/packaging seems to be much more heterogeneous. To see if a specific cargo is more affected than another, we analyzed pro- vs anti-angiogenic molecules, growth factors, tissue factors, chemokines, and cytokines and found that all classes of proteins were decreased in some extent indicating that overall trafficking might have been affected.

Protein levels in V-8^{-/-} platelets (Figure 5.4 and Table 5.1) were not much affected but levels in V-2/3^{-/-} platelets (Figure 5.5 and Table 5.1) were noticeably decreased supporting our earlier report of the role of VAMP-3 in endocytosis and vesicular trafficking in platelets. Loss of all three isoforms further affected the levels. VAMP-2/3/8^{-/-} platelets showed that more than 50% of cargo tested diminished by at least 20% or more indicating that the loss of multiple VAMP isoforms has a global effect on intracellular trafficking (Figure 5.6 and Table 5.1).

Conclusions

The role of SNAREs in intracellular trafficking is well known, however, it is not yet clear how they participate in trafficking in platelets. A recent report shows that VAMP-3 is crucial in platelet endocytosis though the roles of other VAMPs remain unclear. Here using our wide array of VAMP deficient strains, we address the question how the loss of VAMPs affects intracellular trafficking in platelets.

Using antibody arrays, we found that there is a global defect in cargo levels in absence of VAMP isoforms and the extent of this defect increases with the loss of multiple VAMP isoforms. This suggests that individual VAMP isoforms facilitate intracellular trafficking, however, their loss is compensated by other residual VAMP isoforms. $V-8^{-/-}$ and $V-2/3^{-/-}$ platelets show some decrease in cargo levels suggesting that the functional redundancy among VAMPs may have dampened the effects of their loss. However, deletion of three major isoforms in $V-2/3/8^{-/-}$ cannot be efficiently compensated for by residual VAMP-7, which is also reduced by about 50%. This leads to smaller and more numerous alpha-granules with remarkably diminished cargo levels in $V-2/3/8^{-/-}$ platelets.

Thus, findings from these studies show that VAMPs are indispensable for trafficking and further studies are needed to specifically determine roles of each isoform. The results from these studies will have a major impact not only hemostatic functions of platelets but also immunological roles of platelets that are newly emerging.

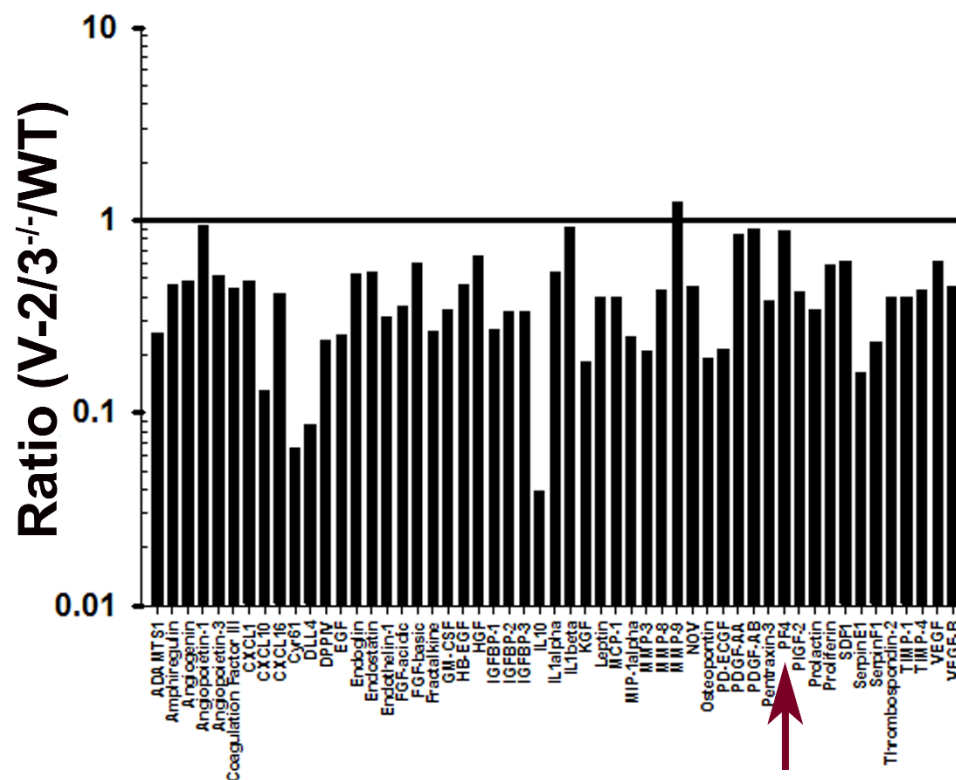


Figure 5.5. Granule cargo deficiency in V-2/3^{-/-} platelets

Platelet extracts were prepared from WT or V-2/3^{-/-} mouse and probed using duplicate Mouse Angiogenesis Antibody Arrays (see Methods). The fluorescence intensity from 2 individual experiments was used to calculate a V-2/3^{-/-} /WT ratio for each protein detected on the array. The average of the 2 calculated ratios is plotted. Note that the y-axis is a log scale.

The red arrow on the x-axis shows the normalized levels of PF4.

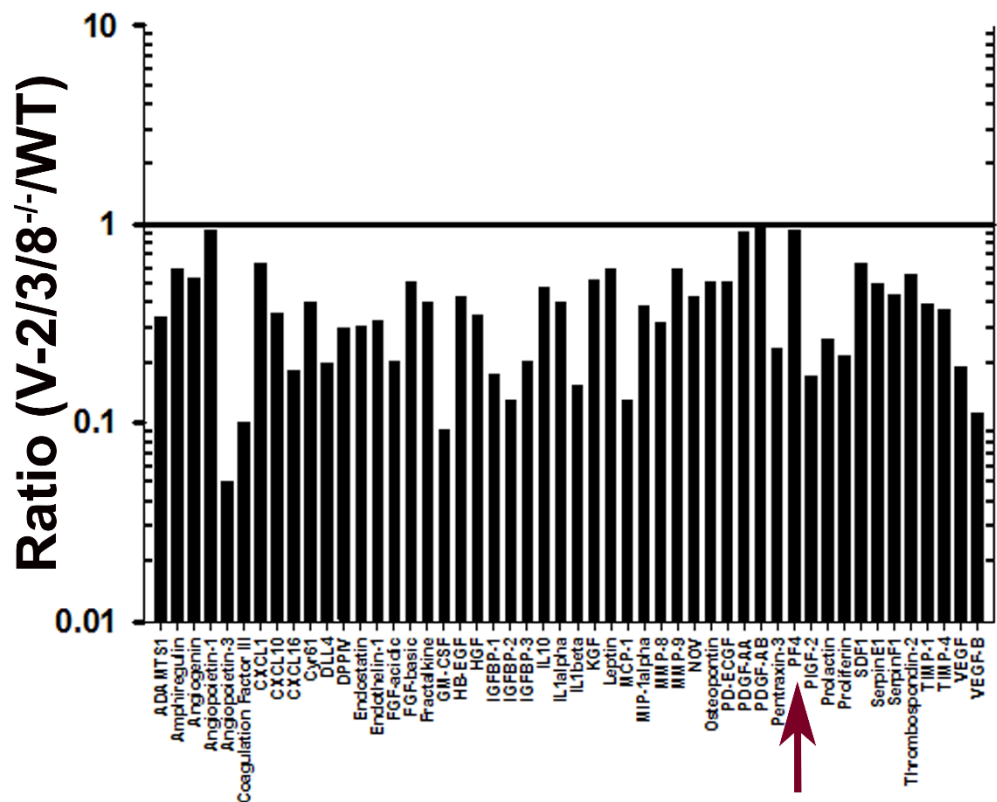


Figure 5.6. V-2/3/8^{-/-} platelets have reduced granule cargo levels

Platelet extracts were prepared from WT or V-2/3/8^{-/-} mouse and probed using duplicate Mouse Angiogenesis Antibody Arrays (see Methods). The fluorescence intensity from 2 individual experiments was used to calculate a V-2/3/8^{-/-} /WT ratio for each protein detected on the array. The average of the 2 calculated ratios is plotted. Note that the y-axis is a log scale.

The red arrow on the x-axis shows the normalized levels of PF4.

Table 5.1 Summary of cargo levels in V-8^{-/-}, V-2/3^{-/-} and V-2/3/8^{-/-} platelets

Amount of cargo	V-8^{-/-}	V-2/3^{-/-}	V-2/3/8^{-/-}
25% or less	0	1(2%)	5(9%)
25-50%	14(26%)	22(42%)	26(49%)
50-75%	27(51%)	23(43%)	20(38%)
75-100%	11(21%)	6(11%)	2(4%)
100% and above	1(2%)	1(2%)	0
Total	53(100%)	53(100%)	53(100%)

Chapter 6 Discussion

Overview

Cardiovascular diseases/CVDs contribute to more than 30% of all deaths worldwide (American Heart Association Statistics, 2017). Previously thought to be diseases of affluence, CVDs have noticeably permeated the developing world and has become the number one contributing factor to morbidity and mortality, causing one out of every four deaths. Despite impressive advances in anticoagulant and antiplatelet therapies in the last few decades, the current antithrombotic repertoire still lacks precision at which occlusive thrombosis could be prevented without risking spurious bleeding (Khurram, Chou et al. 2006, Holmes, Kereiakes et al. 2009, Berger, Bhatt et al. 2010, Hira, Kennedy et al. 2015). The ongoing debate on the efficacy of combined antiplatelet drugs such as aspirin and clopidogrel with potent anticoagulant like warfarin suggests that new armamentarium of antiplatelet drugs that will achieve this much desired hemostatic balance is urgently needed (Dewilde and Berg 2009, Gao, Zhou et al. 2011, Zhao, Zheng et al. 2011). Platelets are indispensable in maintaining the vascular integrity and therefore, a clear understanding of the optimal level of platelet secretion will aid in the management of anti-thrombotics without unwanted bleeding.

Platelet secretion at the site of vascular injury decides the fate of thrombus formation and subsequent recovery. A plethora of research has been done on a wide range of molecules that play roles in platelet secretion. These molecules include, but are not limited to, SNAREs- (both v and t - SNAREs) (Ren, Barber et al. 2007, Koseoglu, Peters et al. 2015), SNARE regulators (STXBP5/Tomosyn), Rab effectors (granuphilin), tethering factors (Munc 18b, Munc 13b), GEFs (Cal DAG) and GAPs (RASA3), trafficking molecules (Rab 27b) and endocytosis players (dynammin). These molecules have been attributed to a wide range of hemostatic roles and could be used as potential therapeutic targets. However, uncertainty about how much secretion from platelets is necessary to balance hemostasis occludes their therapeutic promise.

Platelet granule release is a remarkably dynamic event. These small cellular fragments are expected to rapidly form a dense aggregate under high-pressure blood

flow in a closed vascular system to stop bleeding. Localized release at the site of vascular injury to stop bleeding is inefficient considering the high shear force present in vessels. To overcome this challenge, platelets are armed with various strategies that help control the nature of the fusion event and the cargo release. It is essential to appreciate this complexity to control platelet function and find an ideal hemostatic balance.

With advancing technologies, our understanding of platelet secretion has significantly improved in the last two decades, however, on a cellular level, many mechanistic details of cargo release are still under investigation. First, what determines the rates and the extent of granule cargo release? Do these factors have a similar effect on all granule release events, *i.e.* dense, alpha and/or lysosomal release? Does the nature of fusion events- simple vs compound- contribute to determining the rate and the degree of granule secretion? Does the composition of granule cargo, solubility and/or a presence of a scaffold protein decide the nature of fusion event? Could these characteristics of the rate and the degree of secretion be manipulated to control thrombosis upon vascular injury? This Chapter describes how my work addresses these important questions. The first part of this dissertation addresses the questions about how much granule secretion is necessary to attain hemostatic balance where occlusive thrombosis is prevented without triggering spurious bleeding while the second part describes how granule cargo release is dependent on multiple factors that ensure the optimal effect of diverse cargo at the site of vascular injury.

Part I- Titrating VAMPs to achieve hemostatic balance

The nature of platelet secretion is a decisive factor in maintaining hemostatic balance. Multitudes of reports indicate that defective or diminished platelet secretion affects hemostasis in varying degrees. These defects either disturb platelet granule formation as noted in granule storage pool deficiencies/SPDs or they influence cargo trafficking and or granule fusion machinery (Joshi and Whiteheart 2017). Additionally, GWAS studies indicate that SNPs in some platelet secretion machinery such as VAMP-8 (Shiffman, Rowland et al. 2006, Shiffman, O'Meara et al. 2008) and microRNAs that

control their expression (Kondkar, Bray et al. 2010), contribute in hyperreactive platelets leading to increased susceptibility of these individuals to cardiovascular events. Though its importance is well established, the exact nature of platelet secretion remains elusive.

To decode the nature of platelet secretion we addressed the following series of questions. We asked how the rates and the extent of cargo release are controlled. How the nature/mode of granule fusion affects the secretion. How the dense granule vs alpha-granule release regulates the progression and the architecture of thrombus formation. And most importantly, how much platelet secretion is necessary to achieve hemostatic balance.

To address these questions, we turned to the platelet secretion machinery. Though t-SNARE usage syntaxin-11, syntaxin-8, and SNAP23 are imperative for platelet secretion, v- SNARE usage was not clear. Using V-8^{-/-} animals and tetanus toxin treated platelet permeabilization assays, we showed that in platelets, VAMP-8 is a primary v-SNARE while VAMP-2 and -3 play accessory roles (Ren, Barber et al. 2007). Recently, VAMP-7 has been shown to play a role in platelet secretion as well (Koseoglu, Peters et al. 2015). Based on these data, we decided to create a system by altering the types and amounts of various VAMP isoforms in platelets. *Our hypothesis was that each VAMP isoform differentially contributes to granule secretion process. By manipulating the levels and types of these isoforms, we could determine the threshold level of secretion that will inhibit occlusive thrombosis without triggering unwanted bleeding.*

Quantification of VAMPs in murine strains

Using the quantitative data based on western blots, we could estimate how much of total VAMP isoforms are left in each of these VAMP-deficient strains. There are multiple studies on platelet proteomics, both human (Burkhart, Schumbrutzki et al. 2012) and mouse (Zeiler, Moser et al. 2014) platelets with minor differences. We used quantitative blot data (Graham, Ren et al. 2009) to compare and analyze various results presented here (Table 6.1 and Figure 6.1 A). Based on this quantitation, we show that our mouse models display an impressive, wide array of VAMP isoforms and amounts in

mouse models. These include amounts from 100% in WT to about 13% in V-2/3/8^{-/-} animals.

Secretion and hemostatic balance

The loss of a primary v-SNARE, VAMP-8, leads to a significant secretion deficiency that is mainly manifested in alpha and lysosomal release other than in dense granule release (Figure 6.1 C). These deficiencies do not lead to any bleeding defect but instead delay and diminish thrombus formation (Graham, Ren et al. 2009). On the other hand, VAMP-2/3^{-/-} platelets secrete normally and do not have any hemostatic or thrombotic deficiencies indicating their ancillary roles in platelet secretion (Figure 6.1 C and D). V-2/3/8^{-/-} platelets show significantly reduced secretion from all three granules but predominantly affect alpha and lysosomes. These animals represent an extreme imbalance in hemostasis with increased bleeding and failure to form a thrombus. The only major VAMP isoform that is present in these strains is VAMP-7. The Flaumenhaft group showed that V-7^{-/-} platelets had ATP secretion defects as well as alpha-granule secretion deficiency (Koseoglu, Peters et al. 2015). In fact, the loss of VAMP-7 displays similar patterns of dense and alpha-granule secretion deficit as observed in V-8^{-/-} platelets (Table 6.1). However, these defects do not translate into elevated bleeding times or defective thrombus formation post laser injury indicating that VAMP-7 loss could be well compensated by VAMP-8 and the secondary VAMPs (Koseoglu, Peters et al. 2015). These data beg the question of what causes this difference in hemostasis and thrombosis in V-7^{-/-} vs in V-8^{-/-} animals.

Mass action and/or intrinsic property

Since both VAMP-7 and VAMP-8 are critical for platelet secretion and are abundantly present in platelets, the question arises of whether their abundance makes them vital for secretion or whether there is an intrinsic property of these isoforms that is central to their roles in secretion. Based on our quantitative analysis and its correlation with secretion events and hemostasis, an abundance of VAMP isoforms is not the only decisive factor in secretion process (Figure 6.1). It is mainly evident in case of V-8^{+/-}(2/3)^{-/-} platelets that harbor only about 30% of total VAMP isoforms (Table 6.1)

and yet do not have any secretion deficiency or hemostatic defects (Figure 3.5 and Figure 6.1).

To address this question, we compared protein sequences of mouse VAMP-2, -3, -7 and -8 and using alignment analysis studied how identical all these isoforms are from each other. We found that VAMP-2 and -3 share about 70% identity on the entire protein sequence (Figure 6.2 and Table 6.2) and about 98% identity within SNARE domains, which could explain their redundant roles in secretion (Figure 6.3 and Table 6.3). On the other hand, VAMP-7 and -8 share only about 30% amino acid sequence identity and their SNARE domains share only about 40% identity with each other (Figure 6.3 and Table 6.3). Despite these different SNARE domains, VAMP-7 and -8 have almost similar rates and extents of cargo release. One possibility might be that the differences in SNARE domain and transmembrane/TM domains influence fusogenicity of these isoforms. The role of TM domains of v-SNAREs has been vigorously studied. Replacement of the VAMP-2 TM domain with membrane spanning lipids led to SNARE complex formation but not fusion (McNew, Weber et al. 2000). Similarly, point mutations in TM domains failed to fuse, underlining the importance of TM domains in granule fusion (Fdez, Martinez-Salvador et al. 2010, Shi, Shen et al. 2012). Though most of these studies focused on VAMP-2, it is reasonable to expect a similar pattern with other VAMP isoforms. These data suggest that minor differences in TM domains may become instrumental in the transduction of force on lipid bilayer that changes the outcome of a fusion event.

Table 6.1 Summary of characterization of VAMP KO strains

Mouse Strain	% Remaining	Alpha (%)	Dense (%)	Lysosome (%)	Bleeding Time (sec)	Thrombosis Time (min)	PS exposure	Spreading
WT	100	100	100	100	250.07±86.82	6.55±2.48	100	
V-3 ^{-/-}	89.5	100	100	100	275.08±92.27	NA	NA	Increased
V-7 ^{-/-}	87	55	66	NA	NA (no defect)	NA	NA	Decreased
V-8 ^{+/-}	80	100	100	100	288.27±104.42	7.16±2.53	100	NA
V-2/3 ^{-/-}	53	100	100	100	279.85±107.06	7.12±1.87	70	No change
V-8 ^{+/-} (2/3) ^{-/-}	33	100	100	100	269.05±108.98	6.75±2.9	70	NA
V-8 ^{-/-}	60	62	85	70	281.23±71.24	21.27±8.68	100	Decreased
V-2/3/8 ^{-/-}	13	12	49	50	571.74±72.61	28±5.29	40	Decreased

NA- Not Available

Percent of VAMPs remaining is based on quantitative blotting data from (Graham, Ren et al. 2009).

The table is based on the characterization of V-3^{-/-} (Schraw, Rutledge et al. 2003), V-8^{-/-} (Ren, Barber et al. 2007) and V-7^{-/-} (Koseoglu, Peters et al. 2015) while V-2/3^{-/-}, V-8^{+/-}(2/3)^{-/-} and V-2/3/8^{-/-} are characterized in this dissertation work. The quantification of granule secretion percentage is based on 0.05U/mL thrombin stimulation for 2 min (see figure 3.4)

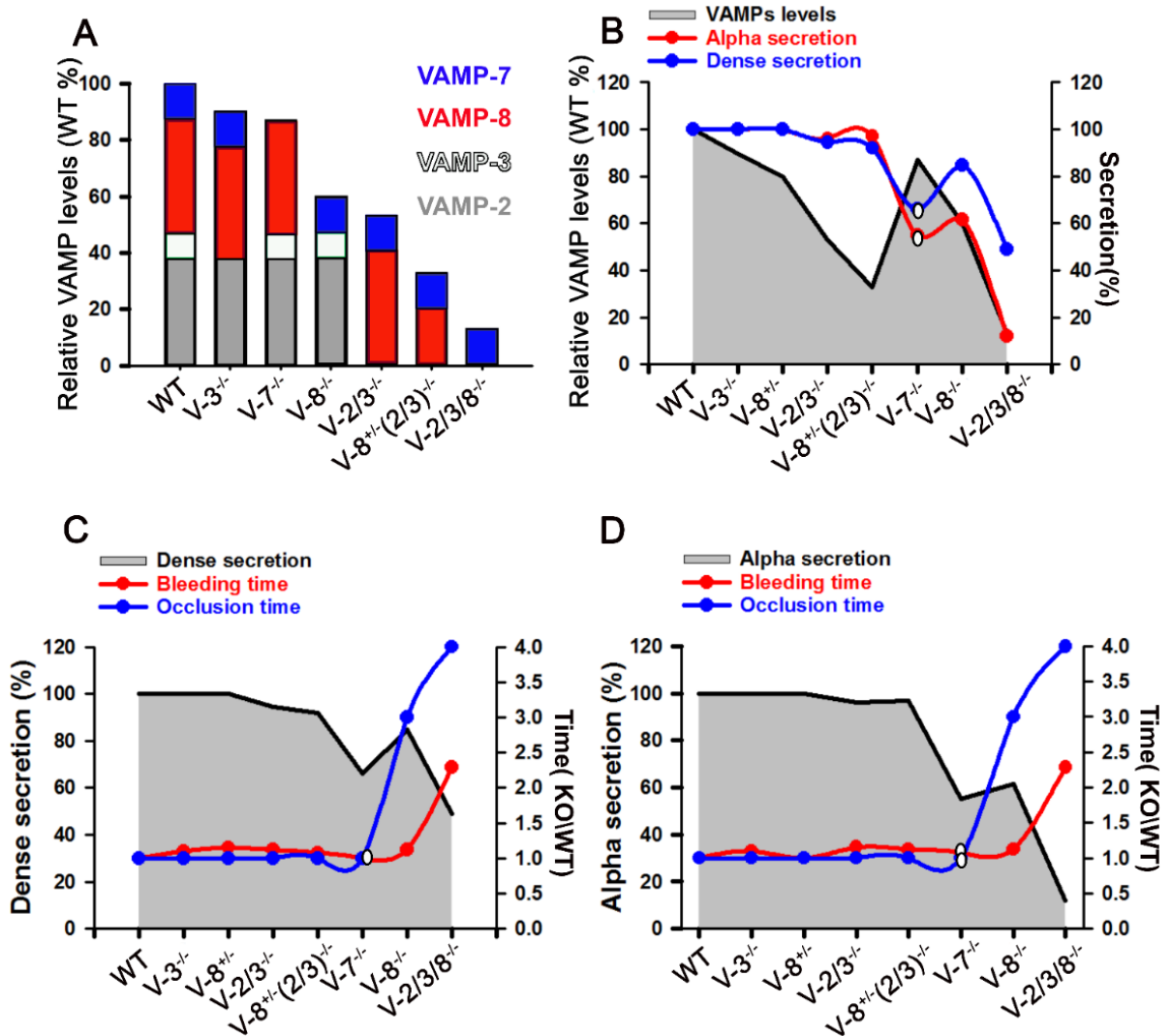


Figure 6.1. Correlating VAMP-mediated exocytosis to platelet function

VAMP levels (per molecule), secretion, and function are graphically correlated using our data and that from (Koseoglu, Peters et al. 2015). (A). Predicted VAMP levels remaining, relative to WT, in each VAMP-deficient strain were calculated from data in (Graham, Ren et al. 2009). (B). The VAMP levels in each strain are plotted as gray fill and the red line indicates alpha-granule secretion and the blue line indicates dense granule secretion. Secretion data are based on platelet stimulation with 0.05 U/mL thrombin for 2 min (see Figure 3.4). Secretion from dense and alpha-granules of V-7^{-/-} platelets (open symbols) are estimated based on ATP release and P-selectin exposure, respectively (Koseoglu, Peters et al. 2015). All values are normalized to WT. (C). Dense granule

release and (D). alpha-granule release values are same as in B and are plotted as gray fill. The red line indicates tail bleeding times and the blue line indicates the occlusive thrombosis times in the FeCl₃ injury model. Data are normalized to WT.

Another possibility for different fusion rates and extents is if VAMPs represent a specific type of granule and regulate its size and composition. It remains to be seen if VAMP distribution on granules is exclusive with only one VAMP isoform decorating the granule. Peters et al., showed that VAMP-3 and VAMP-8 positive granules are centralized and form a granulomere while VAMP-7 positive granules translocate to periphery upon platelet spreading. However, it is not clear if or what percent of a granule population was labeled with multiple VAMP isoforms.

Furthermore, in a recent study, Jena et al., showed that VAMP-3, -7 and -8 specific vesicles isolated from human platelets exhibit distinct sizes and proteomes (Jena, Stemmer et al. 2017). The authors used osmotic lysis of platelets to isolate vesicle/granule populations and immunoprecipitation to further separate specific VAMP-containing vesicles. They performed atomic force microscopy and mass spectrometry to further characterize these vesicles. They found that vesicles harboring VAMP-3 were about 50% smaller than the vesicles displaying VAMP-8 (Jena, Stemmer et al. 2017). Considering smaller spherical vesicles would be more fusogenic than the bigger ones due to high curvature stress, the lack of VAMP-3 should decrease or at least delay the release of these vesicles which is not in agreement with previously published findings and data presented in this work (Schraw, Rutledge et al. 2003). Though these experiments were done in human platelets and there might be some species-specific differences, it seems improbable that granules will be decorated with only one type of VAMP isoform. However, the distribution of VAMP isoforms may offer some variability in the fusion rates. Further studies are underway to study this mechanism and since our animal models have a gradient of various VAMP isoforms and their levels, they will be instrumental in the further investigation.

Thrombus architecture and secretion deficiency

Thrombus architecture has been an active area of research for decades. In a series of reports, the Brass group illuminated how thrombus formation is heterogeneous by using experimental and computational models. They showed that the thrombus core is formed by maximally stimulated platelets (P-selectin positive) while

the shell is composed of minimally stimulated platelets (P-selectin negative) that are barely tethered to each other. The activation gradient from the central core to the outer shell layer controls the thrombus porosity regulating trafficking of agonists and other molecules (Stalker, Welsh et al. 2014, Tomaiuolo, Stalker et al. 2014, Welsh, Stalker et al. 2014, Welsh, Poventud-Fuentes et al. 2017). From our data, we can speculate that low agonist doses and early time points in time course analysis resemble platelet activation and secretion in shell region while data at high agonist concentrations and later time points in time course analysis resemble what occurs in a core region of a thrombus. Based on the correlation between a dense and alpha-granule release with hemostasis and thrombosis, it seems that the loss of VAMP-2, -3 and -8 affected alpha-granule release to a greater extent than it did dense granule release, and this may be a deciding factor in thrombosis (Figure 6.1 and Table 6.1). This is also evident in dense and alpha- granulopathies where patients with HPS and CHS who lack dense granules, have severe bleeding defects, as well as Munc13-4 KO mice which are deficient in dense granule secretion, also have a severe bleeding defect (Ren, Wimmer et al. 2010). In contrast, patients with GPS and ARC syndrome have a wide range from mild to severe bleeding diathesis. This pattern in hemostatic deficiencies in these SPD patients suggests that dense granules are probably more significant for hemostasis than alpha-granules. Alpha-granules are perhaps more critical for thrombosis. From our data, VAMP-8 deficiency falls in a threshold range where the rate and the degree of thrombosis are affected but a stable thrombus does form without causing bleeding (Figure 3.9 and 6.1).

Implications of the rate and extent of granule secretion

Two parameters crucial to decide the fate of thrombosis are how fast/slow the cargo from platelet granules is released and how much of it is released. As discussed earlier, based on dense storage pool deficiencies/SPDs, the extent of cargo release is crucial for thrombosis. Lack of dense granules in HPS and CHS leads to severe bleeding. The extent of alpha-granule secretion does not seem to be as critical since a wide range of bleeding defects- mild to severe are observed in patients with GPS and ARC syndrome, major alpha-granule SPDs. V-8^{-/-} platelets show slower and lower release at

early time points and at lower agonist concentrations, but the extent of secretion increases at later time points and higher agonist concentrations. These animals are protected from occlusive thrombosis and spared from bleeding defect (Figure 6.1). This argues that if the extent of cargo release is within a reasonable range of that of control, the rate of release has a minimal impact on thrombus formation. However, significantly delayed secretion may not be effective to recruit and retain other platelets affecting thrombus growth. The rate of cargo release is important in burst phase because it dictates how fast/slow the thrombus will form. Slower release affects the buildup of the thrombus as seen in $V-8^{-/-}$ animals because of delayed platelet recruitment that limits thrombus growth while, as seen in $V-2/3/8^{-/-}$, the low extent of release may be insufficient to provide the necessary stimulus to recruit and hold on to other platelets. Based on our *in vitro* assays measuring platelet granule secretion and various *in vivo* studies to assessing hemostasis and thrombosis, we show that the loss of about 40-50% of secretion is well tolerated to gain protection from thrombosis without causing bleeding.

Limitations of the study

The observations in this study are mainly based on *in vitro* assays using platelet suspension or *in vivo* assays- tail bleeding and $FeCl_3$ arterial injury assays. Experiments in suspension fail to consider the *in vivo* system where the interaction among platelets, endothelial cells, and blood cells facilitate platelet adhesion to vascular bed and subsequent activation and aggregation. These experiments were unable to account for platelets' ability to sense their environment and respond accordingly (Sakurai, Fitch-Tewfik et al. 2015). In fact, many reports show that minor defects discovered using *in vitro* assays may have a significant impact *in vivo* (Ye, Huang et al. 2014, Ouseph, Huang et al. 2015). This clearly shows that the *in vivo* assays are more sensitive than *in vitro* analysis.

```

VAMP-7 MAILFAVVARGTTILAKHAWCGGNFLEVTEQILAKIPSENNKLTYSHGNYLFHYICQDRI
VAMP-8 -----
VAMP-2 -----
VAMP-3 -----

VAMP-7 VYLCITDDDFERSRAFSFLNEVKKRFQTTYGSRAQTALPYAMNSEFSSVLAQLKHHSEN
VAMP-8 -----MEEASGSAG-----
VAMP-2 -----MSATAATVPPAAPAGEGGPPAPPNL
VAMP-3 -----MSTGVPSGSSAATG-----
                * *

VAMP-7 KSLDKVMEtQAQVDELKGI MVRNIDLVAQRGERLELLIDK TENLV DSSVTFKTTSRNLAR
VAMP-8 --NDRVRNLQSEVEGVKNIMTQNVERILSRGENLDHLRNKTEDLEATSEHFKTT SQKVAR
VAMP-2 TSNRRLQQTQAQVDEVVDIMRVNVDKVLERDQKLS ELDDRADALQAGASQFETSAAKLR
VAMP-3 -SNRRLQQTQNVDEVVDIMRVNVDKVLERDQKLS ELDDRADALQAGASQFETSAAKLR
      . : : * :*: : .** *:: : .*:.*. * ::: * : *::: : : *

VAMP-7 AMCMKNIKLTIIIIIVSIVFIYIIVSLLCGGFTWPNCVKK
VAMP-8 KFWWKNVKMIVIIICVILIIIVILILFATG-----TIPT
VAMP-2 KYWWKNLKMIIILGVICAIILIIIIIVYFST-----
VAMP-3 KYWWKNCKMWAIGISVLVIVIIIIIVWCVS-----
      ** * : * : : : * :

```

Figure 6.2. Sequence alignment of mouse VAMP-2, VAMP-3, VAMP-7, and VAMP-8 amino acids

Table 6.2 Percent identity matrix of mouse VAMP-2, VAMP-3, VAMP-7, and VAMP-8 proteins created by Clustal2.1

	VAMP-2	VAMP-3	VAMP-8	VAMP-7
VAMP-2	100	73.79	35.05	29.31
VAMP-3	73.79	100	34.02	29.13
VAMP-8	35.05	34.02	100	32.67
VAMP-7	29.31	29.13	32.67	100

VAMP-2	-RLQQTQAQVDEVVDIMRVNVDKVLERDQKLELDDRADALQAGASQFETSAAKLKRKYW
VAMP-3	-RLQQTQNQVDEVVDIMRVNVDKVLERDQKLELDDRADALQAGASQFETSAAKLKRKYW
VAMP-7	DKVMETQAQVDEKLGIMVRNIDLVAQRGERLELLIDKTENLVDSVTFKTTSRNLARAMC
VAMP-8	-RVRNLQSEVEGVKNIMTQNVERILSRGENLDHLRNKTEDLEATSEHFKTTSQKVARFW
	.: : * :*: : .** *:: : .*...*. * :::: * : *::: : *
VAMP-2	WKN--
VAMP-3	WKN--
VAMP-7	MKNIK
VAMP-8	-----

Figure 6.3. Sequence alignment of SNARE domains from mouse VAMP-2, VAMP-3, VAMP-7, and VAMP-8

Table 6.3 Percent identity matrix of SNARE domains of mouse VAMP-2, VAMP-3, VAMP-7, and VAMP-8 proteins created by Clustal2.1

	VAMP-2	VAMP-3	VAMP-7	VAMP-8
VAMP-2	100	98.39	37.1	33.9
VAMP-3	98.39	100	35.48	33.9
VAMP-7	37.1	35.48	100	40.68
VAMP-8	33.9	33.9	40.68	100

```

VAMP-7      -ltiiiiivsivf-iyiivsllc-
VAMP-8      ---miviicvivliiviliilfat
VAMP-2      mmiilgvicaiiliii---ivyf-
VAMP-3      -mwaigisvlviivii--iivwcv
              : :   ::: *   :

```

Figure 6.4. Sequence alignment of transmembrane- domains from mouse VAMP-2, VAMP-3, VAMP-7, and VAMP-8

Table 6.4 Percent identity matrix of transmembrane domains of mouse VAMP-2, VAMP-3, VAMP-7, and VAMP-8 proteins created by Clustal2.1

	VAMP-7	VAMP-8	VAMP-2	VAMP-3
VAMP-7	100	42.11	22.22	26.32
VAMP-8	42.11	100	41.18	26.32
VAMP-2	22.22	41.18	100	36.84
VAMP-3	26.32	26.32	36.84	100

Part II- Titrating VAMPs to study the nature of fusion event

Thrombus formation occurs in a closed vasculature under high shear pressure. Rapid release of the entire granule cargo in a bolus would not be able to achieve the desired targeted outcome if it is washed away downstream of vascular injury without any local effect. To circumvent this, the process of granule release is controlled differentially. It is well accepted that dense granules are generally docked and are quick to release upon stimulation (Chicka, Ren et al. 2016). Unlike dense granules, alpha-granule cargo is released in three different waves and these waves are independent of an agonist or the function of the cargo (Jonnalagadda, Izu et al. 2012). While the differential packaging of cargo in different subtypes of alpha granules or specific areas in alpha granules could explain the various rates, these hypotheses could not be supported by more extensive studies (Jonnalagadda, Izu et al. 2012). So, what determines the rates of release?

To address this question, we studied the morphology of stimulated platelets. Our image analysis shows that the decondensation of granule cargo is dependent on agonist concentration and the duration of stimulation. To investigate if this process is secretion-dependent, we employed secretion-deficient VAMP KO mouse strains. We stimulated VAMP deficient platelets at lower and higher concentrations of an agonist and for various time points and studied the morphology of these stimulated platelets. Analysis of intermediate structures that represent variable decondensation of alpha-granule cargo suggested that cargo decondensation follows the granule fusion and is VAMP-dependent.

This work provides a critical piece of the puzzle to address how the rates of granule cargo release differ. Granules form a short neck or a longer pipe to fuse with the plasma membrane/OCS (Pokrovskaya, Aronova et al. 2016). This probably facilitates the influx of solvent inside the granule which is densely packed with abundant proteins. Hydration of these cargo helps reduce the density of the packed material and gives a characteristic appearance to fused granule (Figure 4.1 red and purple arrows). Thus, the rate at which these cargoes are dissolved may regulate their exodus. Second, the

incidence of decondensed granules increases with stronger agonist dose or longer stimulation time while defective fusion, as seen in V-8^{-/-} and V-2/3/8^{-/-}, decrease this incidence. This argues that the rate and the extent of decondensation are dependent on the nature of stimulation and granule fusion to the plasma membrane is required for solubilization of cargo. Furthermore, the fibrous material is always present in decondensed granules with lack of complete fusion with the exterior of the plasma membrane. This may indicate that the cargo scaffold in alpha-granules that hold the cargo together adheres to the platelet plasma membrane and may facilitate cargo release long after fusion is completed.

These findings are instrumental in broadening our understanding of how the kinetics and the extent of alpha-granule cargo release are determined. As discussed in Chapter 3 and earlier sections in this Chapter, the rates and the degrees of granule cargo release govern the thrombus architecture and mechanistic details that control these events are crucial to balancing hemostasis. The findings from this study provide a glimpse of a previously unknown layer of regulation that determines cargo release rates.

Decondensation of granule cargo

Morphological analysis of activated mouse platelets presented heterogenous population of alpha-granules that differ in cargo density (Figure 4.1 red and purple arrows). Further analysis indicated that these granules represent various stages of granule secretion and cargo decondensation. Upon granule fusion external solvent\water enters the granule through the fusion pore and cargo is dissolved\solubilized before leaving the granule. The entry of water could be facilitated by aquaporins on the plasma membrane or granules in platelets. Several studies have reported that aquaporins facilitate water influx in secretory granules which lead to a spontaneous increase in granule volume, followed by a reduction suggesting a cargo release (Kelly, Cho et al. 2004). So far, this mechanism has been studied in zymogen granules (Cho, Sattar et al. 2002, Cho and Jena 2006), synaptic vesicle release (Kelly, Cho et al. 2004), and to some extent in platelets (Lee, Agrawal et al. 2012, Goubau, Jaeken et al. 2013). Goubau and colleagues showed that a homozygous mutation in aquaporin 7

leads to altered platelet morphology and subclinical platelet secretion defects. Human platelets with these mutations are circular in shape and the granules are centrally located. ADP secretion is affected in these platelets. The group showed that aquaporin 7 is colocalized with CD61/LAMP-1 but not with an alpha-granule marker, vWF. Though aquaporin 7 is found in the releasate, it is not clear if it was secreted or if it was associated with the membrane (Goubau, Jaeken et al. 2013). Other than aquaporin 7, only aquaporin 6 has been implicated to exist in platelets. Using mercuric chloride that inhibits all aquaporin isoforms except aquaporin 6, Lee et al. showed that it is present in platelets (Lee, Agrawal et al. 2012). The group used mastoparan to activate G proteins and demonstrated the presence of $G_{\alpha 0}$ and $G_{\alpha i}$ in platelets. The roles of aquaporins in platelets are still under-investigated. In fact, the literature is sparse in various isoforms present in human and mice platelets, their location and functions. Recent mass spectroscopic studies failed to find any aquaporins in mouse platelets (Zeiler, Moser et al. 2014). The mechanistic details of their roles will be crucial to understanding granule secretion.

Granule cargo scaffold

Serglycin is a proteoglycan which is critical for storage of secretory proteins in neutrophils, mast cells, and cytotoxic T lymphocytes. By using a serglycin KO (Woulfe, Lilliendahl et al. 2008) mouse model, Wolfe et al., showed that the lack of serglycin leads to dense and alpha-granule secretion defect. Though P-selectin exposure was not affected in serglycin KO platelets, PF4 levels were significantly reduced. Additionally, these mice had a significant thrombosis defect suggesting that serglycin is critical for protein retention and granule secretion in platelets. These data also suggest that serglycin may function as a platform/scaffold to support granule cargo. Serglycin binds to negatively charged glycosaminoglycans/GAGs such as heparin. Multiple studies have reported that the lack of heparin synthesis or serglycin in mast cells affect granule morphology (Forsberg, Pejler et al. 1999, Humphries, Wong et al. 1999) suggesting a role of serglycin in granulopoiesis.

In our study, though we observed various stages of decondensation, surprisingly we hardly observed any decondensing granule that has fused completely with the plasma membrane. Each decondensing granule had at least some associated fibrous material. This raises the possibility that the granule scaffold such as serglycin holds cargo together. Upon stimulation, the exodus of this cargo and scaffold occurs simultaneously; in fact, the solubilization of scaffold could determine the rate of cargo release. Furthermore, observed fibrous material may present remnants of this scaffold that could adhere to platelet surface and further control the release of cargo still associated with it.

Another interesting possibility is the association of serglycin to porosomes. The porosome is a supramolecular structure shaped like an inverted cup that facilitates granule fusion to the plasma membrane (Schneider, Sritharan et al. 1997). It has been reported that porosomes are about 150 nm in diameter and are present in pancreatic acinar cells (Schneider, Sritharan et al. 1997), growth hormone-secreting cells of pituitary (Cho, Jętkinija et al. 2002), chromaffin cells (Cho, Wakade et al. 2002), β cells of pancreas and nerve terminal where they have smaller about 10-12 nm diameter (Cho, Jeremic et al. 2004). Furthermore, t-SNAREs such as syntaxin-1 and SNAP25 are concentrated in porosomes in mouse insulinoma Min6 cells suggesting that they might provide a preferred site for granule fusion (Naik, Kulkarni et al. 2016). Similarly, syntaxin-11 and SNAP23 are trafficked to lipid rafts upon platelet stimulation and their association to the plasma membrane by acylation is critical for platelet secretion (Zhang, Huang et al. 2018). Taken together, it can be argued that despite the lack of direct evidence, porosomes may exist on the platelet membrane and it will be interesting to see if there is any association between serglycin and porosome-like structures.

The nature of fusion – simple vs compound

Along with the entry of water into the granule and dissolving the cargo, and the composition of cargo scaffold, the nature of fusion event- heterotypic, simple (granule to plasma membrane) or homotypic, compound (granule to granule) may also dictate the rate and the degree of secretion. While simple fusion is certainly faster than the

compound fusion events, compound fusion events are crucial for the extent of release since cargo from multiple granules could be released from a single fusion event.

Using tannic acid staining and Focused Ion beam-scanning electron microscopy/FIB-SEM, Eckly et al., showed that VAMP-8 plays a major role in compound exocytosis as well as a minor role in simple exocytosis (Eckly, Rinckel et al. 2016). Weak stimulation leads to simple fusion events while strong stimulation results in compound fusion. Only single fusion events were observed in the case of dense granules, unlike alpha-granules which fuse with either simple or compound fusion events. Our findings support Eckly et al., in that VAMP-8 is crucial for compound fusion events (Figure 4.7F). Deficiencies of VAMP-2 and -3 did not influence simple or compound fusion events (Figure 4.7E and F). It is still not clear what role VAMP-7 plays in these events. VAMP-7 has been shown to interact with VARP and Arp2/3 in platelets (Koseoglu, Peters et al. 2015). These proteins are important for neurite outgrowth and actin polymerization. Since VAMP-7 is shown to translocate to the periphery when platelets spread, it seems possible that these associations with cytoskeletal elements may enable it to undergo simple fusion events. However, a detailed analysis is warranted to determine which events required VAMP-7.

An intriguing question from these data is what is the sequence of a compound fusion event where one of the granules is fused to plasma membrane? Does simple fusion happen first, followed by compound fusion or after compound fusion, one of the granules fuses to the plasma membrane discharging cargo? Though it is challenging to answer this question directly, it seems that the compound fusion event probably follows simple granule to plasma membrane fusion. Compound fusion events show that the granules are in decondensed state suggesting that there must be some outlet that allows the entry of water and subsequent solubilization and exodus of cargo from these fused granules. It is possible that a simple fusion between a granule and plasma membrane occurs first (Figure 6.5 step no. 3) and then a second condensed granule fuses to fused granule (Figure 6.5 step no. 4).

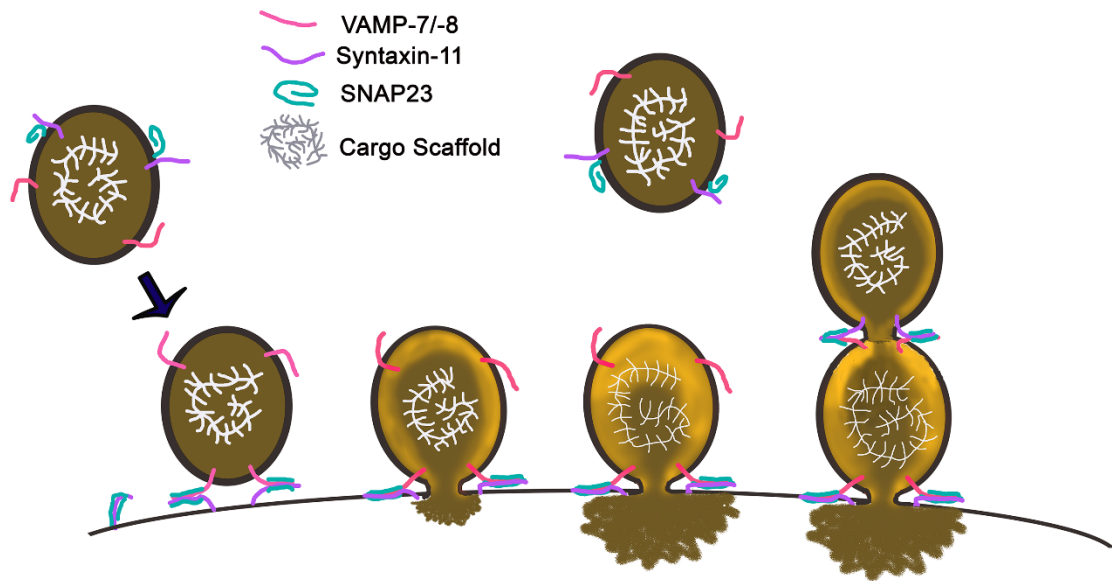


Figure 6.5. Progressive decondensation upon granule fusion decides the rate of granule cargo release

The schematic represents the progressive change in decondensation process. The data presented in this report support following steps-

- 1- An intact granule/condensed granule
2. Granule -plasma membrane fusion/simple fusion event initiating release of cargo in the extracellular environment. Decondensation starts with fusion as depicted by the change in density around inside periphery of the fused granule.
3. The progressive release of cargo changes the density of decondensing granule. Note that the cargo scaffold/matrix also appears to be dissolved.
4. An example of homotypic granule to granule/compound fusion. This may occur before or after simple fusion of one of the granules to the plasma membrane.

Part III- Role of VAMPs in intracellular vesicle trafficking

Platelet granule cargo is instrumental in platelet function. Platelets inherit some of these cargoes from their precursor cells, megakaryocytes, during granule formation. Platelets can synthesize at least some cargo in the ER in addition to endocytosing proteins from plasma. Cargo synthesized in the ER is trafficked to the endosomal system. In the case of dense granules, BLOC complexes (1,2 and 3) process these cargoes and transport them to mature dense granules. In the case of alpha-granules, cargo travels from endosomal system to the multivesicular body along with endocytosed cargo and after progressive maturation, alpha-granules are formed (Figure 6.6).

The role of SNAREs in intracellular trafficking is well studied (Rothman 1994). VAMP isoforms could play roles in any or every one of these events indicated by arrows in Figure 6.6. We recently showed that VAMP-3 is essential for endocytosis (Banerjee, Joshi et al. 2017). As discussed in Chapter 1, VAMP-8 was identified as an endosomal v-SNARE and has been implicated in trafficking in other cell types. However, the loss of VAMP-7 or VAMP-8 did not cause morphological changes in platelets or alter cargo levels (Ren, Barber et al. 2007, Koseoglu, Peters et al. 2015), suggesting that either these VAMPs do not play role in trafficking or the presence of residual VAMPs may have contributed to compensate for them. However, the question remains: which of these trafficking events are carried out by which VAMP isoforms? To address this question, we studied how trafficking occurs in VAMP deficient strains and if we could allocate specific VAMP isoforms to each of the trafficking steps. The data presented here are preliminary and work is ongoing.

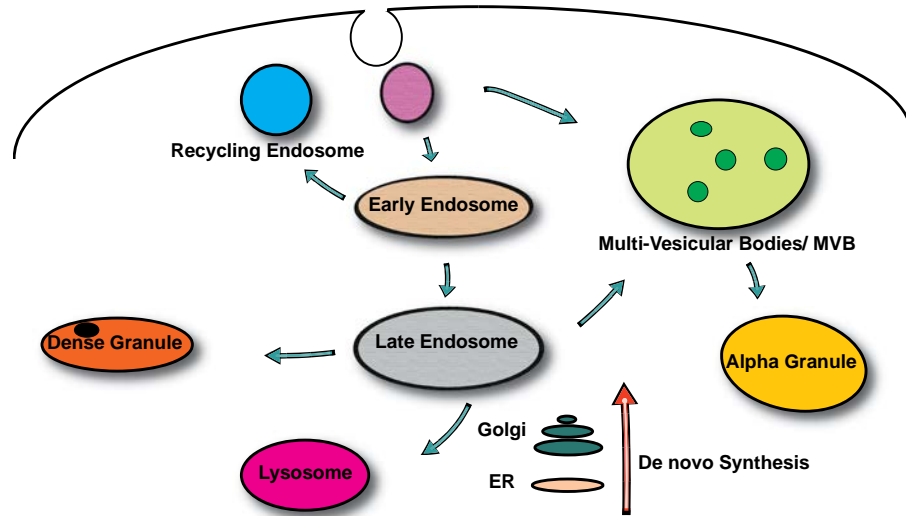


Figure 6.6. Intracellular trafficking in platelets

Platelets endocytose cargo through the endosomal system and traffic the cargo to early and late endosomes. Progressive maturation of cargo occurs in multivesicular bodies before packaging it in alpha granules\dense granules\lysosomes. Moreover, platelets can also synthesize cargo which travels through the endosomal system and may also get transported to multivesicular bodies before getting packaged in various granules. Each trafficking event shown with arrows here potentially can be mediated by one or more VAMPs.

Role of VAMPs in endocytosis

We previously showed that VAMP-3 is crucial for platelet endocytosis (Banerjee, Joshi et al. 2017). $V-3^{-/-}$ platelets showed about a 50% decrease in fibrinogen uptake. Consistently, platelet-specific $V-2/3^{-/-}$ and $V-8^{+/-}(2/3)^{-/-}$ platelets also showed about a 20-30% decrease in fibrinogen levels while $V-2/3/8^{-/-}$ platelets have about a 50% decrease (Figure 3.3). Through the pattern in the reduction of fibrinogen levels is as expected, quantitative differences might be due to different backgrounds of these strains. Additionally, the timing of PF4 Cre expression might have some influence on this process.

Based on our secretion assays we measured serotonin uptake, PF4 and β -hexosaminidase levels (Figure 5.1). None of these levels were affected indicating that serotonin transporters on platelet membranes and dense granules (Vesicle monoamine transporter/VMAT2) are not affected in absence of VAMP isoforms. It also shows that de novo synthesis of alpha-granule cargo is normal in these platelets. Morphological analysis, however, indicated that $V-2/3/8^{-/-}$ platelets have smaller and more numerous alpha-granules. These data imply that the loss of VAMP-2, -3 and -8 disturbs trafficking in platelets. Surprisingly, instead of a compensatory increase in VAMP-7 levels which is the only residual VAMP isoform still present in these platelets, its levels decreased remarkably by about 50%. This again shows that the trafficking or targeting of cargo is affected.

To understand the global impact of defective trafficking in these platelets, we used antibody arrays. In these arrays, 53 different molecules (including- pro- and anti-angiogenic factors, growth factors, tissue factors, cytokines, chemokines, metalloproteases *etc.*) were probed in duplicates. The data were normalized to WT levels. These data hint at various levels of biogenesis/trafficking defects

PF4 levels could be used as an internal control since based on ELISA assays, we know that these are not changed. In our arrays, we find PF4 levels close to WT levels, validating our results. The loss of VAMP-8 did not affect cargo levels as much, though

about 25% proteins were reduced by about 50% or more (Figure 5.4 and Table 5.1). In V-2/3^{-/-} platelets, cargo levels were significantly reduced as more than 40% of proteins were reduced by 50% or more (Figure 5.5 and Table 5.1). These defects were further aggravated in absence of all three isoforms in V-2/3/8^{-/-} platelets (Figure 5.6 and Table 5.1). About 60% of proteins were reduced by 50% or more. In fact, only 4% of proteins were at the level of 75% or above compared to WT levels indicating that the loss of all three isoforms has a significant impact on cargo levels. Noticeably, though the levels were reduced variably in these VAMP KO strains, all cargoes were present, albeit at different levels. This argues that though intracellular trafficking may have been impacted to some extent, the effect was not absolute. The loss of VAMP isoforms may be overcome by other trafficking factors to execute these events. More detailed studies are necessary to validate these results and determine trafficking steps mediated by VAMPs.

Chapter 7 Summary and future directions

Summary of the work

The work done in this thesis indicates following findings-

1. Various VAMP isoforms play redundant roles in regulating the rates and the extents of granule secretion.
2. Titration of the types and amounts of VAMP isoforms provide crucial insight into the process of secretion and how it affects *in vivo* hemostasis and thrombosis.
3. Based on *in vivo* studies, we argue that the therapeutic window is represented by about 40-50% loss of secretion which could offer protection from thrombosis without triggering unwanted bleeding. Beyond 50%, a reduction in secretion leads to a severe hemostatic deficiency and defective thrombosis.
4. Upon granule fusion to the plasma membrane, outside solvent enters the fused granule and dissolves the cargo. This aids in the exodus of cargo from granule. The rate at which the cargo is dissolved could determine the rate of their release.
5. The process of granule decondensation follows granule fusion and it is dependent on the strength of platelet stimulation- potency of an agonist, the dose of an agonist and the duration of stimulation -all factors contribute in determining the rates and the degrees of granule cargo release.
6. Platelet granules appear to contain a scaffold and it aids in retention of cargo inside a granule. The interaction of this scaffold with cargo and the rate of solubilization of scaffold may play a role in regulating the rate of cargo release. The presence of residual scaffold outside fused granule may also indicate alternative mechanism through which this scaffold could control the cargo release.
7. VAMP isoforms are crucial for intracellular trafficking. Their loss has a global impact on cargo levels. Alpha granules are much smaller in absence of VAMP-2, -3 and -8 suggesting defective granule formation.

Importance of the work

These findings are important on many levels-

1. This is the first, systematic study of VAMP redundancy *in vivo*, showing how secretion events alter in the presence or absence of primary/secondary VAMPs.
2. This is the first quantitative study targeting the contribution of platelet granule secretion in thrombosis. It argues that manipulating the types and amounts of VAMPs and systematic characterization of secretion profiles enable us to roughly pinpoint the range (40-50%) of acceptable secretion deficiencies that could help prevent occlusive thrombotic events responsible for cardiovascular diseases without causing bleeding. More than 50% loss of platelet granule secretion leads to severe hemostatic deficiency and thrombotic defects. This insight could be used to develop novel therapeutics to combat thrombotic diseases given our understanding of multiple new players found to contribute to platelet secretion and related processes. Additionally, this therapeutic range could also be used to manage current antithrombotic regimen more effectively by maintaining the platelet secretion level in this range.
3. This is the first orchestrated study that uses genetic tools and biochemical analysis with morphological evidence to understand the fine mechanics of platelet granule secretion process.
4. The strains generated for this study- $V-2/3^{-/-}$ and $V-2/3/8^{-/-}$ have been used in deciphering platelet autophagy (Ouseph, Huang et al. 2015). Additionally, these animals could be immensely useful as invaluable reagents to study the role of platelet secretion in various pathologies including but not limited to metabolism, wound healing, angiogenesis, immunity, infection, and inflammation.

Future directions

First, it remains to be seen what role VAMP-7 plays in diverse exocytotic events. Though the loss of VAMP-7 leads to secretion deficiencies like $V-8^{-/-}$ platelets, it does not

affect thrombosis (Koseoglu, Peters et al. 2015). These data argue that perhaps the secretion deficiency in absence of VAMP-7 is different than the secretion defects observed upon VAMP-8 loss. However, it is not clear how. Peters et al., showed that VAMP-7 positive alpha-granules travel to the periphery while VAMP-3 positive and VAMP-8 positive granules form a central granulomere (Peters, Michelson et al. 2012). These findings also support the role VAMP-7 plays in platelet spreading. What is not yet clear is whether VAMP-7 is preferentially important for simple and/or compound fusion events that would explain the rates and extents of secretion in these animals. It will also provide insight into the secretion pattern that is expendable. More investigation is necessary to study this mechanism.

Second, it remains to be seen if VAMP isoforms are distributed differentially on granules. Based on some preliminary data from the Storrie laboratory, it is unlikely that specific VAMP/VAMPs decorate granules exclusively but is it possible that the ratio of types of VAMPs present on vesicles may decide the fate of that granule and the nature of its secretion events. Fusogenicity of SNARE complexes is contingent upon the v- and t- SNAREs forming it. We speculate that in platelets, VAMP-8 and VAMP-7 containing complexes may be more fusogenic than the ones containing VAMP-2 and VAMP-3 since the abundance of VAMP-2 and VAMP-8 is comparable but only the loss of VAMP-8 leads to secretion deficiency (Ren, Barber et al. 2007). This could explain the rates and the extents of granule secretion we observed in various VAMP deficient strains. Alternatively, the distribution of VAMP isoforms may also contribute to secretion of granules they reside on. It remains to be seen if VAMP distribution on granules is random or if they are distributed to certain regions bestowing polarity to granules.

Third, though platelet granule secretion is a well-studied process, its potential is not much appreciated beyond hemostasis. Given the dynamic roles of platelets in a wide variety of physiological processes, not all cargo molecules that are released from platelets have been accounted for their location in platelets or the nature of their release. One example is an important ER resident protein-protein disulfide isomerase/PDI which is mainly known for its chaperone activity. PDI has been shown to

be essential for thrombosis (Jasuja, Passam et al. 2012) but it is not clear how it is released from platelets. Is it released in the SNARE-dependent manner? Does release from other intracellular organelles which are not granules depend on SNAREs? The second example is CMP sialic acid and sialic acid transferases. These enzymes and their substrates are found in platelets, but their role is not yet clear. Jones et al., showed that the CMP sialic acid release from platelets is crucial for B cell-independent sialylation of IgG. It will be interesting to see where exactly these enzymes are located and if their release is SNARE-dependent (Jones, Oswald et al. 2016).

Fourth, the role of VAMPs in intracellular trafficking should be studied in detail. As preliminary data presented in Chapter five suggest, both endocytosed and synthesized megakaryocyte originated cargo levels are depleted in VAMP deficient strains, indicating possible trafficking defects. The development of super-resolution techniques offers a great tool to study how VAMPs contribute to granule biogenesis and cargo trafficking. These discoveries are crucial not only for hemostatic functions but also immunological functions of platelets (Banerjee and Whiteheart 2016).

Furthermore, platelet trafficking has a broader influence on platelet function. Platelet metabolism is a critical piece of cell biology and physiology of platelets. Roles of VAMPs in glucose transporters/GLUT translocation are well known. In myoblasts, VAMP-2 mediates insulin-stimulated GLUT4 translocation to the plasma membrane (Randhawa, Bilan et al. 2000). Previous reports argue that in adipocytes, GLUT4 is found in two pools of vesicles- endocytic pool (including trans- Golgi network) and non-endocytic pool that consists of special vesicles called GLUT4 storage vesicles (GSVs). These different pools of vesicles release GLUT4 in agonist-dependent and VAMP isoform-dependent manner. VAMP-3 facilitates GLUT4 translocation from the endosomal pool in guanosine-5'-O-(3-thiotriphosphate) (GTP γ S) dependent manner while VAMP-2 is important for insulin-stimulated GLUT4 translocation. VAMP-3 is also associated with both insulin and GTP γ S stimulated GLUT1 translocation contrary to VAMP-2 which does not have any impact on GLUT1 translocation (Millar, Shewan et al. 1999). Recently, it has been shown that in adipocytes, VAMP-8 is important for GLUT4

endocytosis from the plasma membrane (Williams and Pessin 2008). Though it is known that platelets contain GLUT3 transporter and utilizes glucose, it is not clear how the energy needs alter based on resting/activated states and how/which metabolic pathways are employed. The animal models we generated in this study could uniquely help address these questions.

Finally, *in vitro* experiments were used to study the dynamics of the secretion process in VAMP deficient platelets in which a platelet suspension was used. However, there is a possibility that platelet behavior may be altered, had they been on the adherent surface. Some reports suggest that platelets could have some polarity, and this affects how secretion takes place. Supporting evidence comes from multiple reports where mild deficiency in platelet secretion led to robust hemostatic defect (Adam, Kauskot et al. 2010, Ye, Huang et al. 2014). In a growing thrombus under shear pressure from blood flow, platelets form layers of adhesive surface to form the core of thrombus while activating and recruiting more platelets to form the less activated shell. These functions could be differentially executed by polar surfaces. Moreover, some reports suggest that platelets can sense their surroundings and use matrix cargo from alpha granules (such as fibrinogen/fibronectin) to enhance their adhesion on the vascular surface beyond subendothelial matrix (Sakurai, Fitch-Tewfik et al. 2015). Data presented in Chapter three indicate that VAMP-mediated secretion is crucial for platelet spreading. It will be interesting to see if this spreading defect is limited to certain adhesive proteins. Could these platelets spread beyond the matrix?

In summary, the work presented in this thesis offers a valuable insight into the process of platelet granule secretion. We show that about 40-50% loss of secretion could be well tolerated for hemostatic balance, while beyond a 50% reduction, both hemostasis and thrombosis are affected. These results will be instrumental not only in the development of therapeutics but also for the management of current anti-platelet therapies. This window could provide a starting point that is helpful to maintain the hemostatic balance. Using novel microscopic tools, we show that the rate of cargo decondensation follows granule fusion and plays a crucial role in determining the rates

and extents of granule cargo release. This may have a significant impact on the thrombus formation.

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SCIENTIFIC PUBLICATIONS

- **Joshi S.**, and Whiteheart S. W., *The nuts and bolts of the platelet release reaction*. Platelets, 2016.28(2): p. 129-137.
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- **Joshi S.**, Banerjee M., Zhang J., Kesaraju A., and Whiteheart S. W., *Secretory machinery levels define granule release kinetics and set thresholds for platelet modulation of thrombosis and hemostasis* (Submitted)
- Pokrovskaya I*, **Joshi S***, Whiteheart S. W., and Storrie B., *α -Granule matrix decondensation/membrane fusion as intracellular targets for hemostasis versus thrombosis* (* equal contribution) (Submitted)
- **Joshi S***, Banerjee M*, Qi Z, Mitchell J. M., Lin P., Warmoes M. O, Sun Q., Yang Y., Fan T. W. M., Moseley H. N. B., Whiteheart S. W. and Wang Q. J. *Platelet autophagy and metabolism.* (* equal contribution) (In preparation)
- Pokrovskaya I., **Joshi S.**, Whiteheart SW., and Storrie B., *Three-dimensional reconstruction of murine and human platelets.* (In preparation)