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Rania A. Al Hawas, Student

Dr. Sidney Whiteheart, Major Professor

Dr. Doris Baker, Director of Graduate Studies

REGULATION OF PLATELET EXOCYTOSIS AND ITS ROLE IN DISEASES

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DISSERTATION

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A dissertation submitted in partial fulfillment of the  
requirements for the doctorate degree in the  
College of Health Sciences at the University of Kentucky

By

Rania A. Al Hawas

Lexington, Kentucky

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

Lexington, Kentucky

2012

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## ABSTRACT OF DISSERTATION

### REGULATION OF PLATELET EXOCYTOSIS AND ITS ROLE IN DISEASES

In addition to their role in hemostasis, platelets appear to contribute to vascular inflammatory diseases. Platelets achieve this through the secretion of various pro-thrombotic and pro-inflammatory molecules. Platelet secretion is mediated by integral membrane proteins called Soluble NSF Attachment protein REceptors (SNAREs). SNAREs come from both granule/vesicle membranes (v-SNAREs) and target membranes (t-SNAREs) to form a *trans*-bilayer complex that promotes membrane fusion and subsequent granule cargo release. The work described in this dissertation dissects various, yet related aspects of platelet secretion in both physiological relevant and pathological circumstances.

Atherosclerosis is a leading cause of death in the westernized countries and a major contributor to heart attacks and strokes. Given the potential involvement of platelets in atherosclerosis and previous work from our laboratory showing that VAMP-8 is the primary v-SNARE for platelet secretion, one part of this dissertation focuses on the role of VAMP-8- mediated secretion in atherosclerosis. The data showed that the deletion of VAMP-8 in the ApoE<sup>-/-</sup> null model of chronic atherosclerosis attenuated plaque development compared to the wild type littermates. Aged (50 week) VAMP-8<sup>-/-</sup>/ApoE<sup>-/-</sup> mice showed a reduction in lesion size compared to ApoE<sup>-/-</sup> controls, as measured by Oil Red-O staining of the plaques in the aortic sinus and by *en face* analysis of plaques in the aortic arch. These data show that the loss of VAMP-8 attenuates the development of atherosclerotic plaques and suggest that platelet secretion contributes to atherosclerosis.

Considering the vital role of platelet secretion in both physiological and pathological conditions, it is essential to understand how it is regulated. SNARE proteins are controlled by a range of regulatory molecules that affect where, when, and with whom they form *trans*-bilayer complexes for membrane fusion. One family of such regulators is the Munc18 family: platelets contain three (Munc18a-c). The second part of this dissertation focuses on the role of Munc18b/STXBP2. Mutations in the Munc18b/STXBP2 gene underlie Familial Hemophagocytic Lymphohistocytosis type 5 (FHL5), which is a life- threatening disease caused by dysregulation of the immune system. Platelets from two biallelic FHL5 patients had almost undetectable levels of Munc18b/STXBP2 levels; the levels of Munc18a increased slightly and Munc18c levels were unaffected. Syntaxin 11 levels were affected but the levels of other secretory machinery proteins were normal. Platelet secretion from dense and alpha granule in two



biallelic patients and the one heterozygous patient was decreased. The release of serotonin from dense granules, and platelet factor 4 (PF4) from alpha granules was profoundly affected in the biallelic patients and partially affected in the heterozygote heterozygous patient. Lysosome release was affected only from the platelets of the biallelic patients. These data indicate that Munc18b plays a key role in platelet secretion.

Ras is the prototypical member of a family of low molecular weight, GTP-binding proteins. It affects various cellular functions by cycling between an active, guanine triphosphate (GTP) and an inactive guanine diphosphate (GDP) -bound state. Little is known about the role of Ras activation in platelets. The third part of this dissertation focuses on what could be learned about Ras' role by analyzing platelets from patients with Noonan Syndrome. Specific mutations in the genes encoding elements of Ras signaling pathways are associated with Noonan Syndrome. Platelets from Noonan Syndrome patients with a mutation in kRas (F156V) were analyzed and shown to have a defect in aggregation in response to low levels of agonist. These data suggest that Ras may play a functionally relevant role in platelet activation.

In summary, the experiments presented in investigations of this dissertation support a role for platelet secretion in several pathological conditions and suggest that platelet secretion assays may serve as useful as diagnostic tools for some genetic diseases. In addition, these studies elucidate the importance of understanding the regulation of platelet exocytosis, to drive the development of new antithrombotic therapeutics.

KEYWORDS: Platelet Secretion, Atherosclerosis, VAMP-8, FHL5  
Munc18b, Noonan Syndrome, Ras Protein

Rania Al Hawas

06-23-2012

REGULATION OF PLATELET EXOCYTOSIS AND ITS ROLE IN DISEASES

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06-23-2012

To My Beautiful Children  
Khaled, Sara, Maram, and Yasmine

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## **Chapter One**

### **Introduction**

#### **Thesis Overview**

In this thesis research, various yet related aspects of platelet secretion have been discussed. Platelets are small, discoid, cellular fragments that circulate in the blood stream. Circulating platelets play a key role both in hemostasis and thrombosis. Platelets respond to variety of events in the body by secreting numbers of molecules from their granules. Upon vascular injury under physiological conditions, platelets adhere to subendothelial collagen, become activated, and undergo cytoskeletal rearrangements that allow them to aggregate and secret their granule content to form a clot. This activation of platelets can be pathological: inappropriate thrombosis can lead to heart disease or stroke (Ruggeri 2002). The secretion of platelet granule content is a complex and important process that can influence several physiological and pathological conditions, *e.g.* wound healing, inflammation, coagulation and malignancy. These effects raise several questions that are central to this dissertation, how the platelet secretion machinery is regulated and how can platelet secretion influence physiological and pathological processes.

#### **Platelet Biology and Structure**

Wright described the production of platelets from the bone marrow in the 20<sup>th</sup> century as “detachment of plate-like fragments or segments from pseudopods” of the megakaryocytes (Wright 1910). The maturation of megakaryocytes and the production of platelets are controlled by several cytokines such as thrombopoietin and potentially interleukin (IL)-3, IL-6, and IL-11 (Kaushansky 1995; Kaushansky et al. 2002). Megakaryocytes undergo endomitotic divisions and become polyploid with at least 16N of DNA content (Ebbe 1976; Hata et al. 1993). Once the process of nuclear division is completed, the cytoplasm continues to mature and the cell mass increases (Paulus 1970). At this stage, the cytoplasm of a megakaryocyte will develop a demarcation membrane system (DMS), a dense tubular system (DTS) (Daimon et al. 1982), and granules (Ebbe 1976). In megakaryocytes, the DMS serves as a reserve for proplatelet formation and membrane extension. In mature platelets, the DMS develops into the open canalicular

system (OCS) (Radley et al. 1982). DTS is the site of prostaglandin synthesis in platelets, and doesn't stain with surface membrane tracers such as ruthenium red. The process of granule biogenesis starts during the development of megakaryocytes. There are three types of secretory granules:  $\alpha$ -granules, dense granules, and lysosomes.

The most abundant granules are the  $\alpha$ -granules. Platelets comprise 50-80% of  $\alpha$ -granules. They vary in size between 200 and 500 nm in diameter (Frojmovic et al. 1982). Electron microscopy was used to determine the morphology of  $\alpha$ -granules as a spherical shape with a dark central nucleus (Harrison et al. 1993). Proteomic studies suggest that  $\alpha$ -granules contain and release hundreds of different types of molecules (Maguire et al. 2003; Coppinger et al. 2004). This diversity makes the platelet a potentially multifunctional cell whose functions range from normal hemostasis and wound healing to pathologies such as inflammation. Proteins synthesized by megakaryocytes (Kieffer et al. 1987) and endocytosed proteins from the plasma (Handagama et al. 1987; Behnke 1992) are present in  $\alpha$ -granules. For platelet adhesion,  $\alpha$ -granules contain adhesive molecules, such as fibrinogen (Broekman et al. 1975), thrombospondin (Jaffe et al. 1982), von Willebrand factor (vWF) (Slot et al. 1978), fibronectin (Zucker et al. 1979). Proteins that are involved in the coagulation pathway such as factor V and factor IX and plasminogen activator inhibitor-1 (PAI-1; (Booth et al. 1988; Rendu et al. 2001; Smyth et al. 2009) are also present in  $\alpha$ -granules. P-Selectin and  $\alpha$ IIb $\beta$ 3, which are important for platelet aggregation, are integral membrane proteins that are present in granule membranes and become exteriorized upon activation (Cramer et al. 1990). Inflammatory cytokines, such as RANTES and platelet factor 4 (PF4) (McLaren et al. 1982) and angiogenic (VEGF, PDGF, bFGF) (Nurden et al. 2008) and anti-angiogenic (CXCL4) proteins are also present in  $\alpha$ -granules (Brill et al. 2004).

Human platelets contain three to eight dense granules per platelet (Holmsen et al. 1979). By electron microscopy, dense granules, as their name indicates, are electron dense in whole mounts and appear black after osmium staining (Gunay-Aygun et al. 2004). They are the smallest platelet granules, around 200-300 nm in diameter. Dense granules are formed in early stages of megakaryocyte development and filled with their contents during maturation. Small molecules such as calcium (Holmsen and Weiss 1979),

serotonin (5-HT) (Da Prada et al. 1979), phosphate, adenosine diphosphate (ADP), and adenosine triphosphate (ATP) (Wurzinger 1990) are stored in dense granules. These molecules play a key role in platelet activation. For instance, serotonin release leads to vasoconstriction and enhancement of platelet release (Watts 2002; Walther et al. 2003), while ADP is important for recruiting and activating neighboring platelets and thus enhances the thrombus formation. The release of content from dense granules is the most rapid of the three granule release events.

Lysosome is the third class of granules in platelets, and is intermediate in size between  $\alpha$ -granules and dense granules, and around 175–250 nm in diameter. Lysosomes have been identified in the earliest form of recognizable megakaryocytes. Lysosomes contain many hydrolytic enzymes including  $\beta$ -hexosaminidase, acid hydrolases, glycosidase, heparinase, and cathepsin. These enzymes play an important role in degradation of proteins and lipids, thus might play a role in platelet remodeling (Ciferri et al. 2000). The membrane of the lysosome contains the lysosomal integral membrane protein LIMP (CD63) and the lysosomal-associated membrane proteins, LAMP-1 and LAMP-2 (Metzelaar et al. 1992). These integral membrane proteins are heavily glycosylated at the luminal side of the lysosome, forming a protective coat from the lysosomal granule hydrolytic enzymes (Rendu and Brohard-Bohn 2001). Activated platelets express the lysosomal specific membrane proteins on their surface, which can be used as a marker for detecting platelet function. A summary of the major platelet granule content is presented in (Table 1-1).

### **Platelet Activation**

The primary physiological role of platelet in sensing the damaged vascular endothelium and its accumulation at site of injury to initiate blood clotting was identified in 1865 (Bizzozero 1865). There are several agonists identified to bind their corresponding receptors on the platelet surface, and initiate several signaling cascades that lead to platelet activation. The initiation of platelet adhesion to the damaged vessel wall requires platelets to adhere to exposed collagen, which is one of the primary platelet agonist that is exposed on the vascular lesion and binds to GPVI receptor on platelet surface causing platelet adhesion to the vascular damaged area. Von Willebrand's factor

(vWF) is expressed on the endothelium and subendothelium matrix and binds to GPIb receptor on platelets to enhance platelet adhesion. The adhesion of platelets leads to initiation of signaling events that leads to cytoskeleton changes, and secretion of more than 300 proteins (Rendu and Brohard-Bohn 2001) that causes platelet aggregation.

Secreted agonists by activated platelets, such as ADP, thromboxane A<sub>2</sub>, and thrombin lead to further activation and aggregation of platelets. Once bound to their corresponding G protein coupled receptor, these agonists (Table 1-2) activate phospholipase C (PLC $\beta$ ), which hydrolyzes phosphatidylinositol 4,5-bisphosphate (PI-4,5-P<sub>2</sub>) to diacylglycerol (DAG) and inositol tri phosphate (IP<sub>3</sub>) that causes Ca<sup>2+</sup> channels in the endoplasmic reticulum to open (Lawrence et al. 2010). The increase in intracellular calcium activates protein kinase c (PKC) and PI3K, which cause activation of cytoskeletal proteins such as talin (Petrich et al. 2007) and initiate inside-out signaling in platelets. Activated talin can then bind to the cytoplasmic domain of the  $\beta$ 3 subunit causing a conformational change that leads to the dissociation of the cytoplasmic and transmembrane domains of  $\alpha$ IIb and  $\beta$ 3, which in turn causes the oligomerization of integrin. This conformational change in this receptor leads to its binding to fibrinogen and thus promoting platelet-platelet interaction and aggregation.

The binding of several agonists to their corresponding platelet receptors is very important for platelet function in hemostasis. Defects in platelet receptors can lead to bleeding disorders that involve  $\alpha$ IIb $\beta$ 3 integrin receptor (Glanzmanns thrombasthenia) and GPIb receptor (Bernard-Soulier syndrome; (Nurden et al. 2008).

### **SNARE Proteins (t-SNARE and v-SNARE)**

Soluble N-ethylmaleimide-sensitive factor activating protein receptor (SNARE) proteins were characterized in 1980's and 1990's (Block et al. 1988; Trimble et al. 1988; Sudhof 1989; Bennett et al. 1992; Sollner et al. 1993) as key components of protein complexes that mediate all intracellular membrane fusion. Membrane fusion is an essential process that is required for membrane biogenesis, intracellular trafficking, and cellular exocytosis, including hormone and enzyme secretion, and neurotransmission. The importance of divalent cations, especially Ca<sup>2+</sup>, in membrane fusion was studied in the 1970's by D. Papahadjopoulos *et al* (Newton et al. 1978), throughout the 1980's by

different groups including S. Ohki (Ohki 1982). In the 1990's, James E. Rothman and his research group demonstrated that SNAREs proteins are involved in membrane fusion in mammalian cells (Sollner, Whiteheart et al. 1993; Whiteheart et al. 1993; Weber et al. 1998). There are two classes of SNARE proteins: target membrane proteins (t-SNAREs) on the plasma membrane, and the secretory vesicle-associated membrane proteins (VAMPs or v-SNAREs) on the vesicle membrane. The interaction of the t-SNAREs; two SNAP-25 and a syntaxin on the plasma membrane, with the v-SNARE; VAMP on the vesicle membrane, leads to the formation of a four-helix bundle that spans the two bilayers and mediate the membrane fusion. The assemblies of the SNARE complexes that bridge the two membranes are called *trans*-SNARE complexes, and it is the minimum component required for membrane fusion (Weber, Zemelman et al. 1998). The completion of the membrane fusion is called *cis* complexes, which is a conformation where all the SNAREs are associated with the same membrane (Ungar et al. 2003).

The v-SNAREs, such as VAMPs, are type II integral membrane proteins, with a single transmembrane domain (TMD) that is required to anchor SNAREs to the membrane. The t-SNAREs can be divided to two types: the SNAP23/25 type (SNAP-23, SNAP-25, SNAP-29, sec9p) and the syntaxin type (syntaxin-1 to -19, *etc.*). The SNAP-23/25 like SNARE proteins have two SNARE motifs and no TMD, but they are anchored to the membrane through a thioester-linked acyl group (Ungermann et al. 2005). Syntaxin is a type II integral membrane protein that consists of a SNARE motif and an N-terminal regulatory domain (Dietrich et al. 2003). In SNARE proteins, the SNARE motif is approximately 60–70 residues in length (Weimbs et al. 1997; Jahn et al. 1999) and is adjacent to the C-terminal TMD. Monomeric SNARE motifs are disordered, and their interactions lead to the formation of parallel four-helix bundles. This structure of the assembled SNARE motifs is stabilized by the presence of heptad repeats of the hydrophobic residues on the same side of the  $\alpha$ -helix (Poirier et al. 1998; Sutton et al. 1998; Antonin et al. 2002). This parallel arrangement of SNARE motifs brings both the transmembrane anchors and the two membranes into close proximity to form the *cis*-SNARE complex and mediate fusion (Hanson et al. 1997). Finally, after fusion occurs, in order for SNAREs proteins to be reused for more fusion processes, the *cis*-SNARE complex needs to be disassembled. The process of *cis*-SNARE complex disassembly is



mediated by the chaperone NSF (N-ethylmaleimide-sensitive factor) and the co-chaperone SNAP and uses the energy of ATP hydrolysis (Fasshauer et al. 1997; Fasshauer et al. 2002). The interaction of NSF (hexamer) and three  $\alpha$ -SNAPs with the *cis*-SNARE complex lead to the formation of a transient 20 S complex (Hohl et al. 1998). The ATP hydrolysis by NSF leads to the disassembly of the 20 S complex along with the *cis*-SNARE complex. This allows SNARE proteins to be in the *trans*-complex assembly and the SNARE proteins to be recycled for more membrane fusion events (May et al. 2001) (Figure 1-1).

### **SNAREs in Platelet Secretion**

The presence of SNARE proteins in human platelets was first identified by Whiteheart *et al* (Lemons et al. 1997). There are 17 isoforms of syntaxins in human genome. In platelets the (t-SNAREs) syntaxin-2, -4, -7, and 11 were detected (Lemons, Chen et al. 1997; Flaumenhaft et al. 1999; Chen et al. 2000; Chen et al. 2000). Syntaxin-2 and -4 were found to be important for platelet secretion. Syntaxin-2's role in platelet secretion was determined by using a polyclonal syntaxin-2 inhibitory antibody in permeabilized human platelets. The secretion from the three granules ( $\alpha$ -granules, dense granules, and lysosome) was inhibited. A monoclonal syntaxin-4 inhibitory antibody had an effect on secretion from  $\alpha$ -granules and lysosomes only. A syntaxin-7 antibody had no effect on platelet secretion from permeabilized human platelets. Recently the role of syntaxin-11 in human platelets was defined by our group (Ye *et al.* 2012 submitted). The genetic mutations of the syntaxin-11 gene are associated with the rare autosomal recessive disorder called Familial Hemophagocytic Lymphohistocytosis type 4 (FHL 4). The disease is characterized by a defect in degranulation from cytotoxic T cells and Natural Killer (NK) cells that leads to hyperactivation of the immune system. The manifestations of the disease include hepatosplenomegaly and cytopenias. Variable bleeding diatheses have been reported in these patients. The disease is fatal unless bone marrow transplantation is performed at an early age. Both SNAP-25 and SNAP-23 were identified in platelets. SNAP-23 is known to play a function in platelet secretion. Using anti-SNAP-23 Fab fragment and the SNAP-23 C-terminal peptide, secretion from all

three platelet granules were inhibited (Flaumenhaft, Croce et al. 1999; Reed et al. 1999; Chen, Bernstein et al. 2000; Chen, Lemons et al. 2000; Polgar et al. 2003).

In human genome, there are 9 VAMPs (VAMP-1-8, excluding VAMP-6, sec22, ND YKT6P) (Bock et al. 2001). The v-SNAREs that are present in human and murine platelets include: synaptobrevin/VAMP-2, cellubrevin/VAMP-3, TI-VAMP/VAMP-7, and endobrevin/VAMP-8 (Polgar et al. 2002; Schraw et al. 2003; Ren et al. 2007). In this section, I will focus more on the v-SNARE VAMP-8, since it is part of this thesis project.

The role of VAMP-2 and VAMP-3 in platelet secretion was controversial. *In vitro* experiments using the tetanus toxin endopeptidase showed that P-Selectin exposure was inhibited (Flaumenhaft, Croce et al. 1999). This toxin not only cleaves VAMP-3, but it also cleaves VAMP-2, which was not detected at that time. So it was concluded that VAMP-3 was important for platelet secretion from  $\alpha$ -granules. This work was followed by another experiment using the cytoplasmic domain of VAMP-2, -3, and -8 as inhibitors (Polgar, Chung et al. 2002). VAMP-3 was reported to be important for the secretion from both  $\alpha$ -granules and dense granules. *In vivo* studies with VAMP-3<sup>-/-</sup> mice showed that VAMP-3 was not required for platelet secretion (Schraw, Rutledge et al. 2003). During that time, VAMP-2 was identified in both human and murine platelets. VAMP-2 is important for neurotransmitter release and insulin release from pancreatic cells (Randhawa et al. 2000). Tetanus toxin insensitive VAMP (TI-VAMP)/VAMP-7 has been identified to play a role in epithelial cells (Galli et al. 1998). The N-terminal region of VAMP-7 is called the longin-domain, that has been shown to be important in membrane trafficking and SNARE complex formation (Advani et al. 1999).

The endobrevin/VAMP-8 was first identified as the v-SNARE that mediates fusion between early and late endosomes (Wong et al. 1998; Antonin et al. 2000), by forming a complex with syntaxin-7, -8, and vti1b. Another role of VAMP-8 was reported in mast cell degranulation. VAMP-8 forms a complex with syntaxin-4 and SNAP-23, which plays a role in the regulated secretion of mast cells (Sander et al. 2008). VAMP-8 was found to be a major player in exocytosis in pancreatic acinar cells (Wang et al. 2004). Using VAMP-8<sup>-/-</sup> mice, secretion from the zymogen granules from the pancreatic cells was reduced compared to the wild type. In VAMP8<sup>-/-</sup> mice, the secretion of granzyme A and granzyme B was significantly compromised in cytotoxic T lymphocytes

(Loo et al. 2009). The role of VAMP-8 in platelet secretion was well studied by several groups (Polgar, Chung et al. 2002; Ren, Barber et al. 2007). Our group showed that VAMP-8 is required for platelet secretion from the three granules. Thrombin-stimulated platelets from VAMP-8<sup>-/-</sup> mice had a significant defect in secretion compared to wild type controls. Further work showed that VAMP-8 is the major v-SNARE responsible for platelet secretion in human and mouse. Although the secretion defect is significant, it is not complete; there is a residual secretion. When high levels of thrombin (0.5 U/mL) were added, the secretion defect in platelets was over-ridden. The residual secretion was sensitive to tetanus toxin in VAMP-8<sup>-/-</sup> platelets suggesting that VAMP-2 and/or VAMP-3 might play a secondary role in platelet secretion. The role of VAMP-8 in hemostasis was demonstrated using the *in vivo* laser induced-injury model (Graham et al. 2009). These results show that VAMP-8<sup>-/-</sup> mice are defective in thrombus initiation and growth, but thrombus formation was not ablated, suggesting that the secondary VAMPs mediate enough secretion for reduced clot formation. Since the VAMP-8<sup>-/-</sup> mice exhibited an *in vivo* hemostatic phenotype consistent with their *ex vivo* platelet secretion defect, it appeared that VAMP-8<sup>-/-</sup> mice can be used as a model to study the importance of platelet exocytosis in atherosclerosis.

## **Platelet Pathophysiology**

### **Atherosclerosis and Platelet Secretion**

Atherosclerosis is a chronic inflammatory disorder (Ross 1995), characterized by intimal wall thickening of medium and large arteries (Stary et al. 1995). One theory explaining the development of atherosclerosis is the “Response to Injury” model that Russell Ross proposed over twenty years ago (Ross 1995). In brief, the process of atherosclerosis starts with vascular endothelium injury that leads to accumulation of lipids, in particular low-density lipoprotein (LDL), in the intimal area promoting formation of fatty streaks. As a consequence, many cells are recruited to the lesion site, such as monocytes, which transform into macrophages that engulf the lipid particles and become foam cells (Ross 1999). Platelets are also stimulated, and adhere to endothelial cells. As a result of these events, plaques or “lesions” develop. Rupture of the plaque and

the subsequent occlusive thrombus is responsible for most acute coronary diseases and strokes (Viles-Gonzalez et al. 2004).

One of the major cell types involved in atherosclerosis is the endothelial cell. Once activated, they express a number of surface receptor molecules, *e.g.* intercellular adhesion molecule (ICAM) and vascular cell adhesion molecule (VCAM), and P- and E-Selectins, which promote adhesion of leukocytes and platelets to the lesion area (Lamon et al. 2008). The deficiency of E-Selectins (the endothelial selectin) and P-Selectins (on endothelial cells and platelets) show an 80% and 40% protection in the early and advanced stages of atherosclerosis, respectively (Dong et al. 1998).

The role of macrophage in atherosclerosis has been well documented. The presence of macrophages in the intima of the aorta leads to an influx of inflammatory molecules such as interleukin-1 beta (IL-1 $\beta$ ) and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), which promote the inflammatory response in the area (Popa et al. 2007). Macrophage chemoattractant protein-1 (MCP-1) recruits macrophages to the endothelium (Egashira 2003) and its deletion is protective against atherosclerosis (Boring et al. 1998; Ni et al. 2001). In addition, macrophages can exacerbate the lesion area by secreting several growth factors such as transforming growth factor- $\beta$  (TGF- $\beta$ ) that leads to smooth muscle cell proliferation (Tiwari et al. 2008) and metalloproteinases such as matrix metalloproteinase-9 (MMP-9) that degrade the extracellular matrix (ECM) proteins and cause plaque instability. Plaque rupture activates platelets and coagulation factors which ultimately lead to occlusive thrombus formation (Fuster et al. 1990).

The involvement of platelets in the process of atherosclerosis has been suggested by several studies (Burger et al. 2003; Huo et al. 2003; Huo et al. 2004; Gawaz et al. 2005). Nevertheless, the precise contribution of platelets to this process has been controversial and unclear. While some groups propose that platelets are involved in only the late stages of plaque rupture and thrombus formation, others suggest that platelets play a key role not only in thrombus formation in the end stage of atherosclerosis (Dobroski et al. 1996; Bouchard et al. 2001; Cazenave et al. 2003) but also in the initiation of atherosclerotic lesions (Massberg et al. 2002; Theilmeyer et al. 2002; Chandler et al. 1961; Schonbeck et al. 2000; Langer et al. 2006). Massberg *et al.* showed that in the ApoE<sup>-/-</sup> mice, inhibition of the platelet receptor GPIIb/IIIa affects platelet adhesion

to vascular endothelium before the development of the lesions, while inhibition of  $\alpha$ IIB $\beta$ 3 had only partial effect on platelet adhesion (Massberg, Brand et al. 2002). Huo *et al.* (Huo, Schober et al. 2003) demonstrated that activated circulating platelets exacerbate atherosclerosis in ApoE<sup>-/-</sup> mice.

Currently there is more focus in the literature on platelets as inflammatory cells. Once activated, platelets adhere and interact with endothelial cells, which leads to further activation of platelets and expression of cell surface adhesion molecules and chemokines that are important for recruitment of leukocytes. Once recruited, leukocytes such as monocytes bind and adhere to the vessel wall and migrate into the endothelial cells, and differentiate into macrophages that are critical for the formation of lesions. Thus it is important to understand the importance of platelet exocytosis and its effect on the inflammatory reaction in atherosclerosis.

It has been documented in the literature that platelets serve as a pro-inflammatory mediator, through the secretion of several chemokines from their  $\alpha$ -granules, such as PF4 (also known as CXCL4) which is a specific platelet molecule, and RANTES (CCL5) promoting monocytes recruitment to the lesion and thus intensifying the inflammation in atherosclerosis (Huo, Schober et al. 2003). Furthermore, Pitsilos *et al.* (Pitsilos et al. 2003) demonstrated that PF4 is detected in atherosclerotic human plaque, which demonstrates that platelets secrete their cargo at the site of the lesion. Furthermore, Sachais *et al.* (Sachais et al. 2007) showed that PF4 promotes atherosclerotic lesion development in mice and its deletion had a protective effect.

In addition to the chemokines mentioned above, certain cytokines are released from activated platelets that lead to recruitment of leukocytes into the lesion. The expression of IL-1 $\beta$  was elevated in the arteries of ApoE<sup>-/-</sup> mice (Merhi-Soussi et al. 2005). Activated platelets induce the secretion of the endothelial cell chemokine MCP-1 (important for monocyte recruitment), and it was dependent on IL-1 $\beta$  (Gawaz et al. 2000). Activated platelets, secrete IL-1 $\beta$ , which in turn increases the expression of the endothelial adhesion molecules, such as ICAM-1 (Gawaz, Langer et al. 2005). Although the exact function of IL-1 $\beta$  in platelets is not yet known, its release from the platelets was shown to have an effect on inflammation in atherosclerosis (Toma et al. 2012).

Additionally, platelet  $\alpha$ -granules secrete P-Selectin, which is an adhesion molecule that is expressed on activated platelets and mediates the rolling of monocytes (Dong et al. 2000; Rendu and Brohard-Bohn 2001). This interaction can lead to the transmigration of monocytes into the intima of the artery (Ramos et al. 1999) and thus facilitate atherosclerotic lesion formation. Burger *et al.* (Burger and Wagner 2003) have shown that platelet P-Selectin enhances lesion development in ApoE<sup>-/-</sup> mice, though the role of endothelial cell P-Selectin was deemed more important.

The importance of platelet glycoproteins in atherosclerosis has been documented. Schulz *et al.* showed that in ApoE<sup>-/-</sup> mice, the prolonged administration of soluble GPVI reduced the athero-progression, and in humans, GPVI binding to the plaque core region facilitates platelet adhesion and aggregation, though GPVI binding is not the only factor that activates the plaque formation (Schulz et al. 2008). Despite the fact that Glanzman thrombasthenia patients (Shpilberg et al. 2002), lacking  $\alpha$ IIB $\beta$ 3, are not protected from atherosclerosis, in ApoE<sup>-/-</sup> mice exposed to a wire injury to induce endothelial denudation, the deletion of the  $\alpha$ -subunit of the  $\alpha$ IIB $\beta$ 3 attenuated atherosclerosis, by preventing platelet adhesion to the damaged endothelial cells (Massberg et al. 2005). In addition, recent data suggest that individuals with the single nucleotide polymorphism (SNP), Leu33Pro of the  $\alpha$ IIB $\beta$ 3 have higher risk of atherosclerotic plaque rupture, due to increased platelet activation determined by high level of platelet P-Selectin (Kucharska-Newton et al. 2011).

### **Summary:**

Platelet secretion is mediated by SNARE proteins; the interaction between the t-SNARE on plasma membrane and the v-SNARE on vesicle membrane lead to the formation of a four-helix bundle that spans the two bilayers and mediate the membrane fusion. Among the v-SNAREs in platelets, our group has shown that VAMP-8 is the primary v-SNARE required for platelet secretion from all platelet granule content. Platelet secretion is not only important for hemostasis but also plays a role in inflammatory diseases such as atherosclerosis. Thus in Chapter Three of this thesis, the role of VAMP-8 mediated secretion in atherosclerosis will be deciphered.

### **Regulatory Proteins in Platelet Secretion:**

## **SM (Sec1/Munc18) Proteins**

The process of exocytosis through SNARE proteins is strictly regulated. One of the key regulators is the Sec1/Munc18 (SM) family proteins (Sudhof et al. 2009). The SM proteins are highly conserved through their ~600 amino acids (60-70 KDa) (Misura et al. 2000). SM proteins were first identified by screening the yeast *S. Ceriviseae* secretion mutations (*sec*) (Novick et al. 1979), and the uncoordinated mutants of *C. elegance* (*unc*) (Brenner 1974). The mammalian *unc18* homolog is called Munc18. There are seven of mammalian SM gene family members that have been identified: Munc18a, Munc18b, Munc18c, Sly1, Vps45, Vps33a and Vps33b (Bock, Matern et al. 2001). Munc18 protein's function is involved in intracellular trafficking and regulation of exocytosis (Munc18a, b, and c) (Toonen et al. 2003). Munc18 proteins act as chaperones specific for syntaxin/t-SNARE but they also may play a role in matching v- and t-SNAREs to form fusogenic trans-bilayer complexes. Munc18a (also called Munc18-1, nSec1) is primarily expressed in neurons (Hata, Slaughter et al. 1993; Garcia et al. 1994; Pevsner et al. 1994); Munc18b (also called Munc18-2) is ubiquitously expressed except in the brain; and Munc18c (also called Munc18-3) is also ubiquitously expressed (Hata et al. 1995; Tellam et al. 1995; Riento et al. 1996). Munc18a was first identified to bind syntaxin-1 in rat brain (Hata, Slaughter et al. 1993). Both Munc18a and b can bind to syntaxin-1, -2, and -3, while Munc18c binds to syntaxin-2 and -4 (Hata and Sudhof 1995; Tellam, McIntosh et al. 1995; Tellam et al. 1997).

There are several proposed modes for Munc18a/syntaxin-1 binding. The first mode is called the closed confirmation, in which binding of Munc18a causes the H3 domain of syntaxin-1 to fold back on to the N-terminal Habc domain (SNARE binding motif), blocking it from binding to SNAP-25 and its cognate v-SNARE (Pevsner et al. 1994; Misura, Scheller et al. 2000). Data indicate a negative role of Munc18a on SNARE complex formation, by preventing any premature SNARE complex reassembly and suggest that this binding interaction is important for stabilization of both Munc18a and syntaxin-1 *in vivo* (Verhage et al. 2000; Gerber et al. 2008). The second mode of binding to syntaxin -1 is when it is dissociated from Munc18a in which the H3 domain of syntaxin-1 is open and exposed to bind v-SNARE, and this is called the open conformation (Misura, Scheller et al. 2000; Yang et al. 2000). Recently a distinct

mechanism of the direct interaction between SM and SNARE proteins was suggested. The SM protein can bind to a specific N-terminal peptide sequence in the syntaxin, and this is the third mode (Yamaguchi et al. 2002).

Munc18a plays an important role in neurotransmission; the deletion of Munc18a in mice causes a complete loss of neurotransmitter secretion from the synaptic vesicles, but the brain morphology is normal (Verhage, Maia et al. 2000). The level of syntaxin-1 protein was significantly reduced in Munc18a deficient mice, suggesting that it is a chaperone for syntaxin-1. Additional data confirmed the interaction between Munc18a and its cognate syntaxin-1. In brain lysate the immunoprecipitated Munc18a coprecipitated with high amount of syntaxin-1 and *vice versa* (de Vries et al. 2000). In addition Munc18a was shown to be an important element of large dense-core vesicle (LDCV) release in adrenal chromaffin cells, and its deficiency causes a 10-fold decrease in LDCVs (Voets et al. 2001). Munc18b interacts with syntaxin-3 and thus plays a role in controlling membrane trafficking at the apical plasma membrane of epithelial cells. In addition, cell fractionation studies demonstrated that the majority of Munc18b is associated with the membrane (Riento, Jantti et al. 1996). Munc18b was shown to play a role in the regulation of granule exocytosis by mast cells (Martin-Verdeaux et al. 2003). As mentioned earlier, Munc18c can interact with both syntaxin-2, and -4. The interaction between Munc18c and syntaxin-4 plays a vital role in insulin exocytosis in adipose tissue and muscle cells, in addition to its important role in endothelial cell activation (Tamori et al. 1998; Thurmond et al. 2000; Macaulay et al. 2002).

Three SM isoforms are present in platelets: Munc18a, Munc18b, and Munc18c (Reed, Houg et al. 1999; Houg et al. 2003; Schraw et al. 2003). Using an inhibitory peptide (Pep3) or anti-Munc18 antibodies, our group showed that at least one isoform is important for platelet secretion. In permeabilized platelets, the Munc18c/Pep3 peptide inhibits platelet secretion from the three granules, while using Munc18a/Pep3 peptide and Munc18a/b polyclonal antibody inhibits platelet secretion from  $\alpha$ -granules only. Using a monoclonal anti-Munc18c antibody and a peptide-based inhibitor that disrupted Munc18c-syntaxin-4 interactions, Reed *et al.* showed that Munc18c-syntaxin-4 interactions contribute to platelet secretion and that PKC phosphorylates Munc18c after stimulation with thrombin (Houg, Polgar et al. 2003). Despite these insights, it is not



clear which of the three identified SM protein(s) is/are needed for platelet exocytosis. Recent studies of patients with Familial Hemophagocytic Lymphohistocytosis (FHL) suggest that Munc18b is important for platelet function (Sandrock et al. 2010).

### **Familial Hemophagocytic Lymphocytosis (FHL)**

FHL was first described in 1952 as a rare, invariably fatal, autosomal recessive immune dysfunctional disorder of childhood, named ‘Familial Hemophagocytic Reticulosis’ (Farquhar et al. 1952). FHL is a life threatening genetic disease resulting from cytotoxic T lymphocyte (CTL) and natural killer (NK) cell dysfunction. These cell types contain secretory lysosomes that contain perforin (form pores for granzymes to enter the target cell) and granzymes (the serine proteases enzymes) that are exocytosed to destroy the target cell. It manifests as fever, splenomegaly, with an increase of lymphocytes count. Five defective loci are associated with FHL; the four genes identified are: FHL2-perforin, FHL3-Munc13-4/Unc13D, FHL4-syntaxin-11, and FHL5-unc18b (Filipovich 2011). A similar genetic disease, Gricelli’s Syndrome, is caused by a defect in the Rab27a gene (Menasche et al. 2000). The proteins encoded by these genes are either cargo in cytolytic granules (perforin) or elements of the secretory machinery required for lytic granule exocytosis (Munc13-4, Rab27a, syntaxin-11, and Munc18b).

Our work demonstrated that Munc13-4 is a limiting factor for platelet granule secretion (Ren et al. 2010). Rab27a and b have been shown to be important for platelet secretion (Novak et al. 2002; Tolmachova et al. 2007). These data suggest that platelets employ similar secretory machinery, as do CTL and NK cells.

### **Summary:**

Platelet exocytosis is a highly regulated process that involves several proteins. Among those regulatory proteins is the Munc18 family. The roles of Munc18a and Munc18c have been suggested in the literature; however the role of Munc18b in platelet secretion needs to be further investigated. Chapter Four of this thesis will discuss the approach of using platelets from patients with Familial Hemophagocytic Lymphocytosis disorder that have a genetic defect in Munc18b gene to understand the role of Munc18b in platelet secretion.

## Small GTPases in Platelets

Small GTPases are monomeric guanine nucleotide binding proteins related to the  $\alpha$  subunit of heterotrimeric G proteins, with molecular weights of 21 to 30 kD. Small GTPases belong to Ras superfamily (Parada et al. 1982). The small GTPase superfamily is divided into five major families: Ras, Rho, Rab, Arf, and Ran (Colicelli 2004; Wennerberg et al. 2005). Small GTPases are monomers that function as molecular switches in intracellular signaling. They are present in two forms: GTP-bound (active form) or GDP-bound (inactive form). In response to upstream signaling, the GDP form is exchanged for GTP and this reaction is catalyzed by guanine nucleotide exchange factors (GEFs). The activated GTPases then can interact with one or more effector proteins that lead to activation of downstream signaling pathways. The GTPase activating proteins (GAPs) revert the conformation to the inactive GDP-bound form by stimulating GTP hydrolysis (Bernards et al. 2004).

In platelets, there are members of Ras family (Ras, Ral, and Rap), Rho family (Rho, Rac, and Cdc42), and Rab family (Rab 1, 3, 4, 6, 8, 11, 27, 31, 32) (Karniguian et al. 1993); (Richards-Smith et al. 1999; Wilson et al. 2000). Not all of these have defined functions in platelets. For instance, Rho family GTPases have been shown to play roles in cytoskeletal remodeling and integrin activation in platelets which is important for platelet adhesion, aggregation, and clot retraction (McCarty et al. 2005); (Akbar et al. 2007) (Varga-Szabo et al. 2008). For Arf Family GTPases, our group showed that Arf6 is present in platelets, and it plays a role in platelet activation (Choi et al. 2006). The focus in this thesis will be on the Ras family GTPase, Ras protein.

## Ras Family GTPase

**Ras:** Ras was discovered as an oncogene that is constitutively active in its GTP bound form. In mammalian cells, there are three *ras* genes encoding four Ras isoforms (H-Ras, K-Ras4A, K-Ras4B and N-Ras). Ras proteins are mostly located on the inner leaflet of the plasma membrane and function as molecular switches. In Ras, the GDP exchange to GTP is mediated by the GEF protein SOS. The proline rich domain of SOS binds to the SH3 domain of the effector protein, GRB2. The SH2 domain of GRB2 binds the phosphorylated tyrosine residues on tyrosine kinase receptors and that leads to the

localization of SOS-GRB2 complex to the plasma membrane and activation of Ras (Buday et al. 1993; Rozakis-Adcock et al. 1993). Activated Ras interacts with several effector proteins, such as: RAF, GAPp120, MEKK1 and activates the downstream signaling cascades of the Ras/MAPK pathway. Binding of Ras-GTP to the threonine kinase Raf leads to the activation of the kinases MEK, which activates ERK (Figure 1-2). Activation of ERK regulates transcription factors that lead to cell differentiation, proliferation and survival cells (Omerovic et al. 2008) (Omerovic et al. 2009). Mutations in Ras were detected in variety of cancer cell lines (Colicelli 2004).

In platelets at least one isoform of Ras, the H-Ras, is present; but the presence of K- and N-Ras is not excluded, since anti-pan Ras immunoglobulin, which does not exclude various isoforms of Ras, was used (Shock et al. 1997). Ras was identified in platelets by immunoprecipitation followed by western blotting. Immuno-fluorescence microscopy was used to further confirm the presence of Ras in platelets. Kinases, downstream Ras, such as Raf-1, MEK (MAPK/ERK kinase), and ERK (Tulasne et al. 2002) are all detected in platelets. Upon stimulation of human platelets with the G protein-coupled receptor agonist's thromboxane A<sub>2</sub>, and thrombin, Ras was rapidly activated. Previous work showed that collagen and thrombin induce MEK and ERK activation (Borsch-Haubold et al. 1995). Watson *et al* showed that RAS is activated in platelets in response to thrombin and glycoprotein VI (GPVI) agonists (Tulasne, Bori et al. 2002).

The role of ERK activation in platelet function was examined using ERK specific inhibitors (U0126), and platelet aggregation (Oury et al. 2002) and secretion (Toth-Zsamboki et al. 2003) were affected. Li Z *et al*, showed that agonist activated platelets activate MAPK p38 and ERK pathways leading to platelet integrin  $\alpha$ IIB $\beta$ 3 activation (Li et al. 2006). In addition, the ERK pathway was found to play a role in the activation of store-mediated Ca<sup>2+</sup> entry (SMCE) in human platelets (Rosado et al. 2001). Depletion of the intracellular Ca<sup>2+</sup> stores triggered the Ca<sup>2+</sup> independent activation of the ERK kinase pathway, leading to the activation of SMCE, independent of PI3, or 4-kinases but sensitive to Ras inhibitors. These data suggest that ERK is a downstream effector of Ras proteins in human platelets. ERK was shown to play a role in thromboxane (TxA<sub>2</sub>) generation (Garcia et al. 2007), and it can be affected by inhibiting ERK, though the

effects on aggregation are controversial (McNicol et al. 2003). Therefore, future work has to focus on the exact function and mechanism of action of Ras-dependent signaling during platelet activation.

**Ral:** Present in nucleated cells, Ral GTPases, are members of the Ras subfamily, and consist of RalA and RalB. Upon binding to their effector proteins, Ral GTPase proteins exert a variety of functions such as, exocytosis through recruiting a multi-subunit complex termed as “exocyst complex” that are important for targeting secretory vesicles to specific plasma membrane domains (Bodemann et al. 2008). Ral GTPases are also involved in cytoskeletal organization through filamin (Feig 2003). In platelets, both RalA and RalB were identified and shown to be associated with platelet dense granules (Bhullar et al. 1998). Platelet activation with different agonists, including thrombin, leads to a rapid and strong activation of Ral in a  $Ca^{2+}$  dependent manner (Wolthuis et al. 1998). In permeabilized platelets, the recombinant Ral-interacting domain of Sec5, the downstream effector of RalA in exocyst complex was used and the serotonin release from dense granules was inhibited. These data suggest that Ral-exocyst play a role in regulating platelet dense granule release (Kawato et al. 2008).

**Rap:** Rap GTPases play a key role in integrin activation, the regulation of cell-cell contact, the formation of cell adhesion, the cell polarity, and the control of exocytosis (Frische et al. 2010). The Rap family consists of 2 rap1 genes and 3 rap2 genes. Rap1B and Rap2B are expressed in platelets, in which Rap1B is the most abundant form; consisting of around 90% of total Rab proteins (Torti et al. 1994). Upon stimulation of human platelets with different agonists, including thrombin, Rap1 is rapidly activated. This activation of Rap1 requires an increase in intraplatelet  $Ca^{2+}$  (Greco et al. 2004), and is not dependent on integrin ‘outside-in’ signaling (Franke et al. 2000). In the murine model, Rab1B deficiency results in bleeding and aggregation defect. Stimulated with ADP or PAR4 peptide, platelets lacking Rap1B, had a defect in the activation of Integrin  $\alpha$ IIB $\beta$ 3 and spreading on fibrinogen (Chrzanowska-Wodnicka et al. 2005). Shattil and colleagues defined the importance of Rap1 for  $\alpha$ IIB/ $\beta$ 3 activation. They showed that Rap1-GTP-dependent talin recruitment to  $\beta$ 3 integrin by RIAM is required for  $\alpha$ IIB/ $\beta$ 3 activation (Watanabe et al. 2008).

## Noonan Syndrome and Ras Signaling

Noonan syndrome (NS) is a common, autosomal dominant disorder, found by Jacqueline Noonan in 1968 (Noonan 1968). The estimated prevalence is between 1:1,000 and 1:2,500 live births (Nora et al. 1974; Tartaglia et al. 2011) NS is a genetically and clinically variable condition that is mainly characterized by unique facial features, high forehead, chest deformity, short stature, short neck with excess skin, bleeding diathesis and congenital heart defects (Tartaglia, Gelb et al. 2011). The unique facial features include: a broad forehead, and low set of posteriorly rotated ears. Cardiac defects involve pulmonic stenosis, cardiomyopathy, arterial spetal defects, and ventricle septal defects. Bleeding diathesis is common in patients with NS, due to von Willebrand disease, factor XI and XII deficiencies, and thrombocytopenia (Sharland et al. 1992; Singer et al. 1997). Leukemia, such as juvenile myelomonocytic leukemia (JMML, OMIM 607785) and acute lymphoblastic leukemia (ALL), can occur in NS patients (Fukuda et al. 1997; Choong et al. 1999).

## Molecular and Genetic Aspects of Noonan Syndrome

As mentioned earlier, Ras proteins are small GTPases that work as a molecular switch as they cycle between an active GTP-bound and an inactive GDP-bound form. Upon binding of a growth factor to the receptor tyrosine kinase (RTK) such as the epidermal growth factor receptor (EGFR), the receptor undergoes autophosphorylation and binds to the adaptor protein GRB2 through its *src* homology domain2 (SH2) domain. This results in binding of the GRB2 through its SH3 domain to the GEF protein, son of the seven less (SOS), which in turn will be recruited to the plasma membrane. SOS protein will catalyse the exchange of the Ras GDP-bound to the Ras GTP bound form. The activated Ras GTP binds to its effector molecule, the serine-threonine kinase Raf. Activated Raf phosphorylates MEK, which in turn lead to phosphorylation of ERK. Binding of ERK to the transcription factors results in initiation of many signaling pathways that lead to cell proliferation, differentiation, survival or cytoskeleton rearrangements (Figure 3) (Vetter et al. 2001; Donovan et al. 2002). This occurs in the nucleated cell. The exact role of Ras signaling in platelets is not completely defined.

Mutations in Ras pathway genes (PTPN11, SOS1, Ras, Raf1, MEK) cause NS and its associated disorders.

***PTPN11:*** Ten years ago, the PTPN11 gene was discovered as a major NS disease gene (Tartaglia et al. 2001). PTPN11 is a non-receptor protein tyrosine phosphatase, encodes for the SHP-2 protein that is important for controlling Ras activation. SHP2 structure involves N-terminal *src*-homology 2 domains (N-SH2 and C-SH2), catalytic domain (PTP), and a C-terminal domain with unknown function (Hof et al. 1998). The PTPN11 mutation is the most common, occurring in around 50% of NS patients. In NS patients, the mutation is mainly missense, gain-of-function mutations that lead to disruption of the auto-inhibitory interaction between the N-terminal SH2 domain and the catalytic phosphatase domain; this result in enhanced phosphatase activity and thus the activation of the Ras-MAPK pathway (Tartaglia et al. 2006). Despite the fact that the majority of the cases have germline mutations (Kratz et al. 2005), acquired somatic mutations have been reported in around one third of the patients (Tartaglia et al. 2003).

***K-Ras and N-Ras:*** Monomeric GTPases, that as mentioned above alternate between the GDP bound or “the off” switch and the GTP bound or “the on” switch, that activate downstream signaling. Ras proteins are activated by the GEFs, (SOS1), that lead to the release of the GDP bound, and inactivated by GAPs which lead to increase the intrinsic GTPase activity. The Ras subfamily was reported to be rarely associated with heterozygous germline mutations in each of the three Ras members: H-, K-, and N-Ras (Tartaglia, Martinelli et al. 2006). The incidence of K-Ras mutations is low in patients with NS, around 2-4% and associated with variable but severe phenotype (Noonan 1994; Zenker et al. 2007). There are two splice variants for K-Ras: K-RasA that is tissue-specific and restricted developmentally, and K-RasB, that is ubiquitously expressed. Several K-Ras mutations have been identified in NS patients. These mutations are mainly gain-of-function mutations that lead to increase in the downstream signaling of the Ras/MAPK pathway, either by mutations that decrease the intrinsic and GAP GTPase activity (Noonan 1994; Osio et al. 2005) so Ras is locked in the GTP form, or by mutations that lead to by decrease the affinity of nucleotide binding affinity (Schubbert et al. 2007). These mutations lead to an increase in the GTP-bound “active” form of K-Ras, however, these mutations that lead to the elevation of the downstream signaling cascades,

are less frequent than the mutations occurring in the oncogenic K-Ras that lead to cancer (Osio, Dahlgren et al. 2005). Two germline missense mutations of N-Ras (Thr50Ile and Gly60Glu amino acid changes) have been identified in NS patients (Cirstea et al. 2010). These mutations also enhance the activation of Ras/MAPK pathway.

**Sos:** A member of GEFs proteins that mediates the activation of Ras proteins after the activation of tyrosine kinase receptors. They facilitate the conversion of the inactive GDP-bound form to the active GTP-bound form of Ras, by catalyzing the release of GDP from Ras (Quilliam et al. 1995). In Ras signaling, there are two isoforms: SOS1 and SOS2. Mutations in SOS1 gene are considered to be the second largest common mutations in NS patients, accounting for around 20% (Tartaglia et al. 2007; Zenker et al. 2007). Around half of the SOS1 mutations are amino acid substitution in the short helical linker that connects the plekstrin homology (PH) and the Ras exchanger motif (REM) domains. Other mutations identified are located within the PH domain, and the last cluster of mutations has been identified at the interacting regions of the Dbl homology domain and REM domains. Most of these mutations affects the structure which in turn affect the maintenance of the catalytically autoinhibited conformation, leading to gain of function and enhancing Ras and ERK activation (Roberts et al. 2007; Tartaglia, Pennacchio et al. 2007). Heterozygous NS patients with SOS mutations show distinctive phenotypes of ectodermal abnormalities that are associated with some cognitive disabilities and short stature (Roberts, Araki et al. 2007; Zenker, Horn et al. 2007).

**Raf1:** There are three members of effector proteins have been identified: Raf1, ARaf and BRaf (Wellbrock et al. 2004; Leicht et al. 2007). Once activated by the tyrosine kinase receptor, they phosphorylate and activate the downstream signaling pathway of Ras/MAPK, MEK1 and MEK2, which activates ERK1 and ERK2. Somatic mutations occur mostly in BRaf since it has more kinase activity, and mainly occur in malignant melanomas, thyroid, colorectal and ovarian cancers, while ARaf and Raf1 missense mutations are observed rarely in malignancies. In NS patients, Raf1 mutations occur between 5-15% (Pandit et al. 2007; Razzaque et al. 2007). These mutations in Raf1 affect residues located in three different regions of the protein. The first group of mutations, which account for the majority of the mutations, involve the N-terminal consensus 14-3-3 recognition sequence or residues near it. The second group involve residues within the

activation segment region of the kinase domain. The third group of mutations involve two adjacent residues located at the C-terminus. Most of these mutation lead to increase in the activation of Ras/MAPK pathway (Pandit, Sarkozy et al. 2007; Razzaque, Nishizawa et al. 2007), while the mutations in the activation segment lead to impairment of the Ras\MAPK pathway. These mutations in BRaf are clinically associated with a defect in neonatal growth and difficulties in feeding, and in cognitive function (Sarkozy et al. 2009). Their phenotype is more severe than those caused by PTPN11 and SOS1 mutations. There are more than 40 germline missense BRaf mutations have been identified to date. They are clustered in the cysteine-rich domain and in the amino-terminal domain and in the activation segment of the kinase domain.

**MEK1:** MEK1 and MEK, the effectors of Raf proteins are kinases that are essential to phosphorylate ERK proteins at tyrosine and serine/threonine residues (Zheng et al. 1993). One germline missense mutation in MEK1 has been reported in two unrelated patients with a phenotype that fits NS (Nava et al. 2007). There is no data on the effect of this mutation on MEK1 function and Ras\MAPK pathway.

### **Summary:**

In platelets, the contribution of several Ras GTPases proteins has been identified. Upon agonist activation such as thrombin or collagen, platelets are activated and the GTPases proteins then initiate cytoskeletal remodeling and activation of integrin. These functions are very important for maintaining the physiological function of platelets in adhesion and aggregation and thus in retaining the integrity of the vessel wall. However the exact role of Ras proteins in platelets is not fully understood to date. Therefore using NS patients that have mutations in Ras signaling pathway gives us a novel tool to understand Ras function in platelets. Chapter Five of this thesis will focus on that.

### **Thesis Significance**

The focus of this thesis is on understanding the mechanism of platelet secretion and its related pathological outcomes. In Chapter Three, the importance of the v-SNARE VAMP-8 in atherosclerosis will be discussed. VAMP-8 is known to play an important role in platelet secretion, both *in vitro* and *in vivo*, by being the primary v-SNARE required for platelet secretion. The aim is to study the role of VAMP-8-mediated



secretion in atherosclerosis. The following chapter focuses on the SNARE regulatory protein, Munc18b. The exact role of Munc18b in platelet secretion was not fully characterized in the literature; therefore we sought to understand if Munc18b plays a role in the regulation of platelet exocytosis. In Chapter Five the focus is on the role of the Ras GTPase in platelet secretion using NS patients as a tool to understand the signaling cascade affected by mutations of this protein and its related upstream and downstream-effectors and the effect of that on platelet function.

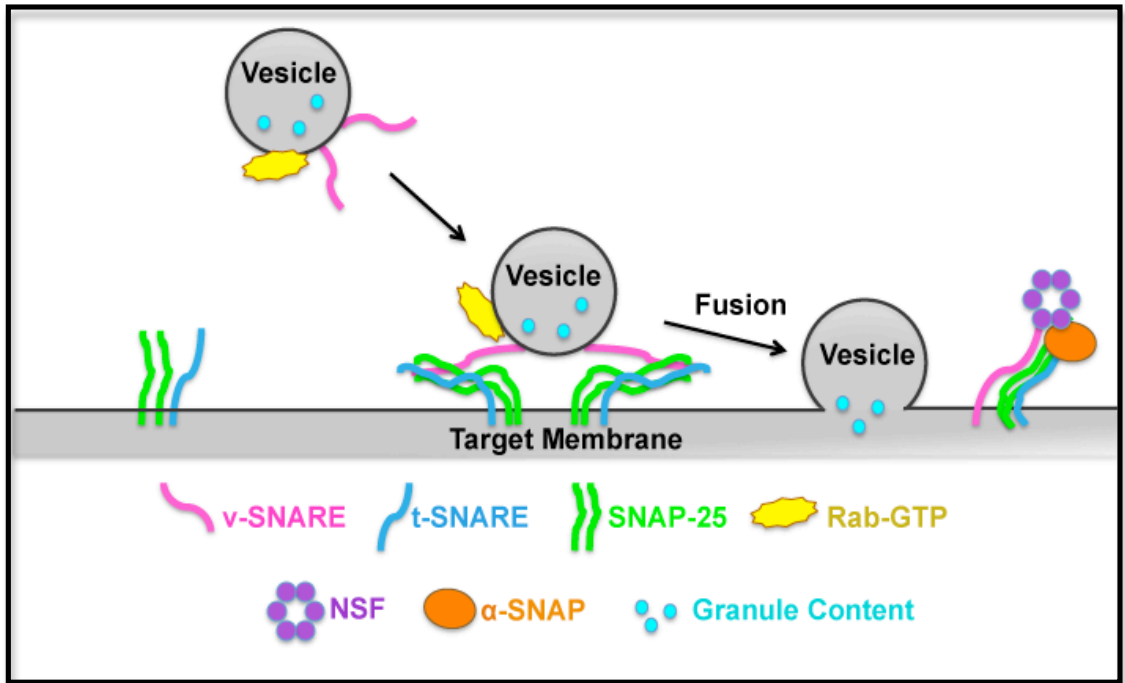
Dense Granule	$\alpha$ -Granule	Lysosome
Nucleotides:	Adhesive glycoproteins:	Acid phosphatase
- ATP	- Fibrinogen	
- ADP	- Fibronectin	$\beta$ -hexosaminidase
	- thrombospondin-1	
Amines:		Acid hydrolases
- Serotonin (5-HT)	Platelet specific:	
- Histamine	- Platelet factor 4 (PF4)	$\alpha$ -fucosidase
	- $\beta$ - Thromboglobulin ( $\beta$ -TG)	
Bivalent cations:		
- Calcium	Angiogenic factors:	
- Magnesium	- VEGF	
	- PF4 (inhibitor)	
	Hemostatic factors:	
	- Fibrinogen, factor V, VIII, XI	
	- Plasminogen	
	- Plasminogen activator inhibitor-1	
	Membrane proteins:	
	- P-Selectin	
	- CD63 (LAMP-3)	
	Others:	
	- IgG, IgM	
	- Albumin	

**Table 1-1: Platelet Secreted Granule Content.** (Modified from Smyth *et al.* (Smyth, McEver et al. 2009). Listed are some examples of platelets granule content from dense granules,  $\alpha$ -granules, and lysosomes.

Platelet Agonist	Platelet Receptor
Thrombin	PAR1 and PAR4
ADP	P2Y <sub>12</sub> and P2Y <sub>1</sub>
Thromboxane A <sub>2</sub>	Thromboxane
Collagen	GPVI
Fibrinogen	$\alpha$ IIb $\beta$ 3

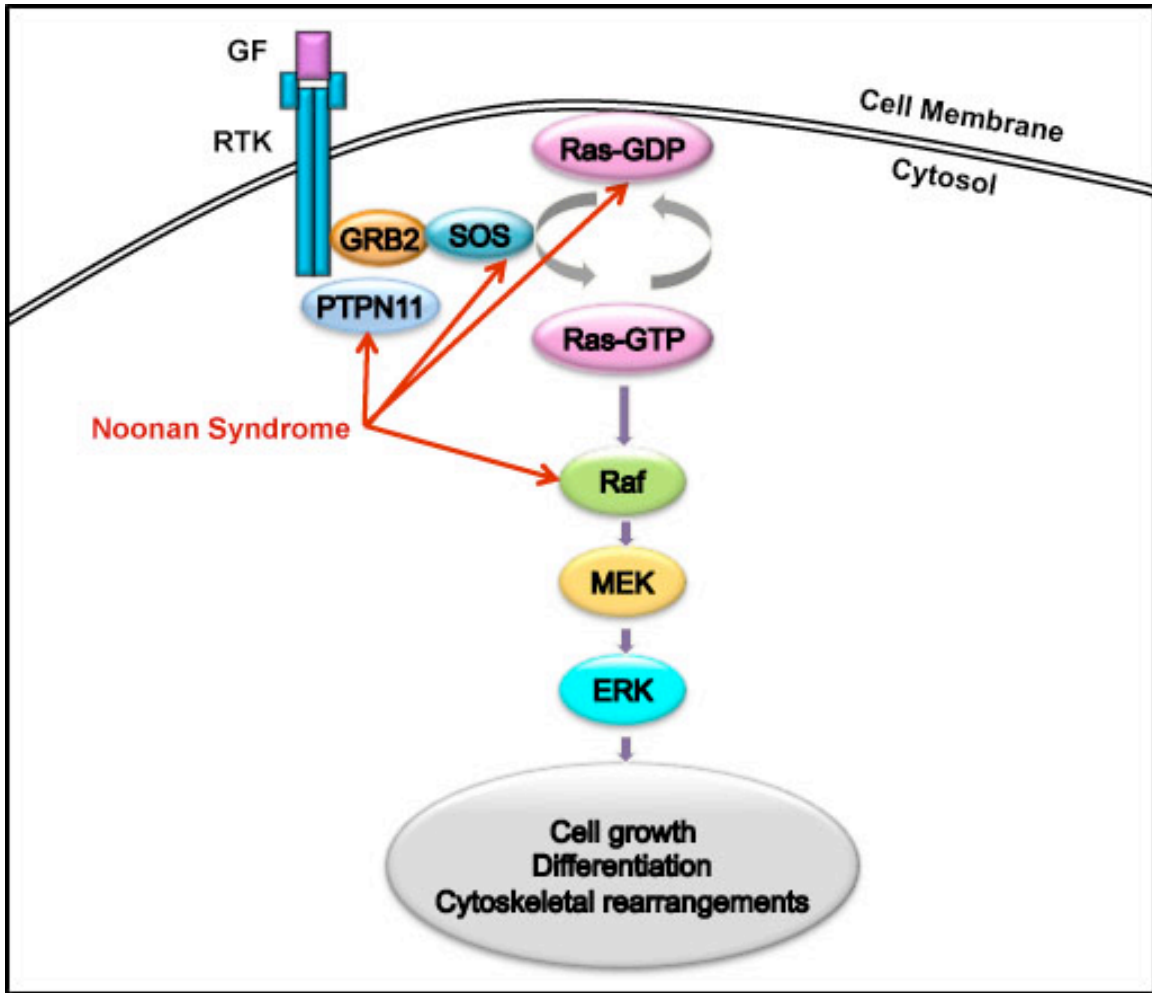
**Table 1-2: Platelet Agonists and their Corresponding Receptors.**

Presented in this table are some examples of platelet agonists and their corresponding receptors.



**Figure1-1: Molecular Machinery of SNARE-Mediated Membrane Fusion.**

Membrane fusion occurs in several steps. Briefly, Rab-GTPase proteins (yellow) mediate the trafficking of the vesicle to the target membrane. The v-SNARE, VAMP (pink) on the vesicle membrane interacts with the t-SNARE, syntaxin (blue) and SNAP-25 (green) on the target membrane to form a heterotrimeric (7S) complex that spans the two bilayers and mediates membrane fusion and release of vesicle (granule) content (cyan). After membrane fusion, NSF (purple) disassembles the 7S complex, and SNAREs proteins are then recycled for more rounds of fusion events.



**Figure 1-2: Ras-MAPK signaling pathway and associated mutations with NS.**

The binding of a growth factor to the receptor tyrosine kinase (RTK) leads to phosphorylation of GRB2, which in turn binds SOS (guanine nucleotide exchange factors (GEFs)) leading to the exchange of the GDP to GTP and thus activation of the Ras protein. The activated Ras-GTP in turns activates RAF and downstream effectors MEK and ERK, which lead to activation of several signaling pathways that are, involved in cell proliferation, differentiation and cytoskeleton rearrangements.

## **Chapter Two**

### **Materials and Methods**

#### **Antibodies**

##### **Antibodies to SNARE Proteins**

Rabbit anti-human endobrevin/VAMP-8 polyclonal and rabbit anti-human VAMP-7 polyclonal antibodies were generated in our laboratory using recombinant version of the following antigens respectively: cytoplasmic domain of VAMP-8 (1-73 aa) and the longin domain of human VAMP-7 (1-120 aa). Anti-synaptobrevin VAMP-2 monoclonal antibody (CL69.1) and rabbit anti-endobrevin/VAMP-8 polyclonal antibody was from (Synaptic System, Goettingen, Germany). Sheep anti-cellubrevin/VAMP-3 polyclonal antibody was a generous gift from Dr. Jeffery Pessin (Stony Brook University, Stony Brook, NY). Anti-SNAP-23, anti-syntaxin-2 and -4 polyclonal antibodies were generated in the Whiteheart laboratory as described in (Chen, Bernstein et al. 2000).

##### **Antibodies to SNARE Proteins Regulators**

Munc18a/STXBP1 monoclonal antibody was purchased from Synaptic System. Rabbit anti-human Munc18b/STXBP2 polyclonal antibody was generated in our laboratory using the full recombinant protein of Munc18b. Rabbit anti-Munc18c/STXBP3 polyclonal antibody was generated in our laboratory.

Rabbit anti-Munc13-4 A and anti Munc13-4 C polyclonal antibodies were generous gifts from Dr. Christian Wimmer (Basel Institute of Immunology, Basel, Switzerland). They were generated by, injecting the rabbits with maltose binding protein (MBP), tagged domain A (36-349 aa), and tagged domain C (703-975 aa) respectively. Anti Munc13-4 monoclonal antibody was from (Synaptic System). Anti-mouse Rab27a monoclonal antibody was from (BD Biosciences, San Jose, CA).

##### **Other Antibodies**

Monoclonal anti-mouse Ras antibody was purchased from Millipore (Billerica, MA). Monoclonal anti- $\beta$ -actin antibody was from Sigma-Aldrich (St. Louis, MO). A paired monoclonal platelet factor 4 (PF4) antibodies were used for the Enzyme-Linked

Immuno Sorbent Assay (ELISA); a monoclonal anti-human PF4 antibody that was used to assay human PF4, was used as the capture antibody (R&D Systems, Minneapolis, MN), and the biotin conjugated rabbit anti-human PF4 polyclonal antibody was used as the detection antibody (Leinco Technologies Inc., St. Louis, MO). Fluorescein isothiocyanate (FITC) labeled anti-human P-Selectin monoclonal antibody (AK-4) and FITC-labeled anti-mouse P-Selectin monoclonal antibody (RB 40.34), were from BD Pharmingen<sup>TM</sup> (BD Bioscience, San Jose, CA). Alkaline phosphatase conjugated secondary anti-mouse, anti-rabbit, anti-rabbit protein-A, anti-sheep, anti-goat, IgGs were purchased from (Sigma-Aldrich, St. Louis, MO). FITC conjugated anti-mouse, anti-rat and anti-rabbit IgGs were from Vector Laboratories (Burlingame, CA).

## Reagents

Acid citrate dextrose (ACD) tubes used for blood collection (BD Vacutainer<sup>R</sup>) were obtained from BD Diagnostics (Sparks, MD). Formaldehyde (70%) was purchased from Electron Microscopy Sciences (Hatfield, PA). EDTA-free protease inhibitor cocktail was purchased from Roche (Indianapolis, IL). Platelet activation inhibitor, prostaglandin I<sub>2</sub> (PGI<sub>2</sub>) was obtained from Cayman Chemical (Ann Arbor, MI). Agonists used for platelets activation, human plasma thrombin and ADP were purchased from Chrono-log (Havertown, PA), while convulxin, the snake venom toxin was purchased from Centechem Inc. (Norwalk, CT). Mouse cytokines detection kit was obtained from Millipore (Billerica, MA). The Avidin Biotin Complex (ABC) immunostaining kit was from Vector (Burlingame, CA).

## Methods

### Mouse Strain Construction and Genotyping

VAMP-8<sup>-/-</sup> mice on a mixed genetic background (C57BL/6J and 129Sv) were generously provided by Dr. Wanjin Hong (Institute of Molecular and Cellular Biology, Proteos, Singapore). ApoE<sup>-/-</sup> mice (C57BL/6J) were obtained from the Jackson laboratory (Bar Harbor, Maine). Four experimental groups were produced by crossing VAMP-8<sup>-/-</sup> and ApoE<sup>-/-</sup> mice: ApoE<sup>-/-</sup> (control for atherosclerosis), VAMP-8<sup>-/-</sup> (control for secretion defect), VAMP-8<sup>+/+</sup>/ApoE<sup>+/+</sup> (wild type), and VAMP-8<sup>-/-</sup>/ApoE<sup>-/-</sup> (test group). Mice were



maintained on normal chow for 50 weeks. A 2-millimeter piece of each mouse-tail was used to extract the DNA. Genotyping was performed using Polymerase Chain Reaction (PCR). VAMP-8 genotyping was described in (Wang, Ng et al. 2004), wild type and mutant primers were used as follows:

Wild type primers: 5'-GATCCTCTGCTGCCCCGGCCTAAT-3' and 5'-CCGTGGCTTCCAAGTCCTCTGTC-3'

Mutant primers: 5'-GATCCTCTGCTGCCCCGGCCTAAT-3' and 5'-GCCTGCAAAGGGTCGCTACAGAC-3'.

ApoE genotyping was performed as previously described (Zhang et al. 1992). Wild type and mutant primers were used as follows:

Forward primer: 5' –TGTGACTTGGGAGCTCTGCAGC-3'

Neo NTR: 5'-GCCGCCCCGACTGCATCT-3'

Reverse primer: 5' –GCCTAGCCGAGGGAGAGCCG-3'

For the acute studies, VAMP-8 mice were backcrossed to C57BL/6J background.

VAMP-8<sup>+/-</sup>/ApoE<sup>+/-</sup> progeny were selected based on the genotype and the color (brown mice are excluded). These mice were again crossed with C57BL/6J mice and the selection process was for a total of 10 generations alternating male and female C57BL/6J mice at each breeding time. The resulting VAMP-8<sup>+/-</sup>/ApoE<sup>+/-</sup> progeny was then crossed to make the strains needed for the acute studies. At 8 weeks of age, mice fed chow supplemented with saturated fat 42% and 0.15% cholesterol (diet no. TD88137; Harlan Teklad, Indianapolis, IN). Then, lesion size was examined at 15 weeks. Both male and female mice were included in the study. All procedures were carried out under a protocol approved by the University of Kentucky Institutional Animal Care and Use Committee (IACUC).

### **Human Platelets Preparation**

Washed human platelets were isolated either from fresh banked platelets obtained from Kentucky Blood Center (Lexington, KY), or from whole blood that was diluted 2:1 with HEPES- Tyrode's buffer (20 mM HEPES/KOH, pH 6.5, 128mM NaCl, 2.8 mM KCl, 1 mM MgCl<sub>2</sub>, 5mM D-glucose, 12 mM NaHCO<sub>3</sub>, 0.4 mM NaH<sub>2</sub>PO<sub>4</sub>), with the addition of 0.37 units/mL apyrase (Sigma-Aldrich), 100 ng PGI<sub>2</sub> (Sigma-Aldrich), and

1mM EGTA, by centrifugation at 150 x g for 10 minutes at room temperature to remove red blood cells. Platelets pellet was collected by centrifugation at 900 x g for 10. The pellet then was washed with HEPES- Tyrode's buffer (pH 6.5) containing 0.37 units/mL apyrase, 100 ng/mL PGI<sub>2</sub>, and 1mM EGTA. Washed platelets were then suspended with HEPES- Tyrode's buffer (pH 7.4).

### **Murine Platelets Preparation**

To collect blood from the mice heart, mice were euthanized by carbon dioxide inhalation. Then the heart was exposed, and the blood was withdrawn from the right ventricle, using a 26-gauge needle attached to a 1 mL syringe (BD Diagnostics, Sparks, MD) containing 100 µL of 3.8% sodium citrate. The blood (1 mL) then was pooled and diluted with an equal volume of phosphate buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.5 mM KH<sub>2</sub>PO<sub>4</sub>, (pH 7.4)) containing 0.37 units/mL apyrase and 100 ng/mL PGI<sub>2</sub>. To collect the PRP, whole blood was centrifuged at 250 x g for 10 minutes. Washed platelets were collected by centrifugation at 120 x g for 10 minutes in presence of 0.37 units/mL apyrase and 100 ng/mL PGI<sub>2</sub>. Followed by washing platelet pellet with HEPES-Tyrode's buffer (pH 6.5) containing 0.37 units/mL apyrase, 100 ng/mL PGI<sub>2</sub>, and 1mM EGTA. Platelets were then suspended with HEPES- Tyrode's buffer (pH 7.4).

### **Platelet Counting**

To count platelets, the Z2 coulter counter (Beckman Coulter, Inc., Miami, FL) was used. Washed platelets prepared as described above, were diluted in HEPES-Tyrode's buffer (pH 7.4) 1:20.000. The final count was based on the average of three counts. The concentration of platelets was adjusted as indicated in each experiment.

### **Platelet Aggregometry**

Aggregation of washed platelets prepared as described above, was measured using a Chrono-Log Model 460VS Lumi-aggregometer (Havertown, PA). Using 500 µL and 250 µL of human and mice platelets respectively, siliconized glass cuvette with a metal stirring bar (Chrono-log), spinning at 800 rpm for 3 minutes at 37°C. Agonists were

added for indicated period of time. The aggregation traces were monitored using Model 810 Aggro/Link computer interface and Aggro/Link software (Chrono-log).

### **Platelet ATP Release**

Using Chrono-Log Model 460VS Lumi-aggregometer (Havertown, PA), ATP release from platelets dense granule was measured. Washed platelets, 500  $\mu\text{L}$  and 250  $\mu\text{L}$  of human and mice platelets respectively, were incubated with luciferin/luciferase reagent (Chrono-Log) according to the manufacturer's instructions. The mixture was incubated at 37°C for 2 minutes with stirring prior to the addition of agonist or ATP standard. Upon platelets activation, the ATP released by the platelet dense granules binds with luciferin-luciferase and generates light with 560 nm peak. Luminescence traces were acquired simultaneously with aggregation on the Chrono-Log system.

### **Platelet Secretion Assay**

Washed human platelets were labeled with 2  $\mu\text{Ci/mL}$  [ $^3\text{H}$ ]5-HT (Perkin-Elmer Cetus Life Sciences, Boston, MA) for 45 minutes at 37°C, followed by washing with a calcium-free HEPES/Tyrode's buffer (pH 7.4) in the presence of 3  $\mu\text{g/mL}$  apyrase. Platelets pellet were resuspended with HEPES/Tyrode's buffer (pH 7.4), and the concentration of platelets were adjusted to  $2.5 \times 10^8/\text{mL}$ .

For thrombin titration experiments, different concentrations of thrombin (Chrono-Log) were added to stimulate platelets as indicated. To stop the reactions, a twofold excess of hirudin (Sigma-Aldrich) was added. For the time-course experiments, 0.05 U/mL thrombin was added for the indicated periods of time, and to stop the reaction, 0.1 U/mL hirudin was added. The samples were centrifuged at  $13,800 \times g$  for 1 minute. After removing the supernatant, platelet pellets were lysed with an equal volume of lysis buffer (PBS, pH 7.4, 1% Triton X-100) and kept on ice for 1 hour.

Platelet secretion assays were performed using equal volumes of the supernatant and the pellet, from the three granule contents, using the following markers: [ $^3\text{H}$ ]5-HT for dense core granules (DC), platelet factor 4 (PF4) for alpha granules, and  $\beta$ -hexosaminidase for lysosomes as previously described (Schraw, Rutledge et al. 2003). For the dense granule release, 25  $\mu\text{L}$  of platelet supernatant and of the pellet were added

to 5 mL of scintillation cocktail solution, and the analysis of the samples were carried out using Tri-Carb2100TR liquid scintillation analyzer (Beckman, Fullerton, CA). For  $\alpha$ -granule secretion, PF4 was assayed using ELISA method according to the manufacturer's instruction (R&D Systems). Lysosomal secretion was detected by measuring the enzymatic activity of hexosaminidase. Using 96 well plates, 6  $\mu$ L of supernatant and 3  $\mu$ L of the pellets were mixed with 100  $\mu$ L of citrate-phosphate buffer (53.4 mM citric acid, 93.2 mM  $\text{Na}_2\text{HPO}_4$ , pH 4.5) in the presence of 10 mM *p*-nitrophenyl-N-acetyl- $\beta$ -D-glucosaminide). The plate was sealed and incubated at 37°C for 18 hours. The reaction was stopped by adding 100  $\mu$ L of 0.08 N NaOH. To determine the optical density of each well, a Biotek Elx808 plate reader (BioTek Instruments Inc., Winooski, VT) was used at 405 nm. The secretion assays data were analyzed by dividing the supernatant fraction by the total fraction (supernatant plus pellet) to determine the percent of release for each granule marker.

### **Flow Cytometry Analysis**

Washed human platelets (20  $\mu$ L) were incubated with (4  $\mu$ L) FITC-conjugated PAC-1 or PE-conjugated anti-CD41a monoclonal antibodies for 15 minutes at 37°C. Platelets were stimulated with thrombin (0.1 U/mL) for 3 minutes at room temperature. The reaction was stopped with the addition of a 2-fold excess of hirudin. The platelets were 10-fold diluted by adding HEPES-Tyrode's buffer (pH 6.5). The samples were transferred into polystyrene Falcon™ tube (BD Biosciences). Fluorescent intensity was determined using FACScan™ flow cytometer. The voltages for forward light scattering (FSC; E-01/linear) and side light scattering (SSC; 450/linear) were optimized to measure platelet population. The voltages for excitation of blue (FITC) and red (PE) channels were adjusted to detect the fluorescence intensity. The data was plotted as a histogram with statistical values using CellQuest™ (BD Biosciences) analysis software.

### **Electron Microscopy of Platelet Morphology**

Washed platelets prepared as described above, were used either as resting or stimulated with 0.1 U/mL of thrombin for 20 minutes at room temperature. To stop the reaction, an equal volume of 6% glutaraldehyde (Electron Microscopy Sciences) in 0.2 M

Sorenson's phosphate buffer (16.2 mg/mL  $\text{KH}_2\text{PO}_4$ , 3.76 mg/mL  $\text{Na}_2\text{HPO}_4$ , pH 8.0) was added to the platelet suspension as previously described (Chen, Bernstein et al. 2000; Ren, Barber et al. 2007). The samples were incubated at 4°C for 1 hour, followed by washing three times for 5 minutes with 0.1 M Sorenson's buffer by spinning the samples at  $3,000 \times g$  for 2 minutes. Platelet pellet was then resuspended in 300  $\mu\text{L}$  of 1%  $\text{OsO}_4$  dissolved in 0.1 M Sorenson's buffer and incubated for 30 minutes on ice.

Samples were washed twice with 0.1 M Sorenson's buffer and dehydrated in a serial ethanol washes (50%, 70%, 80%, 90%, 100%, and absolute ethanol) for 5 minutes at room temperature. The platelets were incubated with propylene oxide and infiltrated overnight in a 1:1 mixture of propylene oxide and Spurr's resin (10 g vinyl cyclohexane dioxide (VCD), 6 g DER epoxy resin, 26 g nonenyl succine anhydride (NSA) with final addition of 0.4 g dimethylaminoethanol (DMAE)). Samples were washed in Spurr's resin for 1 hour at room temperature and then embedded in 200  $\mu\text{L}$  Spurr's resin and polymerized in a 60°C incubator for two days. After the blocks were polymerized, they were sectioned and mounted on copper grids. The sections were then stained with uranyl acetate and examined using Philips Tecnai 12 transmission electron microscope (FEI, Hillsboro, OR) and images were captured with Gatan Digitalmicrograph software (Pleasanton, CA).

Alternatively, for better identification of platelet dense granules (Schraw et al. 2004), a slight modification of the procedure was employed in the fixation step. An equal volume of 0.1% glutaraldehyde in White's saline (120 mM NaCl, 5 mM KCl, 2.3 mM  $\text{MgSO}_4$ , 3.2 mM  $\text{Ca}(\text{NO}_3)_2$ , 6.5 mM  $\text{NaHCO}_3$ , 0.42 mM  $\text{Na}_2\text{HPO}_4$ , 0.19 mM  $\text{KH}_2\text{PO}_4$ , 0.0005% phenol red) to the platelet suspension for 15 minutes at 37°C. The platelets were centrifuged at  $3,000 \times g$  for 2 minutes and incubated in ice-cold 3% glutaraldehyde in White's saline at 4°C for 1 hour. After three washes with White's saline and the platelets were osmicated in 1%  $\text{OsO}_4$  in White's solution. The rest of the steps were the same as described above. This modification was known to generate a stable fixation of granule contents and provides a better identification of dense granules (White 2004).

### **Dissection of Mouse Aorta and Heart**

Mice were anesthetized using intraperitoneal injection (*i.p.*) of ketamine/xylazine; 100 mg/kg (Fort Dodge Animal Health, Fort Dodge IA) . Mice were then placed on their

dorsal side where the skin can be cut from the base of the abdomen to the underside of the chin. To visualize the heart, two cuts made under each side of the ribs, which was later lifted up. The blood was withdrawn as described above, for lipid analysis. Perfusion of the heart and aorta was performed using a cannula in the left ventricle with 20 mL of PBS to clear the heart. The entire aorta and the heart were dissected and processed with either *en face* or Oil Red O staining techniques for quantifications.

### **Total Cholesterol Measurement**

One hundred microliters of blood was collected by cardiac puncture from anesthetized mice as described above. Serum was separated to measure the concentration of total cholesterol and triglycerides using a commercially available enzymatic assay kit, Cholesterol E enzymatic kit (Wako Chemical Company, Richmond, VA). The distribution of lipoprotein cholesterol was measured using, fast protein liquid chromatography (FPLC) gel filtration (GE Healthcare, Piscataway, NJ) on a single Superose 6 column was used as described previously (Daugherty et al. 2000). The collected fractions were assayed with the Cholesterol E enzymatic kit (Wako Chemical Company, Richmond, VA).

### **Plasma Glucose Measurement**

The level of plasma glucose was measured using the quantitative coupled reaction enzymatic kit from Sigma-Aldrich as described by Raabo *et al.* (Raabo et al. 1960). Glucose was oxidized to gluconic acid and hydrogen peroxide by the addition of glucose oxidase. The substrate, *o*-dianisidine was added to the reaction, and it interacted with hydrogen peroxide to form a colored product in the presence of peroxidase enzyme. Concentrations of the glucose were proportional to the intensity of the pink color product that was measured at 425 nm. The 2X stock peroxidase-glucose oxidase (PGO) enzyme was prepared by dissolving one capsule in 50 mL of distilled water. The substrate, *o*-dianisidine dihydrochloride (50 mg) was dissolved in 20 mL distilled water. The working solution was prepared with 10 mL distilled water, 10 mL 2X PGO enzyme solution, and 0.32 mL of *o*-dianisidine dihydrochloride solution. Using 96-well plate, the

reaction started after the addition of 250  $\mu\text{L}$  of working solution, 24  $\mu\text{L}$  of distilled water and 1  $\mu\text{L}$  of plasma, and incubated for 30 minutes at 37°C then read at 425 nm.

### **Quantification of the lesions in the aorta using *en face* technique**

Whole aorta from each mouse was dissected, and aortic tissue was removed from the ascending aorta to the ileal bifurcation and fixed in 4% paraformaldehyde in Phosphate Buffer Saline (PBS) overnight at room temperature. Adventitial tissue was removed under dissecting microscope and the intimal part of the aorta was exposed after performing a longitudinal cut through the entire aorta. The *en face* method was used to quantify the lesions in the intimal area of the aorta (Daugherty et al. 2003). Briefly, the opened aorta was pinned on a black background and images of the atherosclerotic lesions in the aortic arch to the upper thorax (around 3mm) were captured to computer using a digital camera that is connected to the dissecting microscope. Atherosclerotic lesions on the aortic arch and thoracic area were outlined using Image-pro software (Media Cybernetics, Silver Spring, MD) and are represented as the sum of the lesion areas divided by the area of the vessel for each region.

### **Quantification of the lesions in the aorta using Oil Red-O staining technique**

The heart was dissected as described above for quantifications of the lesions in the aortic root. After PBS perfusion in the left ventricle of the heart, approximately 70% of the lower ventricular mass was severed and the upper cardiac portion was embedded in, a tissue freezing medium O.C.T. (Optimal Cutting Temperature; Electron Microscopy Sciences, and frozen at -20°C. Serial sections (10  $\mu\text{m}$ ) were collected on 10 slides containing 9 sections each that are 80  $\mu\text{m}$  apart. The sections were stained with Oil Red O (neutral lipid stain) and counterstained with hematoxylin and eosin (Vector), then mounted with glycerol/gelatin (Sigma-Aldrich). The surface area covered by lesions was measured using Image-Pro software (Media Cybernetics, Silver Spring, MD). To obtain the medial area, the inner elastic lamina (intima-medial border) was marked and subtracted from the outer elastic lamina (medial-advential border). Mean medial area was measured from 9 to 15 sections per mouse. All measurements for the ascending aorta were calibrated to a 1 mm section of a hemacytometer/40 x magnification.

## **Statistics**

Statistical analyses were performed using SigmaStat program (SPSS Inc.). The measurements for the atherosclerosis studies are represented as the mean  $\pm$  S.E.M; using one Way ANOVA for the four different mice genotypes.

## **Tissue immunostaining**

The frozen serial sections of aortic root were obtained as described above. For immunostaining, the sections were fixed in chilled absolute ethanol for 10 minutes, as previously described (Lu et al. 2007). A Fisher Microprobe system was used for immunostaining. The slides were incubated with indicated different concentrations of the primary rabbit anti-endobrevin/VAMP-8 polyclonal antibody (Synaptic Systems) for 30 minutes at 37°, followed by incubation with the secondary goat anti rabbit biotinylated antibody (1:500) (Vector) for 30 minutes at 37°. The antigen-antibody binding was determined by AEC chromagen substrate using ABC kit (Vector). Controls included, no primary, no primary and no secondary, and non-immune sera or IgG. Images were acquired with a 20x objective lens of a Nikon Eclipse E600 scope and a digital camera DXM1200F (Nikon).

## **Preparation of GTPase binding domain recombinant protein**

Plasmid containing GTPase binding domain, pGEX-4T-3/Raf-Ras binding domain (RBD) was from Johan Bos Netherlands (University of Groningen, Groningen, Netherlands). The plasmid was transformed into the Rosetta<sup>TM</sup>(DE) *E coli* strain that harbor a plasmid (pRARE) contains a rare tRNA sequences (Novagen, Gibbstown, NJ). After adding 20 mL of frozen stocks to 2 x 50 mL Luria-Bertani (LB) broth with antibiotics (ampicilin: 100 mg/mL and chloroamphenicol: 35 mg/mL), the culture was incubated overnight. The culture was centrifuged and the pellet was resuspended in 10 mL LB media, followed by transferring to 2 x 2L LB media and incubating it at 37°C in the presence of antibiotics until OD<sub>600</sub> reached to 0.8 to 1.2. The recombinant protein was induced for 4 hours in the presence of 1 mM isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG). The cells were then harvested by centrifugation using the SLC-4000 rotor



(Thermo Scientific Waltham, MA) at 5000 rpm for 7 minutes. The bacterial pellet was resuspended in 200 mL of ice-cold 1X PBS, and transferred to 500 mL bottles and centrifuged again at 6000 rpm for 7 minutes, and stored at -80°C. The bacterial pellet was resuspended in ice-cold PBS (4 mL per 1 g in weight) with 1X protease inhibitor cocktail plus 1 mM DTT. The cells were lysed using three passages of French Press (12,000 psi, 700 - 1500 cell pressure at high ratio) and centrifuged at 4°C for 1 hour at 50,000 rpm in the Beckman Ti70 rotor with low break. The supernatant was filtered using 0.2 mm nylon filter (Fisher Scientific, Hampton, NH). The recombinant proteins were stored at -80°C after 10% sterile glycerol was added and rotated for 10 minutes at 4°C. The binding affinity was determined by, glutathione agarose beads binding assay. Different concentrations of recombinant proteins (10, 20, 50, 100, 200, 300) were incubated with 10 mL beads for 1 hour at 4°C, followed by washing of the beads complex for three times with HEPES wash buffer. The proteins were eluted by the addition of 2× SDS sample buffer and boiling the samples. The binding was analyzed by 10% SDS-PAGE.

### **Ras Pull-Down Assay**

For platelets preparation, washed human platelets (500 µL), were incubated at 37°C using Aggregometry for 3 minutes followed by the addition of different concentrations of agonist as indicated in the experiment, for 5 minutes. The reaction was stopped by, adding cold 2x lysis buffer (20 mM HEPES-KOH, pH 7.4, 128 mM NaCl, 9 mM MgCl<sub>2</sub>, 2% Triton X-100, 20% glycerol, 2 mM benzamidine, 2x protease inhibitor cocktail). Platelet lysate in the cuvette was sealed with parafilm and instantly frozen in ethanol-dry ice chamber and stored at -80°C, not longer than 24-48 hours before the pull down assay experiment. For resting platelets lysate, the same steps above were followed except for the addition of the agonist.

For pull down assay, after thawing the frozen platelet lysate on ice, they were transferred to 1.5 mL cuvette tubes. To clear the lysates from the cytoskeleton components, lysates were centrifuged at 13,000 rpm for 5 minutes at 4°C. The clear lysate was then incubated with 10 µL glutathione-agarose beads bound to glutathione-S-transferase (GST)-fusion protein, which has a specific domain to bind to the GTP-bound form of Raf-Binding-Domain (RBD) for human Ras, for 1 hour at 4°C, and slowly

rotated. The complex above was centrifuged at 500 x g for 10 seconds, followed by washing with HEPES-wash buffer (20 mM HEPES, pH 7.4, 128 mM NaCl, 2 mM MgCl<sub>2</sub>, 1% Triton X-100, 10% glycerol) for three times. The samples were eluted using 2x SDS sample buffer (130 mM Tris, pH 6.8, 4% SDS, 10% glycerol, 2% β-mercaptoethanol, 0.02% bromophenol blue), and boiled for 10 minutes. Samples were stored at -20°C to be analyzed by western blot.

### **Western Blotting**

After solubilizing washed platelet lysate in 2x SDS sample buffer, the protein in these samples will be separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Ornstein 1964) at room temperature for 2 hours at 100 volts or 1 hour at 200 volts. Proteins were separated based on their molecular weight, then blotted on polyvinylidene difluoride (PVDF, Milipore, Bedford, MA) at room temperature for 1 hour at 100 volts. To block any non-specific interactions between the membrane and the antibody, blots were then blocked with 5% non-fat dried milk in Tris Buffer Saline with Tween-20 (TBST; 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Tween-20 (Sigma-Aldrich) at room temperature for 1 hour. The blocked membranes were incubated with the indicated primary antibody and incubated at room temperature for 2 hours or at 4°C overnight. The membranes then were washed three times with TBST to remove the unbound antibody. The washed membranes were then incubated with secondary antibody that matches the species of the primary antibody, and attached to Alkaline Phosphatase (AP) enzyme. The interaction of the primary and secondary antibody detected visualized using enhanced chemifluorescent substrate (ECL; Peirce ECL detection kit), in which the AP enzyme cleaves the chemiluminescent and the signal was acquired with the Typhoon 9400 phosphoimager and quantified with ImageQuant 5.2 software.

### **Immunoprecipitation of Platelet SNAREs and its Regulatory Proteins**

Immunoprecipitation (IP) assay was performed; using washed banked platelets, in resting or in stimulated condition. To stimulate platelets, 0.1 U/mL of thrombin was added to 500 μL of platelets for 3 minutes, with constant shaking by hand at room

temperature. For Munc18b IP, resting and stimulated platelets were incubated with 2x IP lysis buffer (2% n-Octyl- $\beta$ -glucopyranoside; 2 mM EGTA; 2 mM EDTA; 100 mM HEPES; 150 mM NaCl; 2 mM Na<sub>3</sub>VO<sub>5</sub> and protease inhibitor cocktail (pH 7.4)) on ice for 30-60 minutes to lyse the cells. Platelet lysates were centrifuged at 13,000 rpm for 15 minutes at 4°C to pellet down the cytoskeleton proteins. To clear any non-specific binding, platelet lysate were incubated with 5  $\mu$ g/mL of rabbit IgG (Sigma-Aldrich, St. Louis, MO) for 5 minutes at 4°C on the rotator, then 50  $\mu$ L of Protein A-sepharose beads were added, and the mixture was incubated for 30 minutes at 4°C on the rotator. To clear the bead complex, the tubes were spun down at 500 x g for 12 seconds. The cleared supernatant was then incubated with 20  $\mu$ L of Munc18b polyclonal rabbit antibody for 2 hours at 4°C on the rotator, followed by the addition of 60  $\mu$ L of Protein A-Sepharose beads for 2 hours at 4°C on the rotator. The mixture was washed three times with 1x cold IP lysis buffer at 500 x g for 12 seconds. The protein was eluted with 2x SDS sample buffer by boiling for 10 minutes. Samples were frozen at -80°C and processed within 48 hours. Western blot technique was utilized to detect Munc18b protein, and for the coimmunoprecipitation, syntaxin-11, Munc13-4, Rab 27a, VAMP-8, Munc18a, Munc18b, syntaxin-2 and syntaxin-4 antibodies were used.

Syntaxin-11 IP was performed as indicated above, except in the 2x IP lysis buffer, 2% Triton X-100 was used instead of 2% n-Octyl- $\beta$ -glucopyranoside. For the coimmunoprecipitation, Munc18b, Munc13-4, Rab 27a, VAMP-8, syntaxin-2 and syntaxin-4 antibodies were used.

### **Generation of Munc18b antibody**

New Zealand white rabbits were used to generate Munc18b antibody. Human full Munc18b protein (1mg) was emulsified in 1 mL Freund's complete adjuvant (Sigma-Aldrich, St. Louis, MO) and subcutaneously injected into 10 different sites on both sides of the back of the rabbit (100  $\mu$ L/site). The rabbit was boosted 2 weeks later with 0.25 mg of Munc18b protein emulsified in Freund's complete adjuvant (Sigma-Aldrich) as described above. Two weeks following the boost, 30-35 mL of blood was drawn from the rabbit ear vein to test for the antibody. The rabbit was boosted again every 2 weeks for 7

cycles, and bled 2 weeks after each boost. The blood was centrifuged at 35,000 rpm for 10 minutes, and the serum was stored as aliquots at -20°C.

## Chapter Three

### The Role of VAMP-8 in Atherosclerosis

#### Introduction

Atherosclerosis, the leading cause of heart attack, stroke, and peripheral vascular diseases, is a complex disease that involves a plethora of cells, such as white blood cells, endothelial cells and platelets. Platelets, the first responder of the vasculature, gained this expression not only because they are small, discoid cells, that sense the vascular injury, and form a clot to stop bleeding, but also due to their secretion of several pro-inflammatory molecules that lead to manifestation of numerous pathophysiological processes in the body, such as atherosclerosis. There is growing evidence of a role for platelets in atherosclerosis (Burger and Wagner 2003; Huo, Schober et al. 2003; Gawaz, Langer et al. 2005; Sachais, Turrentine et al. 2007). Platelets contain three granules (alpha, dense granules, and lysosomes). Secretion of pro-inflammatory and thrombotic proteins from these granules contributes to atherosclerosis.

Platelet secretion is mediated by the soluble N-ethylmaleimide-sensitive factor activating protein receptor (SNARE) proteins (Lemons, Chen et al. 1997; Flaumenhaft, Croce et al. 1999; Chen, Bernstein et al. 2000). Platelets express two subclasses of SNARE proteins, t-SNAREs on the target/plasma membrane, and v-SNAREs on the vesicle membrane, and their interaction brings the two membranes together and lead to fusion and secretion of the granule content. Among platelets v-SNAREs, our laboratory demonstrated that the endobrevin/VAMP-8 is the primary v-SNARE for platelets secretion and thrombosis *in vitro* and *in vivo*, using VAMP-8 knock out mouse model (Polgar, Chung et al. 2002; Schraw, Rutledge et al. 2003; Ren, Barber et al. 2007; Graham, Ren et al. 2009). Given the known role of platelets in hemostasis and the proposed role of platelet in atherosclerosis, we sought to determine whether the deletion of VAMP-8 affects the development of atherosclerosis. For these studies, ApoE<sup>-/-</sup> mice, which are susceptible to atherosclerosis, were crossed with VAMP-8<sup>-/-</sup> mice to obtain four different experimental groups. There are three control groups: ApoE<sup>-/-</sup> (control for atherosclerosis), VAMP-8<sup>-/-</sup> (control for secretion defect), ApoE<sup>+/+</sup>/VAMP-8<sup>+/+</sup> (normal control), and ApoE<sup>-/-</sup>/VAMP-8<sup>-/-</sup> (test group). Male littermates from each group were evaluated for the development of atherosclerosis at 50 weeks of age using *en face*

examination of the aorta and Oil Red O analysis of the aortic root. The data demonstrate that plaque size was significantly decreased in the aortic root ( $P= 0.009$ ) in the ApoE<sup>-/-</sup>/VAMP-8<sup>-/-</sup> mice compared with their respective control groups, while in the aortic arch there was a trend towards having decreased lesions that did not reach the significant difference ( $P= 0.097$ ). These data show that the loss of VAMP-8 decreased the development of atherosclerotic plaques and suggests that VAMP-8 may play a role not only in the late stages of the disease but also in the initiation of atherosclerosis.

## **Results:**

### **Mice Characteristics and Diet**

Our laboratory showed that the endobrevin/VAMP-8 is important for platelet secretion *in vitro*, and *in vivo*. VAMP-8<sup>-/-</sup> mice platelets, stimulated with thrombin, showed a secretion defect from the three granules. In addition, Graham *et al.* (Graham, Ren et al. 2009) showed that VAMP-8 is important for thrombus formation and stability. Thus we sought to determine if VAMP-8-mediated secretion plays a role in atherosclerosis. VAMP-8<sup>-/-</sup> mice on a mixed genetic background (C57BL/6J and 129Sv) were crossed with ApoE<sup>-/-</sup> mice (C57BL/6J) to generate a double knock-out mouse model: ApoE<sup>-/-</sup>/VAMP-8<sup>-/-</sup>. ApoE<sup>-/-</sup> mice were used as a positive control for atherosclerosis. In addition, VAMP-8<sup>-/-</sup> was used as a negative control, and all were compared to wild-type mice. Fifty week old, male mice (Table 3-1) were maintained on normal chow and evaluated for the development of atherosclerosis. This was considered a chronic model of atherosclerosis.

Another group of mice were backcrossed onto a C57BL/6J background for 10 generations to resolve the mixed background issue. They were maintained on chow supplemented with saturated fat (milk fat 21% wt/wt) and cholesterol (0.15% wt/wt, diet no. TD88137; Harlan Teklad) for 15 weeks. These two models were compared because it was not clear whether the high-fat diet model might overwhelm any effect that might be caused by VAMP-8-deletion. We had originally assumed that the VAMP-8 role could be subtle and thus be more apparent in a chronic model of atherosclerosis. However, in the acute model, there were several breeding difficulties and the experiments were performed only on the ApoE<sup>-/-</sup> control group for atherosclerosis. Mouse genotyping was carried out

as described in Chapter Two. A 2 mm piece of DNA was used to establish the genotype of each mouse using a Polymerase Chain Reaction (PCR).

### **Quantification of Lesions in Aortic Arch**

As mentioned earlier, VAMP-8 mediated secretion is important for platelet function, and the literature points to the fact that platelet secretion is associated with atherosclerotic lesions and some granule cargo, such as P-Selectin and PF4 may contribute to plaque development (Dong, Brown et al. 2000; Sachais et al. 2004; Sachais, Turrentine et al. 2007). Thus VAMP-8 mediated secretion might play a role in atherosclerosis. The mice generated for the chronic studies, were examined for plaque development in the aortic arch of the heart. In Figure 3-1B, deletion of VAMP-8 reduced the development of lesions in the aortic arch, compared with the control group (Figure 3-1A), as shown by *en face* dissection and computer-assisted measurements from micrographs. The wild type (Figure 3-1C) and VAMP-8<sup>-/-</sup> (Figure 3-1D) mice groups had no atherosclerotic plaques as expected.

The size of the plaques in these mice was measured (Figure 3-2) using Image-pro software and the data was analyzed using Sigma plot software. There was a difference in lesion size (P=0.097) between the ApoE<sup>-/-</sup>/VAMP-8<sup>-/-</sup> mice (open triangles) and the control group of ApoE<sup>-/-</sup> (closed triangles). These data suggest that VAMP-8 mediated secretion events are important for atherosclerosis.

### **Quantification of Lesions in Aortic Root**

An additional measure to evaluate the lesion size in the mice is Oil Red O staining of the aortic root. As shown in Figure 3-3B, there was a significant reduction in the size of atherosclerotic lesions in ApoE<sup>-/-</sup>/VAMP-8<sup>-/-</sup> mice compared to the ApoE<sup>-/-</sup> control group (Figure 3-3A). Both the wild type (Figure 3-3C) and VAMP-8<sup>-/-</sup> (Figure 3-3D) mice groups had no detected lesions.

The quantification data for the lesion size in the aortic root (Figure 3-4) indicated that there is a significant difference (P=0.009) between the ApoE<sup>-/-</sup>/VAMP-8<sup>-/-</sup> mice (open triangles) compared to their respective control (closed triangles). The data suggest that the loss of VAMP-8 suppresses the development of atherosclerotic lesions in two

regions of the aorta and suggest that VAMP-8-mediated secretion plays a role not only in thrombosis but also in the development of atherosclerosis.

### **VAMP-8 Deletion Did Not Have an Effect on the Total Cholesterol**

It was clearly illustrated above that the deletion of VAMP-8 had an effect on the lesion size in atherosclerotic mice. This phenotype might be due to the effect of VAMP-8 deletion on cholesterol metabolism, thus explaining why the lesions are smaller. To rule out this possibility, total cholesterol level was measured. Figure 3-5 shows that there is no difference in cholesterol levels between ApoE<sup>-/-</sup>/VAMP-8<sup>-/-</sup> mice (open triangles) and their respective control of ApoE<sup>-/-</sup> mice (closed triangles), suggesting that the apparent phenotype of decrease lesion size is due to the effect of VAMP-8 on secretion and not the cholesterol metabolism.

### **VAMP-8 Deletion Did Not Have an Effect on Glucose Metabolism**

Previous studies regarding the effect of VAMP-8 deletion on glucose metabolism was controversial. While Hong and colleagues showed that VAMP8 null mice responded normally to glucose challenge and suggested that VAMP8 is not required for insulin secretion (Wang, Ng et al. 2004), others suggested that the deletion of VAMP-8 had an effect on glucose metabolism (Zong et al. 2011). To determine if glucose metabolism was affected and thus contributed to the difference in lesion size, total plasma glucose was measured using the quantitative coupled reaction enzymatic kit from (Sigma-Aldrich). In Figure 3-6, the deletion of VAMP-8 did not have an effect on plasma glucose level in ApoE<sup>-/-</sup>/VAMP-8<sup>-/-</sup> mice (open triangles) and their respective control of ApoE<sup>-/-</sup> mice (closed triangles). This result suggested that the deletion of VAMP-8 did not have an effect on glucose metabolism.

### **VAMP-8 in Other Cell Types**

The endobrevin/VAMP-8 deficiency affects the release from platelets (Ren, Barber et al. 2007), mast cells (Tiwari, Wang et al. 2008), and pancreas (Wang, Ng et al. 2004). Among the major cells involved in atherosclerosis are macrophages and endothelial cells. In order to detect the presence of VAMP-8 in these cells, western blot



analysis was performed. As shown in Figure 3-7A and B, VAMP-8 is expressed in both macrophages and endothelial cells. Data in the literature showed that VAMP-8 is also important for macrophages secretion (Pushparaj et al. 2009). The expression of VAMP-8 is not limited to platelets, and thus the atherosclerotic phenotype demonstrated in this chapter might suggest that other cells, such as macrophages and endothelial cells play a role in atherosclerosis.

### **VAMP-8 Present in the Lesions of the Aortic Root (Immunohistochemistry)**

To demonstrate the presence of VAMP-8 in atherosclerotic lesions, immunohistochemistry was performed using aortic root sections from ApoE<sup>-/-</sup> mice. Using different concentrations of anti-VAMP-8 polyclonal antibody: 1:200, 1:5000, and 1:1000 (Figure 3-8C, D, and E), we showed that VAMP-8 is present in the lesions of aortic root. In addition, macrophages were also present in the lesion area (Figure 3-8A). This data further suggest that VAMP-8 plays a role in atherosclerosis.

### **Discussion:**

Studies in the literature suggested a role of platelet secretion in atherosclerosis, not only in the late stages but also in early stages of atherosclerosis (Sachais, Higazi et al. 2004; Sachais, Turrentine et al. 2007). PF4 was detected in early atherosclerotic lesions, and it was correlated with the severity of the disease. Li *et al.* (Data not published-53<sup>rd</sup> ASH Symposium 2010) showed that the deletion of the platelets P2Y12 receptor decreases the monocyte infiltration in the aortic root and atherosclerosis. These data imply that platelets activation is important for atherosclerosis. In addition the presence of PF4 in the lesions of the aorta in the Sachais *et al.* study reflects the importance of platelets chemokines release in the development of atherosclerosis(Sachais, Turrentine et al. 2007). In addition, previous work from our laboratory showed that among the v-SNAREs present in platelets, VAMP-8 was the primary v-SNARE for secretion and for thrombosis (Ren, Barber et al. 2007; Graham, Ren et al. 2009). VAMP-8 was required for platelet secretion from the three granules, and the stability of thrombus. Putting this evidence together, in this chapter we aimed to identify the role of VAMP-8 mediated

secretion in atherosclerosis. Therefore, ApoE<sup>-/-</sup>/VAMP-8<sup>-/-</sup> mouse model was generated (Table 3-1).

As predicted, the lesion size in the double knockout mice was decreased compared to the control group. This phenotype was observed in both aortic arch (Figure 3-1 and 3-2) and aortic root (Figure 3-3 and 3-4). The double knockout mice showed a significant reduction in the lesion size in aortic root area (P=0.009) compared to the control group (Figure 3-3). A similar phenotype was observed in the aortic arch area; however, this trend did not yet reach statistical significance but may when more ApoE<sup>-/-</sup> animals are added to the study. These data indicate that the deletion of VAMP-8 was protective against atherosclerosis.

Deletion of some genes might have an effect on the body metabolism, and thus might contribute to the phenotype in this study. To investigate this possibility, total cholesterol was measured. There was no difference in cholesterol levels between ApoE<sup>-/-</sup>/VAMP-8<sup>-/-</sup> and the atherosclerotic control of atherosclerosis ApoE<sup>-/-</sup> mice (Figure 3-5). In addition, glucose level was determined to see whether VAMP-8 deletion has an effect on insulin metabolism. The data presented in Figure 3-6, showed no difference between ApoE<sup>-/-</sup>/VAMP-8<sup>-/-</sup> and ApoE<sup>-/-</sup> control groups.

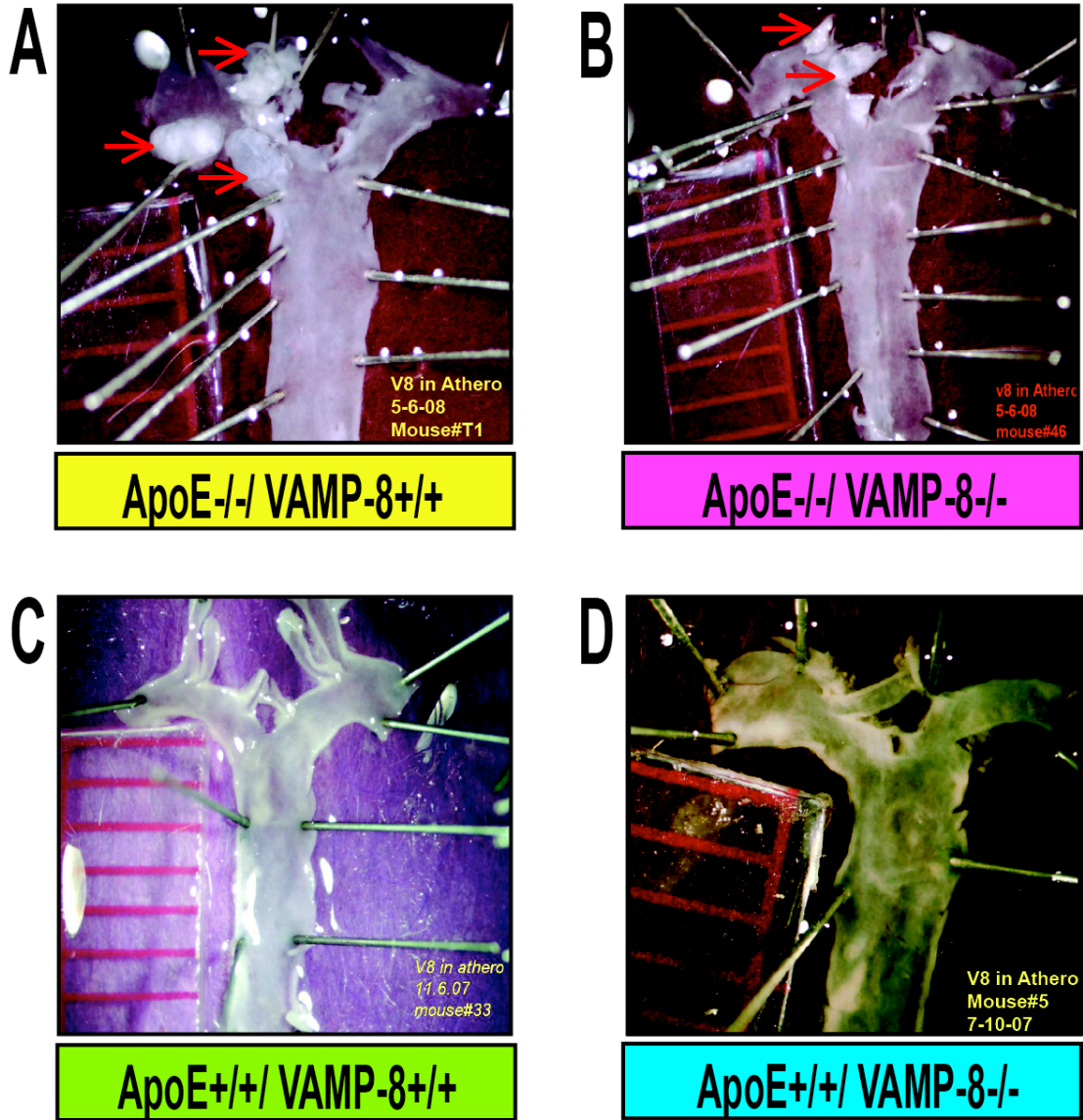
Since the knockout model in this study is global deletion of VAMP-8, it is not certain which of the hematopoietic cells contributed to the present phenotype. Thus we examined the presence of VAMP-8 protein in platelets, macrophage and endothelial cells. VAMP-8 was expressed in all these cell types with the majority in platelets (Figure 3-7). To further characterize the importance of VAMP-8 in atherosclerosis, the immunohistochemistry experiments (Figure 3-8) indicated that VAMP-8 present in the aortic root, indicating that it participates in the process of lesion development through its expression by the cells in that region. To establish the importance of VAMP-8 in those cells we aimed to perform bone marrow transplantation as suggested by some studies in the literature, such as Wagner *et al* (Burger and Wagner 2003). Nevertheless, we did not have the mice at that time since we started the backcrossing to C57BL/6. At the same time, and based on numerous data in the literature, all these cell types (platelets, macrophages, and endothelial cells) found to be important in atherosclerosis. This finding will not change the conclusion of the importance of VAMP-8 in exacerbating

atherosclerotic plaque formation. The data of immunohistochemistry, proved the presence of VAMP-8 in the lesions of the aortic root.

In conclusion, the data in this chapter demonstrated that VAMP-8 plays a role in atherosclerosis. However, more experimentation is needed to further characterize the role of VAMP-8 in atherosclerosis, such as the effect of VAMP-8 deletion on leukocytes recruitments and infiltrations in the lesion area, measuring the levels of cytokines and chemokines release since its been suggested to play a major role in inflammation and atherosclerosis (Sachais, Turrentine et al. 2007). It was recently shown that, the deletion of VAMP8 in mice inhibited the serum level of TNF- $\alpha$  and thus reduced the inflammation (Pushparaj, Tay et al. 2009). The reduction of VAMP-8 activity or expression might be used as a novel therapeutic target to ameliorate lesion development in atherosclerosis.

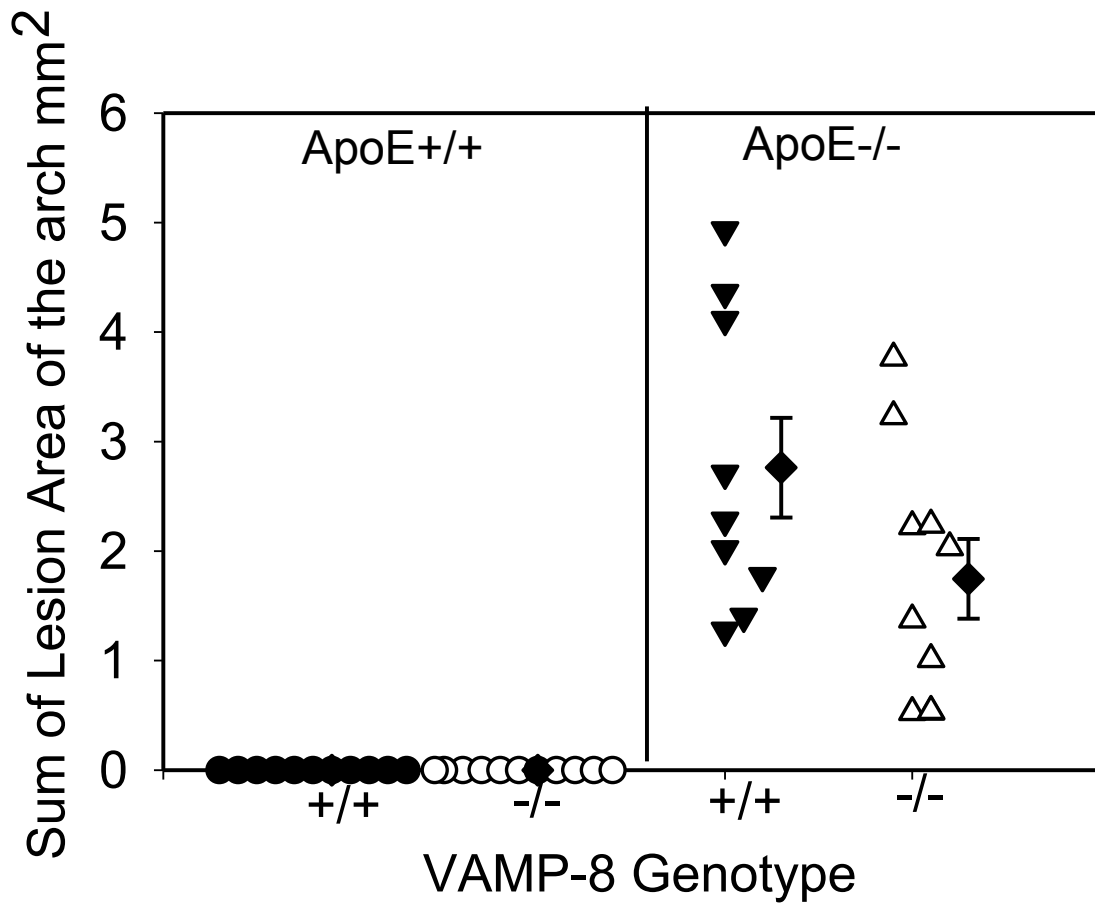
Genotype	Animal Number		Sex	Age in Weeks (average)	Weight in gm
	En Face	ORO			
<b>ApoE<sup>+/+</sup>/VAMP-8<sup>-/-</sup></b>	<b>11</b>	<b>10</b>	<b>m</b>	<b>50</b>	<b>30.9</b>
<b>ApoE<sup>+/+</sup>/VAMP-8<sup>+/+</sup></b>	<b>11</b>	<b>9</b>	<b>m</b>	<b>49</b>	<b>35.5</b>
<b>ApoE<sup>-/-</sup>/VAMP-8<sup>+/+</sup></b>	<b>9</b>	<b>10</b>	<b>m</b>	<b>50</b>	<b>40.1</b>
<b>ApoE<sup>-/-</sup>/VAMP-8<sup>-/-</sup></b>	<b>9</b>	<b>10</b>	<b>m</b>	<b>50</b>	<b>34.7</b>

**Table 3-1. Mouse Characteristics.** ApoE-deficient mice (Apo-E<sup>-/-</sup>) mice (C57BL/6J) were from Jackson Laboratory and VAMP-8 deficient (V8<sup>-/-</sup>) mice (C57BL/6Jx129SvJ) were provided by Wanjin Hong (Institute of Molecular and Cellular Biology, Singapore). Littermates from F2 crosses were used to as the four groups in this study: ApoE<sup>+/+</sup>/VAMP-8<sup>-/-</sup>. (a control for secretion defect), ApoE<sup>-/-</sup>/VAMP-8<sup>+/+</sup> (a control for atherosclerosis), ApoE<sup>+/+</sup>/VAMP-8<sup>+/+</sup> (a wild type control), and ApoE<sup>-/-</sup>/VAMP-8<sup>-/-</sup> (the test group). Polymerase chain reaction (PCR) analysis was used to determine genotypes using DNA from the tail tip. Mice were fed normal chow diet and examined for atherosclerotic lesions at an average of 50 weeks of age.



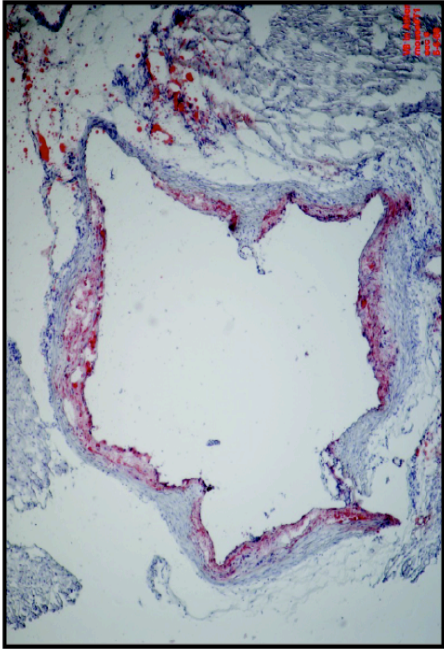
**Figure 3-1. *En-Face* Analysis of Aortic Arch.**

Representative aortic arches from the four study groups are shown in *En-Face* display. The lesions were determined using Image Pro software (Media Cybernetics). The entire aorta for each mouse was collected between subclavian and iliac branches then pinned on dark paper. The plaques are in white color.



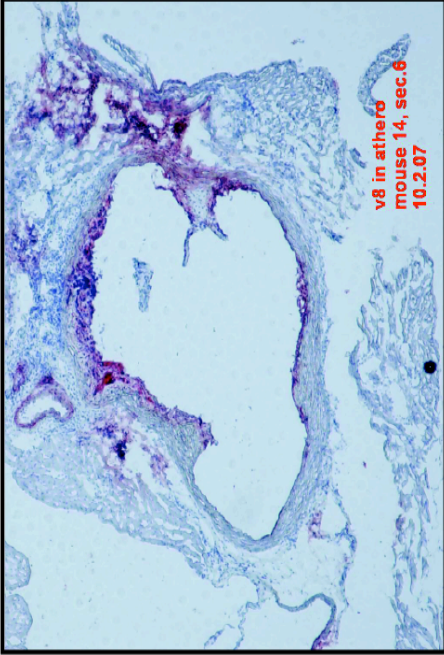
**Figure 3-2. Quantification of the Atherosclerotic Lesions in Aortic Arch.**

Lesion size in the aortic root was quantified by computer-assisted imaging. Closed circles (wild type), open circles (VAMP-8<sup>-/-</sup>), closed triangles (ApoE<sup>-/-</sup>), and open triangles (ApoE<sup>-/-</sup>/VAMP-8<sup>-/-</sup>), represent the values from each mouse, diamonds represent the mean, bars represent ±SEM, (P value=0.097) using one way anova.



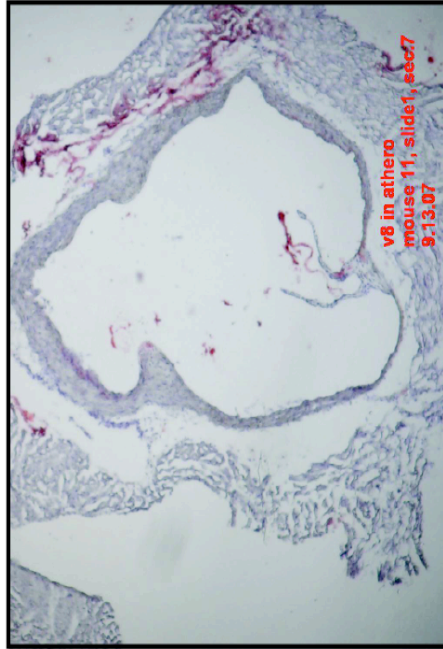
**A**

**ApoE-/- VAMP-8+/+**



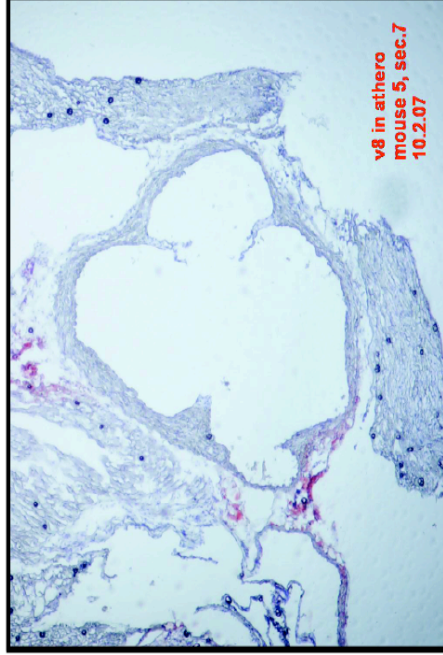
**B**

**ApoE-/- VAMP-8-/-**



**C**

**ApoE+/+ VAMP-8+/+**



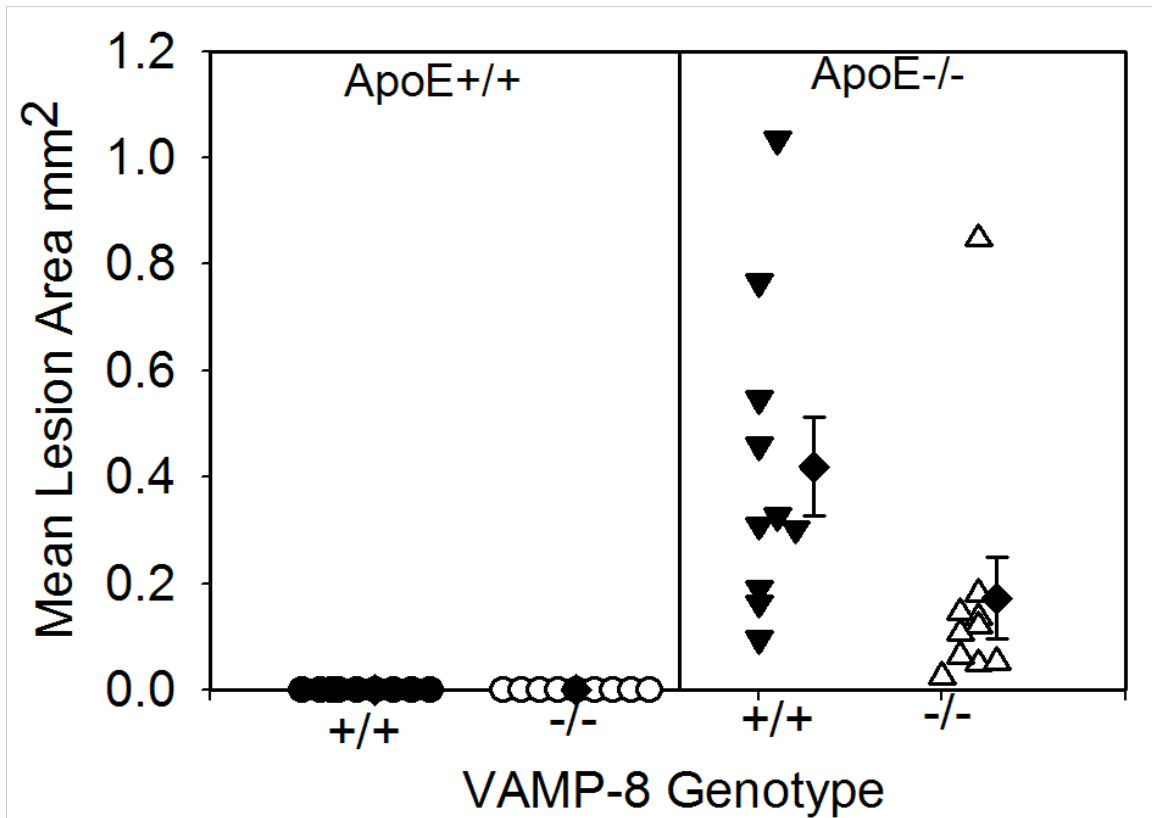
**D**

**ApoE+/+ VAMP-8-/-**



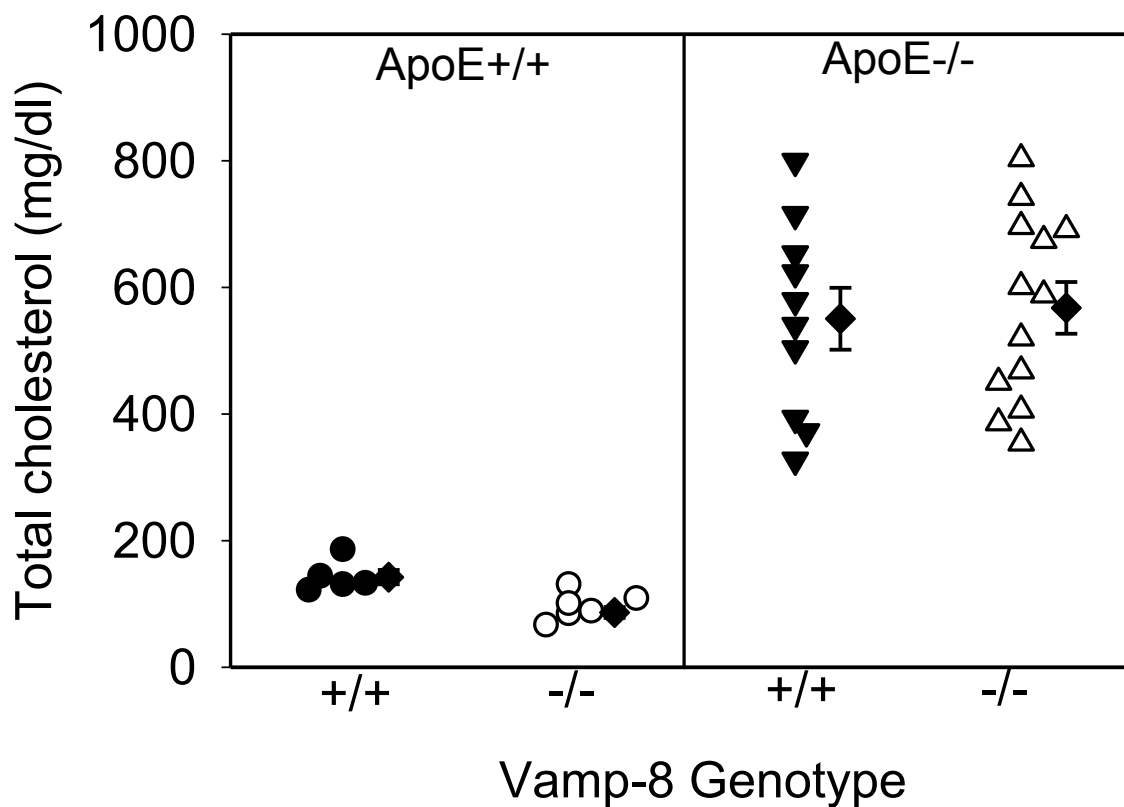
**Figure 3-3. Oil Red O Staining of the Aortic Root.**

Representative sections of the aortic root from the four study groups are shown. The upper cardiac portion of the heart was embedded in OCT compound and sections (10 mm) from comparable regions were taken for analysis. The sections were then stained with Oil Red O (stain the lesions) and hematoxylin (stain the cells) to examine the lesion size.



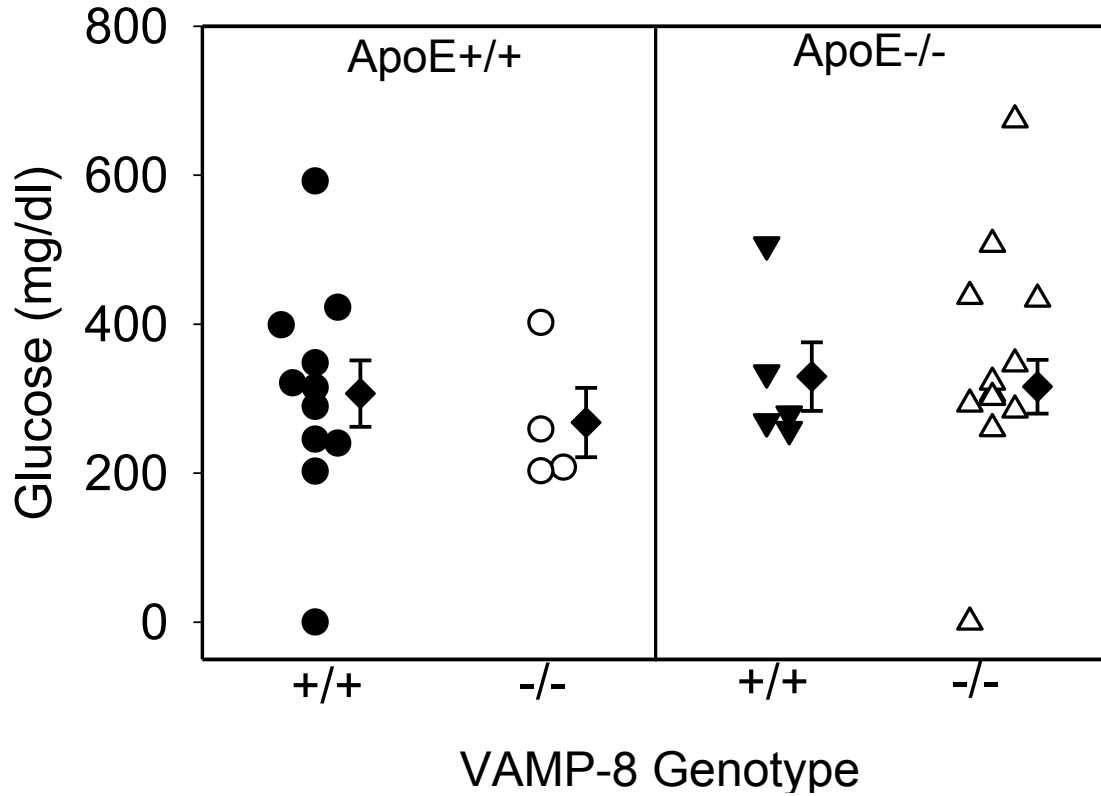
**Figure 3-4. Quantification of Atherosclerotic Lesions in the Aortic Root.**

Lesion size was quantified by computer-assisted imaging. Closed circles (wild type), open circles (VAMP-8<sup>-/-</sup>), closed triangles (ApoE<sup>-/-</sup>), and open triangles (ApoE<sup>-/-</sup>/VAMP-8<sup>-/-</sup>), represent the values from each mouse, diamonds represent the mean, bars represent ±SEM, (P value=0.009) using one way anova



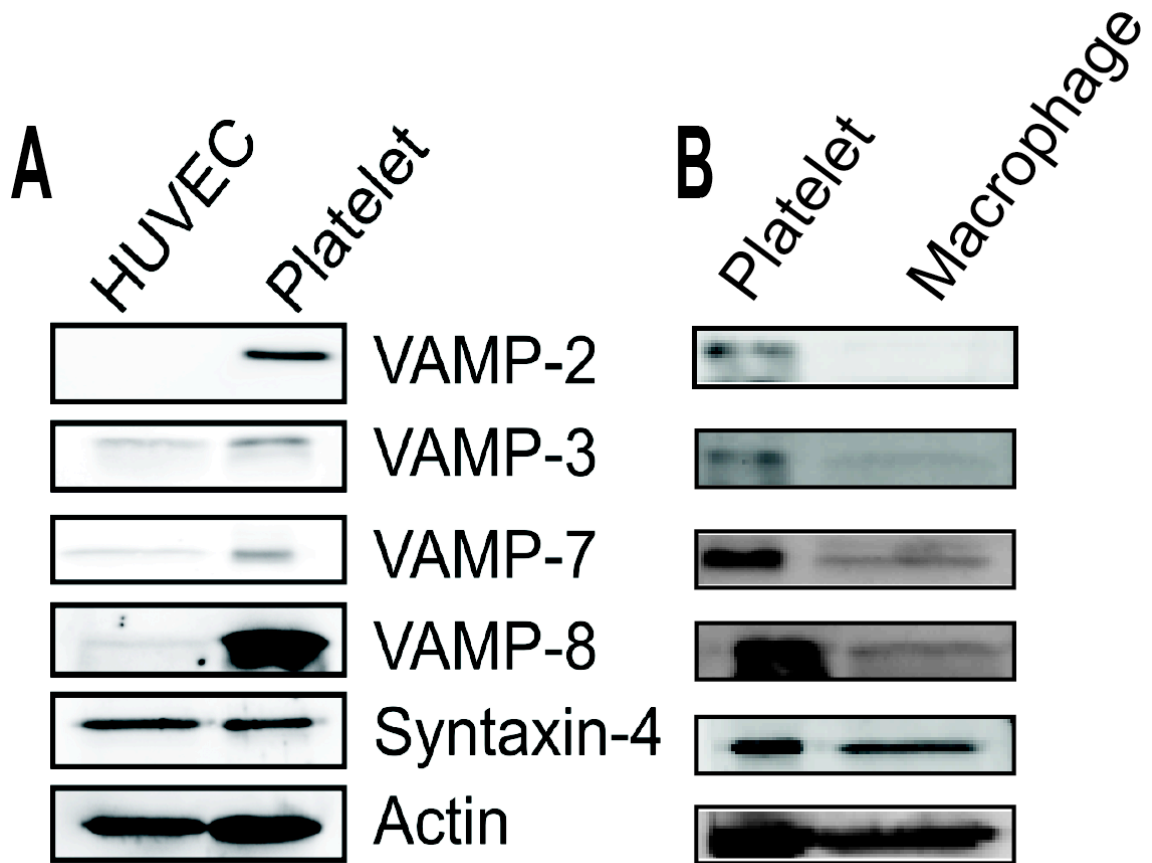
**Figure 3-5. Measurements of Total Serum Cholesterol.**

Total serum cholesterol of individual mouse from the four study groups are shown (in mg/dl). Serum was separated from whole blood by centrifugation at 2000 rpm for 20 minutes. Ten  $\mu$ L of the serum was used in the enzymatic reaction (Wako Chemicals) to measure the total cholesterol. There is no difference between the total cholesterol level of ApoE<sup>-/-</sup> and the double knock out.



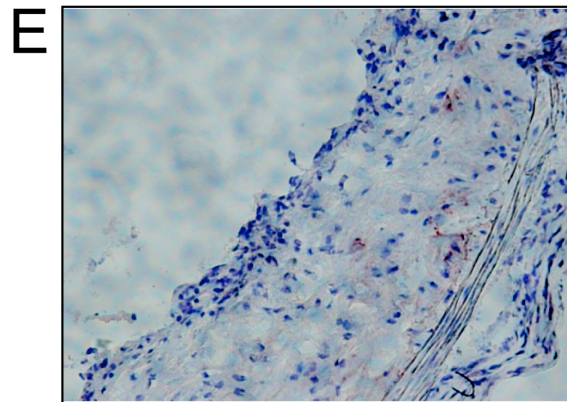
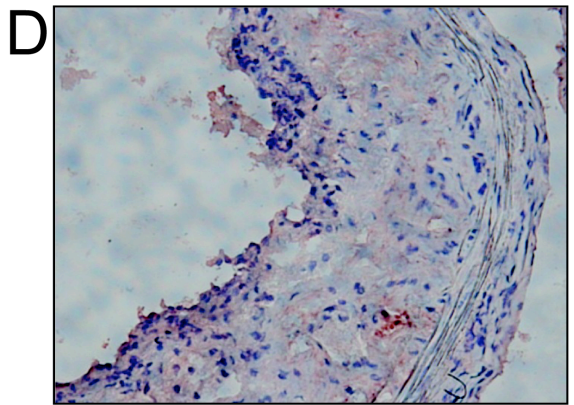
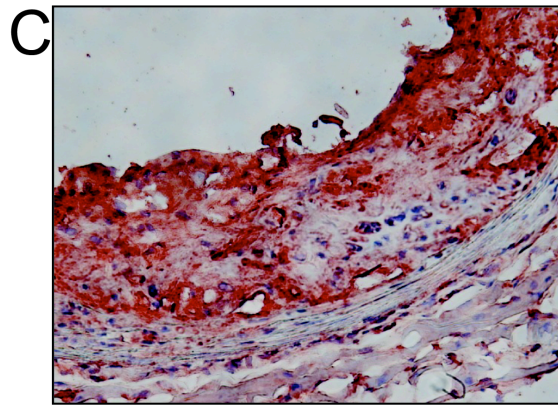
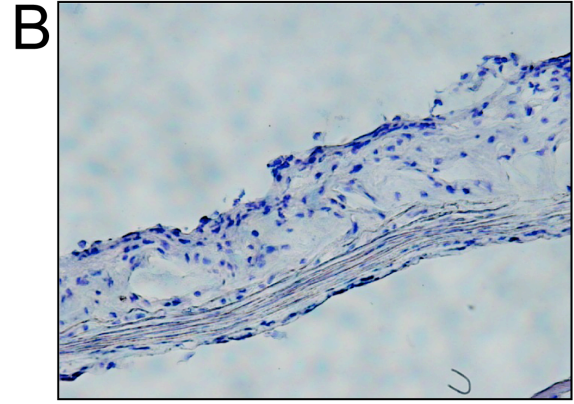
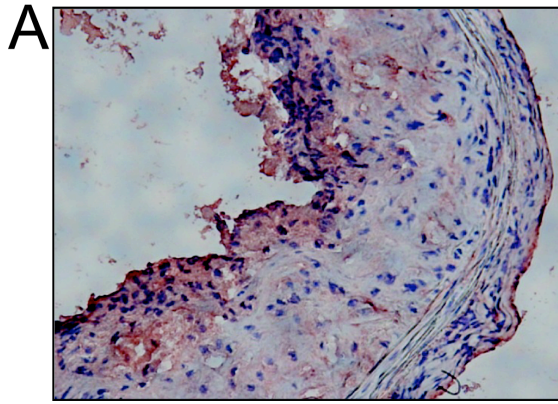
**Figure 3-6. Measurements of Glucose Level.**

The level of plasma glucose determined for mice in the four study groups is shown (in mg/dl). Plasma was separated from whole blood by centrifugation at 2000 rpm for 20 minutes. Plasma glucose was measured using the enzymatic reaction and the intensity of the brown color was measured at 475nm. There is no difference between the glucose level of ApoE<sup>-/-</sup> and the double knock out.



**Figure 3-7. VAMP-8 Expression in Endothelial Cells and Macrophages.**

The HUVEC cell extract was prepared by lysing the culture of HUVEC cells with PBS pH 7.4/1% Triton X-100. For macrophages, mice were euthanized by cervical dislocation and the peritoneal macrophages were collected by washing the abdominal cavity with 0.9% Saline. The protein concentrations for HUVEC and macrophages were determined by Bicinchoninic acid (BCA) assay. 25  $\mu$ g of HUVEC extract, macrophages and mouse platelet equivalents ( $5 \times 10^7$ ) were loaded to a 15% SDS-PAGE gel and analyzed by western blotting for the indicated proteins.



**Figure 3-8: VAMP-8 is Present in Aortic Root.**

Immunostaining of VAMP-8 was performed using, anti-VAMP-8 rabbit polyclonal antibody, in atherosclerotic lesions of aortic root. A) Macrophage rat monoclonal antibody (1:1000) was used as a control. B) Negative control, no primary antibody. Different concentrations of anti-VAMP-8 antibody used C) 1:200, D) 1:500, E) 1:10,000. Images were captured using ImagePro software.

## **Chapter Four**

### **The Role of Munc18b in Platelet Secretion**

#### **Introduction**

Cellular exocytosis is a complex process, mediated by the soluble NSF attachment protein receptors (SNAREs). There are two classes of SNAREs: the v-SNARE (on the vesicle membrane) and the t-SNARE (on the target/plasma membrane). The interaction between the v-SNARE and the t-SNARE forms a four-helix bundle that zips the two membrane bilayers together leading to membrane fusion and release of the granule content. The process of exocytosis through SNARE proteins is strictly regulated. One of the key regulators is the Sec1/Munc18 (SM) family protein (Sudhof and Rothman 2009). Munc18 protein function is either in intracellular trafficking or regulation of exocytosis (Toonen and Verhage 2003). Munc18 proteins act as chaperone proteins, specific for syntaxin/t-SNARE, to regulate membrane fusion. Platelets express three SM isoforms: Munc18a, Munc18b, and Munc18c (Reed, Houg et al. 1999; Houg, Polgar et al. 2003; Schraw, Lemons et al. 2003). Munc18a and Munc18c have been demonstrated to play a role in platelet secretion. This observation was identified either by using specific peptide inhibitors Munc18a/Pep3, and Munc18c/Pep3 (Schraw, Lemons et al. 2003), or a monoclonal anti-Munc18c antibody and a peptide-based inhibitor that disrupted Munc18c-syntaxin-4 interactions (Houg, Polgar et al. 2003). Recent studies of patients with Familial Hemophagocytic Lymphohistocytosis type 5 (FHL5), suggested that Munc18b is important for platelet function (Sandrock, Nakamura et al. 2010). FHL5 is linked to a mutation in the Munc18b/STXBP2 gene. However, it is not yet clear which one of the SM isoforms is the most dominant in human platelets and plays a key role in exocytosis. In this chapter, the role of Munc18b in platelet exocytosis will be dissected using platelets from FHL5 patients and molecular biology techniques to determine the association of Munc18b with other SNAREs complex that mediate platelet exocytosis and thus its function.

#### **Results**



### **Munc18b is the Most Abundant SM Isoform in Human Platelets**

Among the seven SM isoforms, platelets express Munc18a, Munc18b, and Munc18c (Schraw, Lemons et al. 2003). To determine the relative abundance of the three Munc18s, recombinant proteins from cDNA expression constructs were titrated and used to generate standard curve for quantification (Figure 4-1A). There were  $\sim 2.5 \pm 0.1$  ng/ $5 \times 10^7$  of Munc18a ( $\sim 450$  molecules per platelet, n=6),  $56.1 \pm 3.71$  ng/ $5 \times 10^7$  of Munc18b ( $\sim 10,240$  molecules per platelet, n=6) and  $27.8 \pm 0.82$  ng/ $5 \times 10^7$  of Munc18c ( $\sim 5,070$  molecules per platelet, n=6). The specificity of Munc18a, Munc18b, and Munc18c antibodies was demonstrated by comparing their immuno-reactivities to the recombinant Munc18s (Figure 4-1B). The cross-reactivity of anti-Munc18b rabbit monoclonal antibody with Munc18a may be due to the sequence homology between the two proteins. From this analysis, Munc18b is 2-fold more abundant than Munc18c and 25-fold more abundant than Munc18a. This indicates that Munc18b is the most abundant isoform of SM proteins in human platelets.

### **Characterization of Munc18s and SNARE Proteins in FHL5 Platelets**

Work using inhibitory peptides or antibodies suggested a role for Munc18a and Munc18c in platelets (Houng, Polgar et al. 2003); (Reed, Houng et al. 1999; Schraw, Lemons et al. 2003). Munc18a had an effect on secretion from  $\alpha$ -granules, while Munc18c affected the secretion from all three granules. Due to limited availability of some reagents, the exact role of Munc18b could not be detected at that time. FHL5 is linked to a defect in Munc18b/STXBP gene, and provides a unique opportunity to examine the role of Munc18b in platelet function. In Figure 4-2A and B, Munc18b was completely absent in platelets from the two biallelic patients, P1 and P2. The heterozygote, P3, showed a  $\sim 30\%$  reduction. Munc18a increased in both biallelic samples, but this increase was not sufficient to compensate for the loss of Munc18b (Figure 4-2A). Munc18c was unchanged (Figure 4-2A and B). These data indicate that the mutations in the two biallelic patients (P1 and P2) cause the production of an unstable Munc18b protein, which was not detectible in platelets. The mutation in the heterozygous patient (P3) had less of an effect on Munc18b levels.

In the literature, the interaction between Munc18s and their cognate syntaxin has been controversial. While some studies showed that Munc18 binds the short N-terminus of syntaxin (Sudhof and Rothman 2009), others showed that it can bind syntaxin in the closed conformation where the Habc domain is folded back onto the H3 SNARE domain. However the most recent proposed interaction is the binding of Munc18a to the N-terminus of syntaxin, which allows Munc18a binding to the SNARE complex. Often, loss of a Munc18 isoform precipitates loss of a syntaxin isoform and vice-versa. Syntaxins are dependent on the chaperone function of Munc18s (Cirstea, Kutsche et al. 2010); In Munc18a/b double knockdown neurosecretory cells, the levels of syntaxin 1, syntaxin-2 and syntaxin 3 were significantly reduced (Han et al. 2009). A comparable effect was seen when Munc18c was knocked out, there was a significant reduction of syntaxin-4 expression (Houng, Polgar et al. 2003). In Figure 4-2A and B, the loss of Munc18b caused a striking decrease in syntaxin-11 with limited effects on syntaxin-4 (syntaxin-2, 7, and SNAP-23 were unchanged in biallelic P1). Munc18a showed slightly greater enhancement, while VAMP-8, Munc13-4 (FHL3), and Rab27a were unaffected. Munc18b was decreased in the heterozygous patient P3, around 50% of the control, causing syntaxin-11 level to be decreased. Syntaxin, Munc13-4, VAMP-8, and Rab27a were unaffected in the heterozygous P3, however Munc18a was slightly decreased. These data indicate that Munc18b is a chaperone for syntaxin-11 in platelets, and is important to stabilize syntaxin-11 and may play a role in localizing it to the plasma membrane as suggested in Han *et al.* (Han et al. 2004).

### **Thrombin-Stimulated Platelet Secretion is Defective in FHL5 Platelets**

Previous studies showed that peptide inhibition of Munc18a, and Munc18c had an effect on platelet secretion. Munc18a inhibition had an effect on secretion from  $\alpha$ -granules, while Munc18c inhibition affected the secretion from all three granules. Due to limited availability of some reagents, the exact role of Munc18b could not be detected at that time. To investigate the role of Munc18b in platelet function, we analyzed platelets from FHL5 patients lacking Munc18b\STXBP2 gene. This approach provides a unique system to dissect the role of Munc18b in platelet exocytosis. Dose response assays were performed to examine platelet secretion from the biallelic and heterozygous FHL5

patients (Figure 4-3A and B respectively). In figure 4-3A, different concentrations of thrombin were used to stimulate platelet secretions from dense granules,  $\alpha$ -granules, and lysosome. The reactions were stopped after 1 minute using hirudin. In control platelets (dark circles Figure 4-3A), there was a dose dependent increase of secretion from dense granules and  $\alpha$ -granules that was maximum at 0.5 U/mL thrombin. The maximal release of serotonin from dense granules was about 80% and of PF4 from  $\alpha$ -granules was about 60%. However, the maximum lysosomal release in the control platelets was maximal at 0.3 U/mL thrombin. Platelets from the biallelic patient (P1) had a severe secretion defect from both dense granules and  $\alpha$ -granules, despite using high thrombin concentrations (0.5 U/mL).  $\beta$ -Hexosaminidase release from lysosome was also affected compared to the control; however, this defect seems to be corrected when higher concentrations of thrombin were used. The biallelic patient (P2) showed a similar defect. The heterozygous FHL5 patient (P3) showed a partial secretion defect (Figure 4-3B). Serotonin release from dense granules and PF4 release from  $\alpha$ -granules were partially attenuated compared to the control. Lysosome release was largely unaffected, showing similar percent of release as the control.

### **ATP Release and the Exposure of Granule Membrane Proteins were Affected in FHL5 Platelets**

The previous experiment suggests that Munc18b is required for platelet cargo release was demonstrated. As an additional measure the release of ATP from dense granules was measured using luciferin/luciferase assay (Figure 4-4A and B). In Figure 4-4A, a complete loss of Munc18b in the biallelic FHL5 patient (P1) (Figure 4-4A, grey line) resulted in a significant defect of ATP release from dense granules, compared to the control (Figure 4-4A, dark line). The ATP secretion defect was also observed in the heterozygous FHL5 patient (P3) (Figure 4-4B). The level of the ATP release from the biallelic FHL5 patient (P1) (Figure 4-4B, grey line) was around 50% less than the control (Figure 4-4B, black line) further confirming the role of Munc18b in platelet exocytosis.

Activation of platelets not only releases the soluble cargo proteins as investigated above, but also leads to the exposure of membrane proteins. Thus we aim to investigate

the exposure of membrane receptors (P-Selectin and PAC-1) as an additional measure of  $\alpha$ -granules release. Washed control and biallelic FHL5 patient (P1) platelets were either used as resting (Figure 4-5A-B, grey line) or stimulated with 0.1 U/mL thrombin for 3 minutes (Figure 4-5A-B, red color), and the reactions were stopped using 2 fold excess of hirudin. Flow cytometry was used for analysis. The phenotype was robust in the biallelic FHL5 patient (P1) upon platelets stimulation; the fluorescence intensity of P-Selectin expression (Figure 4-5A and C, grey bar) was around 60% less compared to the control. Similar results were observed in PAC-1 expression in the biallelic FHL5 patient (P1) (Figure 4-5B and D, grey bar). From this we conclude that Munc18b is required for platelet secretion.

### **Platelet Morphology is Normal in Platelets From FHL5 Platelets**

Platelet activation leads to cytoskeleton rearrangements that are important for platelet secretion. Since the lack of Munc18b has an effect on platelet secretion, it is possible that this defect is due to a defect in cytoskeleton rearrangements, where platelets undergo shape change without release of their granule content, so the granules will be centralized instead of fused to the plasma membrane. This observation was seen before (Ren 2008, Ren 2010). To investigate this possibility, both resting and thrombin stimulated platelets from control and the biallelic FHL5 patient (P1) were examined by transmission electron microscopy (Figure 4-6). Resting platelets from control (Figure 4-6A) and biallelic FHL5 patient (P1) (Figure 4-6B), showed normal discoid shape, with similar distribution of granules, microtubular network, mitochondria, and OCS. Control platelets stimulated with 0.1 U/mL thrombin, showed shape change with protruding filopodia, and lack of granule contents in the control (Figure 4-6C). This is the typical morphology of platelets after stimulation. Thrombin-stimulated, patient (P1) platelets showed shape-change, however the granules were centralized and with full cargo, indicating a defect in fusion of the granules to the plasma membrane and thus in the secretion of the granule contents. This data indicates that the lack of Munc18b did not affect platelets morphology and certainly is not required for platelets shape change and cytoskeleton rearrangements.

### **Munc18b Association with SNARE Proteins**

It is clear from the previous experiment (western blot data), that the lack of Munc18b from both the FHL5 biallelic patients (P1 and P2) and from the heterozygous (P3) patient highly affected the level of syntaxin-11. These data indicate that Munc18b is a chaperone for syntaxin-11, and that the lack of one protein affects the stability of the other which in turn affects platelet exocytosis. Thus to investigate this further, the association of Munc18b protein with syntaxin-11 and other SNARE proteins was investigated. Resting and thrombin-stimulated platelets were subjected to immunoprecipitation using both anti-Munc18b and anti-syntaxin-11 antibodies, followed by western blot analysis to determine the association with SNARE proteins. In Figure 4-7A, Munc18b was associated with syntaxin-11 in both resting and stimulated platelets lysates. There was no association between Munc18b and other syntaxins, indicating a specificity of the association of Munc18b to syntaxin-11. To characterize this association further, anti-syntaxin-11 rabbit polyclonal antibody was found to associate with Munc18b (Figure 4-7B). This data is consistent with the concept that each SM protein binds to its cognate syntaxins (Garcia, Gatti et al. 1994; Hata and Sudhof 1995; Reed, Houg et al. 1999; Schraw, Lemons et al. 2003). Munc18b was associated with other SNARE proteins: Munc13-4, Rab-27a and SNAP-23, suggesting it plays a role in regulating SNARE complex and thus in platelet exocytosis. Using anti-syntaxin-11 rabbit polyclonal antibody, syntaxin-11 was found to associate with VAMP-8 and SNAP-23. These data suggest that Munc18b is a chaperone for syntaxin-11, and show an association between Munc18b and SNARE complex, which as we show earlier have an effect on platelet secretion.

### **Discussion**

The work in this chapter shows that Munc18b is the major SM protein in platelets compared to the levels of other SM isoforms, Munc18a and Munc18c (Figure 4-1A and B). FHL5 patients lacking Munc18b gene was a perfect *in vivo* model to study the role of Munc18b in platelets exocytosis. Munc18b was completely deficient in biallelic FHL5 patient (P1), while it was partially decreased in the heterozygous patients (P3). This decrease in Munc18b was associated with decrease level of syntaxin-11. These data are

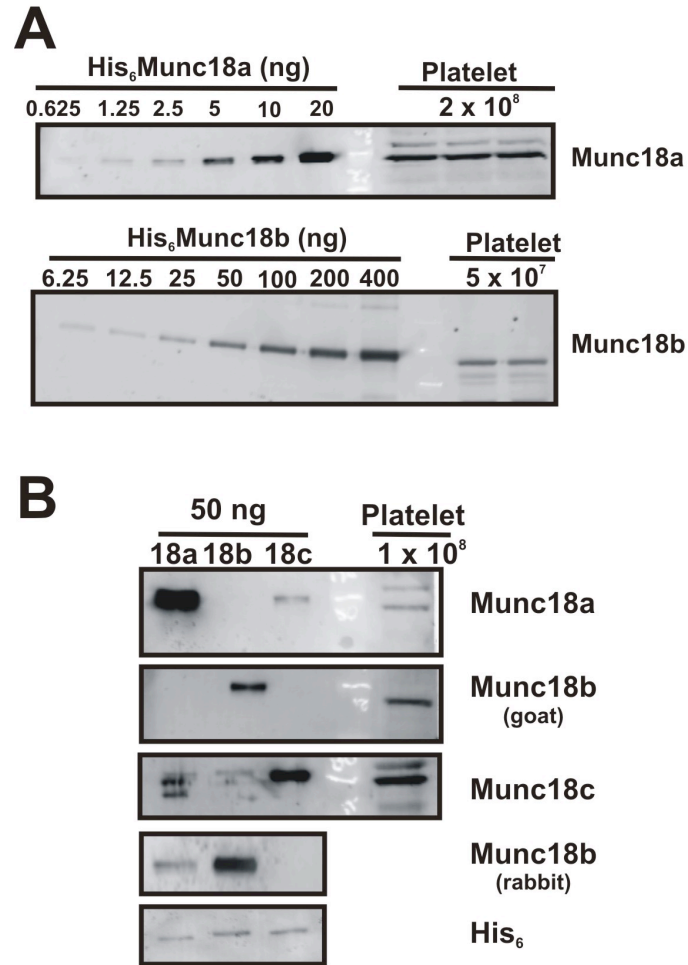
consistent with the role of the SM protein Munc18 as syntaxin chaperone proteins (Garcia, Gatti et al. 1994; Reed, Houg et al. 1999; Misura, Scheller et al. 2000), and suggest that syntaxin-11 could be a required t-SNARE for Munc18b (Ye et al. 2012 submitted). The relative importance of syntaxin-11 cannot be definitively established, but its level is clearly dependent on expression of Munc18b. Their levels were highly compromised and partially decreased in compared to the control.

The secretion phenotype shown in Figures 4-3A and B clearly identified a significant role of Munc18b in platelet exocytosis. Thrombin-stimulated release from the biallelic FHL5 patient (P1) was extremely attenuated from granules and  $\alpha$ -granules (Figure 4-3A), while lysosomal release was moderately affected. In the heterozygous (P3), the release from dense granules and  $\alpha$ -granules was partially compromised. A similar conclusion was demonstrated in the defect of ATP release from the dense granule. This indicates that Munc18b might be a limiting component of the secretory machinery given the partial secretion defect noted in the heterozygous platelets. Our observation of the defect in P-Selectin and PAC-1 expression from  $\alpha$ -granules, in biallelic FHL5 patient (P1) and the heterozygous patient (P3) suggests that the lack of Munc18b might affect platelet signaling and its subsequent events of secretion. This defect in platelet exocytosis was not related to platelet morphology or cytoskeleton rearrangements. The lack of Munc18b had no affect on platelets shape change indicating that signaling required for cytoskeleton rearrangements is still intact. Taken together, these data imply a significant role of Munc18b in platelet exocytosis.

Despite the published results that showed a role of Munc18b in platelets secretion in FHL5 patient (Sandrock, Nakamura et al. 2010), the data was limited to only FACS analysis, without clarifying how Munc18b contributes to the regulation of SNAREs machinery in platelets. Our study, further characterizes the role of Munc18b in platelet secretion by investigating its association with SNARE proteins (Figure 4-5). Munc18b clearly interacted with syntaxin-11 and *vice versa*. In addition, there was an association between Munc18b and other SNARE proteins mediators such as, Munc13-4, Rab27a, and SNAP-23. These results suggest a role of Munc18b in regulation of SNARE machinery and thus platelet exocytosis. Previous studies revealed that Munc13-4 (FHL3) and Rab27

(GS2) are important for platelet exocytosis (Tolmachova, Abrink et al. 2007; Ren, Wimmer et al. 2010).

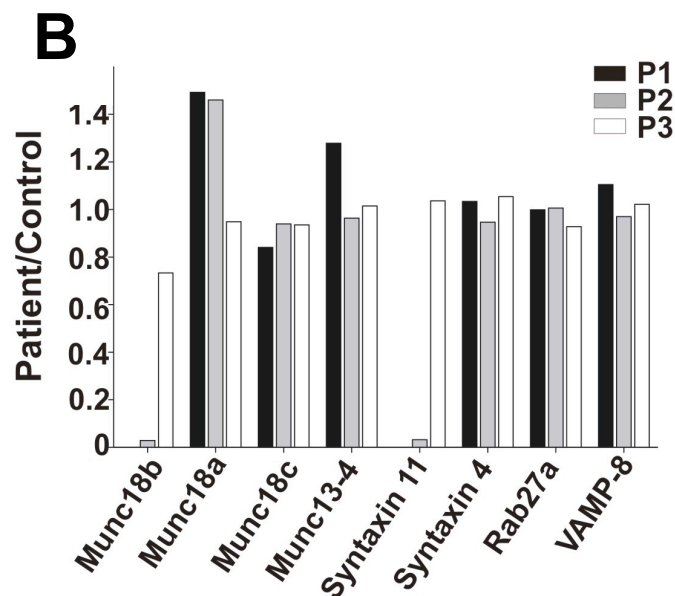
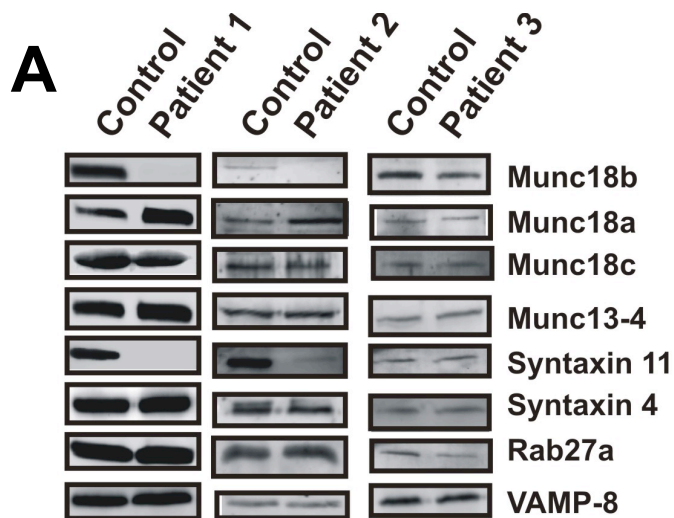
In this chapter, we demonstrate that Munc18b is the dominant SM protein in platelets and that it is important in platelet exocytosis. We also show that Munc18b is a chaperone for the t-SNARE syntaxin-11, and the association between Munc18b and SNARE proteins. However, further work is needed to further characterize the importance of this interaction in SNARE proteins assembly and exocytosis. This work also suggests that platelet function assays may be useful diagnostic tools for FHL patients.



**Figure 4-1. Munc18b is the Major Munc18 Isoform in Platelets and is Missing in Platelets from FHL5/STXBP2 Patients.**

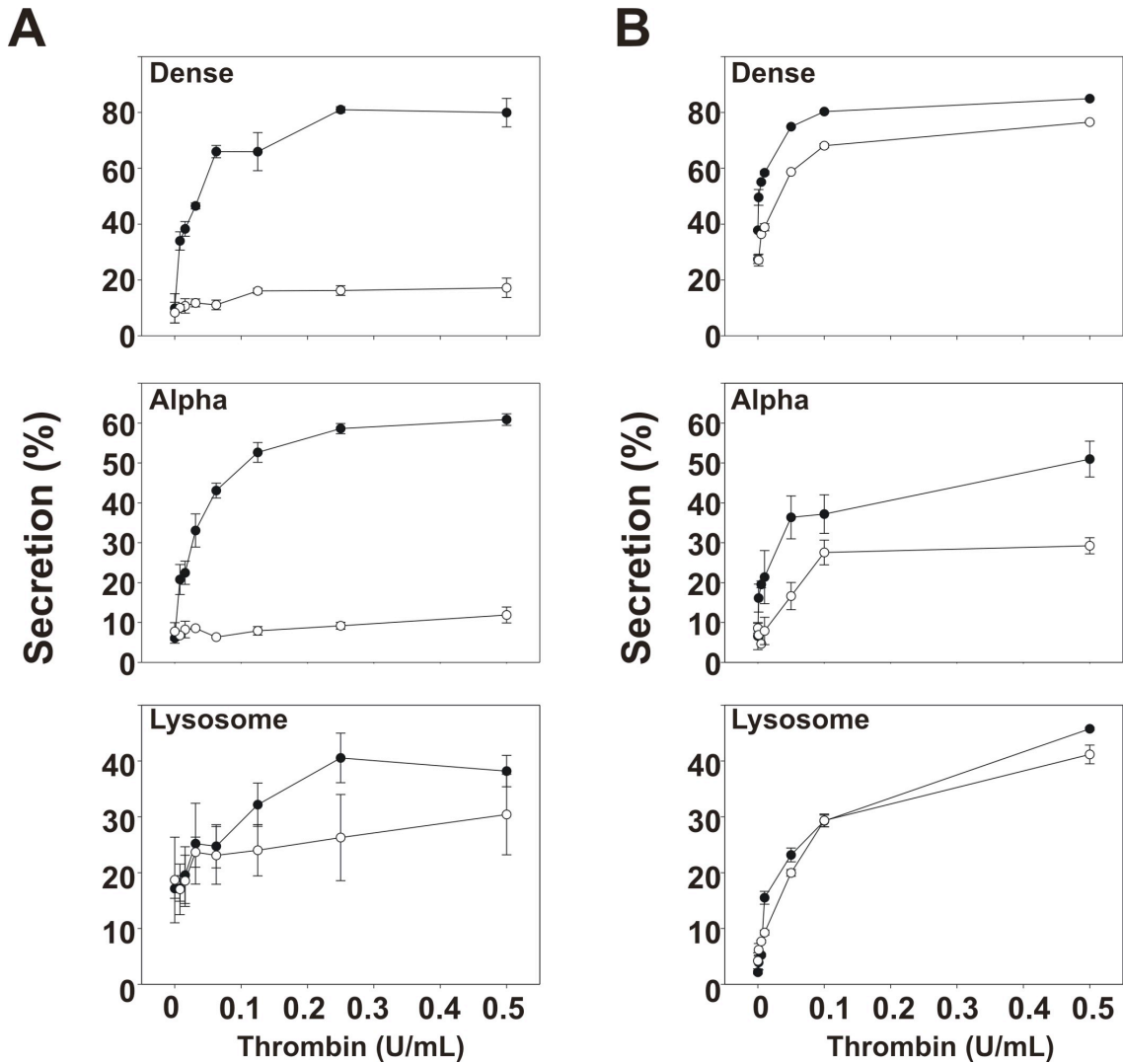
**A)** Recombinant Munc18a, b, and c were used to generate a standard curve for quantification (using Enhanced Chemi-Fluorescence western blotting) of each Munc18 isoforms in the indicated number of human platelets. Quantification of each isoform shows that  $\sim 2.5 \pm 0.1$  ng/ $5 \times 10^7$  of Munc18a ( $\sim 450$  molecules per platelet,  $n=6$ ),  $56.1 \pm 3.71$  ng/ $5 \times 10^7$  of Munc18b ( $\sim 10,240$  molecules per platelet,  $n=6$ ) and  $27.8 \pm 0.82$  ng/ $5 \times 10^7$  of Munc18c ( $\sim 5,070$  molecules per. **B)** Equal amounts of each Munc18 isoform were subjected to western blotting with the antibodies used in this study to evaluate their cross reactivity. (Data in panel A and some of the data in panel B, contributed by Dr. Qiangshen Ren)





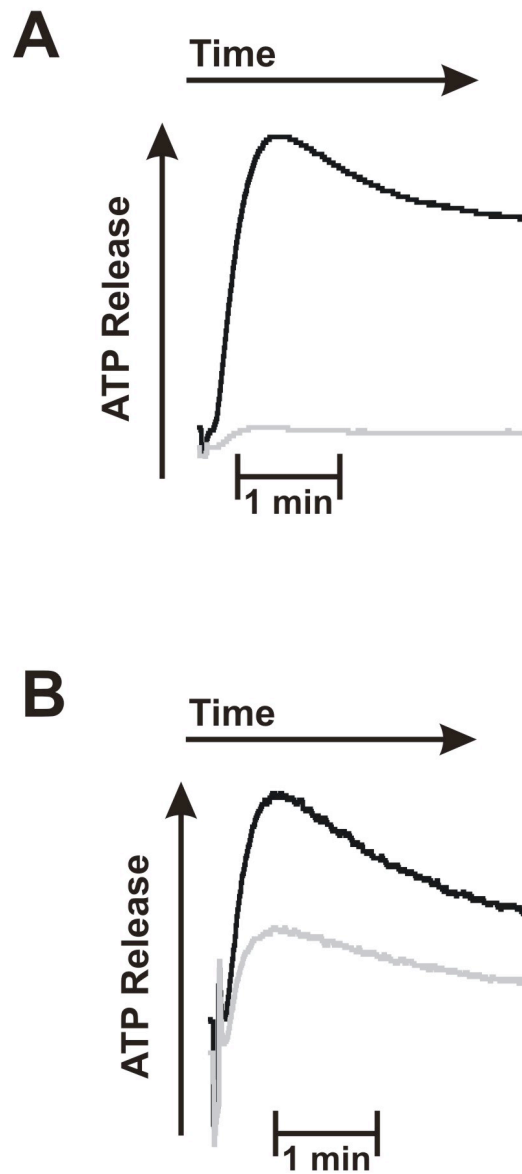
**Figure 4-2. Munc18b is Missing in Platelets from FHL5/STXBP2 Patients.**

**A)** Washed platelet extracts ( $1.0 - 2.0 \times 10^7$  platelets/lane) from control and FHL5 patients were probed by western blotting, using SDS-PAGE, with the indicated antibodies and the ratio of patient to control levels of each protein were measured and graphed (**B**).

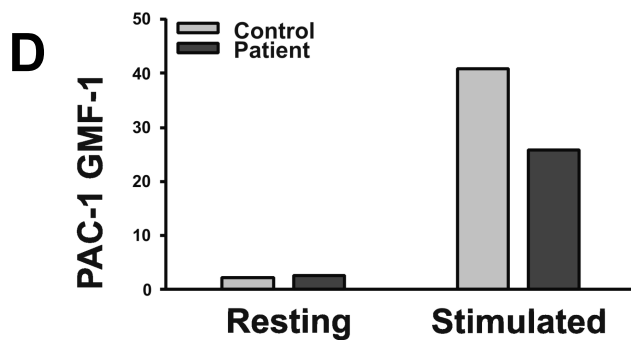
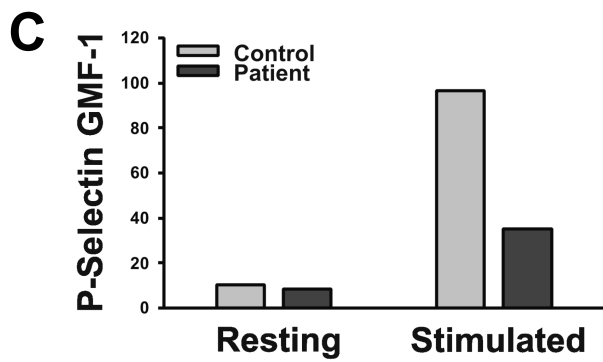
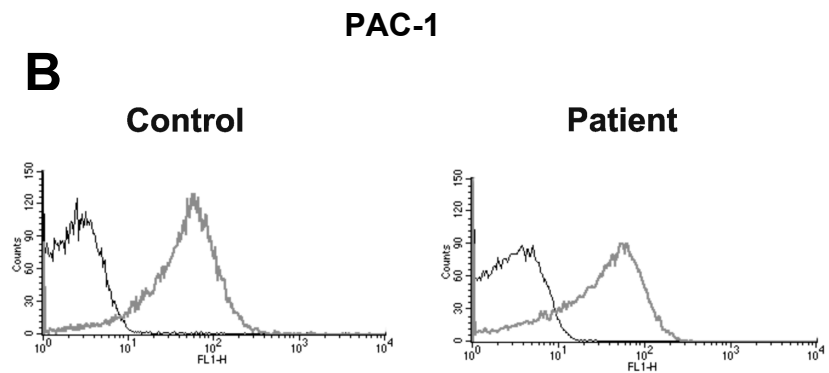
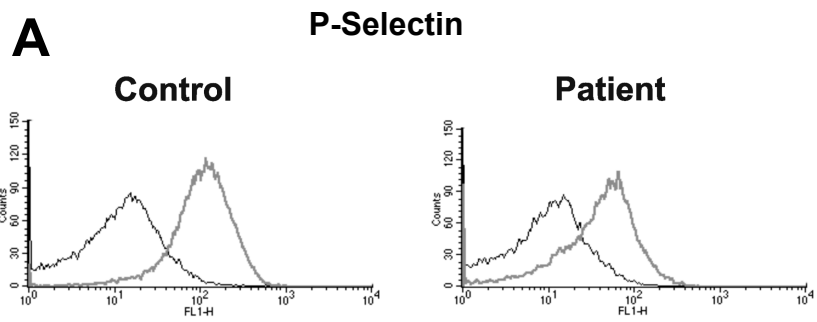


**Figure 4-3. Platelets from FHL5/STXBP2 Patients Have a Secretion Defect.**

Platelet rich plasma (PRP) was generated by centrifugation and labeled with [<sup>3</sup>H]-serotonin. After washing, platelets were stimulated with increasing concentrations of thrombin for 5 minutes and then release of [<sup>3</sup>H]-serotonin (Dense), PF4 (Alpha) and β-hexosaminidase (Lysosomes) were measured as described (Chen, Lemons et al. 2000) (**A and B**). **A**) Show the release profiles for biallelic Patient 1 (open symbols) and a normal control (closed symbols). Patient 2 showed a similar profile. **B**) Show the release profiles for heterozygous Patient 3 (open symbols) and a normal control (closed symbols). Contributed by Drs. Qiangshen Ren and Shaojing Ye.

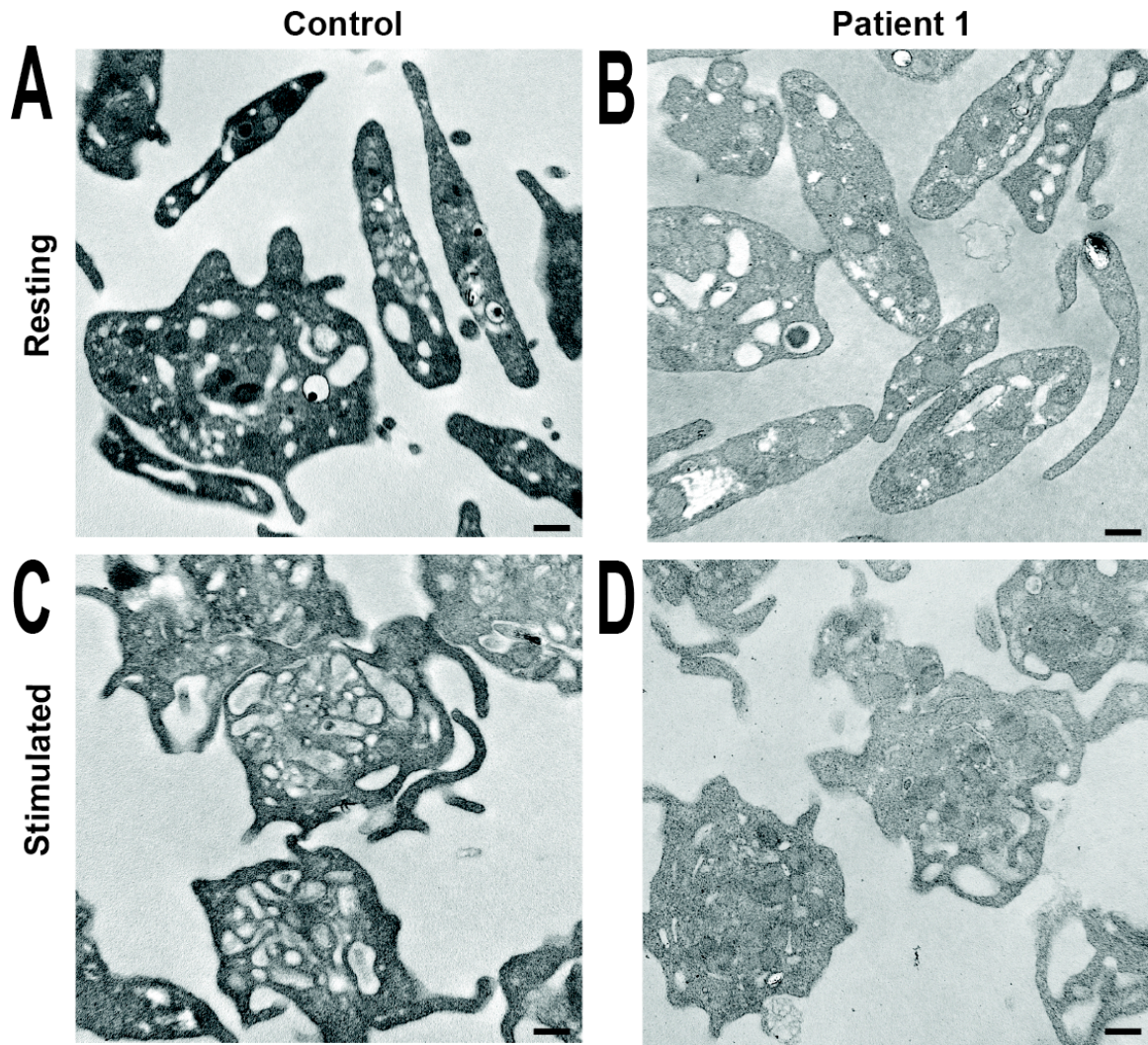


**Figure 4-4. Platelets from FHL5/STXBP2 Patients Have a Defect in ATP release.** Washed platelets, stimulated with thrombin-induced (0.1 U/mL) release of ATP from dense granules were measured in a lumi-aggregometer as described (Ren, Wimmer et al. 2010) (**A and B**). The ATP release profile from a normal control (black line) and biallelic Patient 1 (**A** gray line) and the heterozygous Patient 2 (**B** gray line) are presented. Data contributed by Dr. Zubair Karim.



**Figure 4-5. Platelets from FHL5/STXBP2 Patients Have a Defect in P-Selectin and PAC-1 Exposure.**

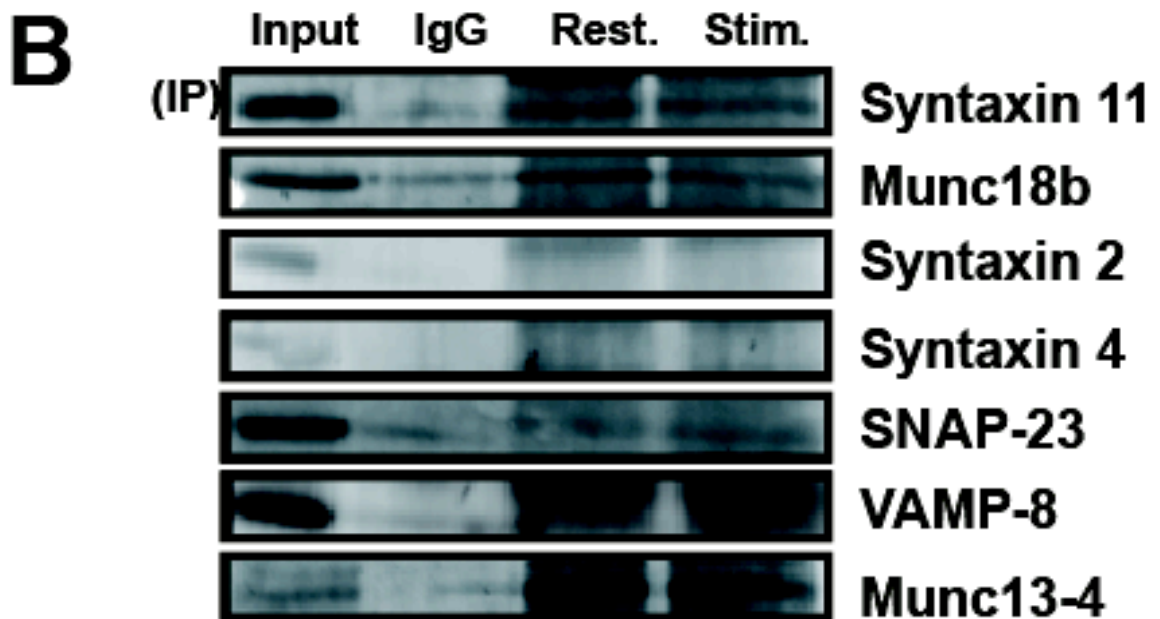
Thrombin stimulated washed platelets from control (gray line/filling), or biallelic Patient 1 (black line/filling) were incubated with FITC-conjugated P-Selectin antibodies (**A and C**) for 1 minute at room temperature. The reactions were stopped with hirudin and data was analyzed by flow cytometry. A similar experiment was repeated (**B and D**) using PAC-1 antibody. Data contributed by Dr. Zubair Karim.



**Figure 4-6. Platelets Ultrastructure is Normal in FHL5/STXBP2 Patient.**

Platelets were prepared as described in Chapter Two. Platelet concentration was adjusted to  $2.5 \times 10^8$ /mL and 0.7 mM  $\text{CaCl}_2$  was added. Platelets from control or biallelic Patient 1, were either in resting (**A and B**) or stimulated (**C and D**) with 0.1 U/mL of thrombin. Samples were then fixed and images were analyzed using transmission electron microscopy. Scale bars represent 0.5  $\mu\text{m}$ . Data contributed by Dr. Zubair Karim





**Figure 4-7. Munc18b Association with Syntaxin-11 and Other SNARE Proteins.**

Washed platelet extracts, resting or stimulated, were lysed with 2% n-Octyl- $\beta$ -glucopyranoside for Munc18b immunoprecipitation (IP), or with Triton-X100 for syntaxin-11 IP, with protease inhibitor cocktail. The extracts were incubated either with anti-Munc18b rabbit polyclonal antibody (**A**), or with anti-syntaxin-11 rabbit polyclonal antibody (**B**), or IgG control (**A and B**), then cross linked with Protein A- Sepharose beads for 2 hours at 4°C. Association of Munc18b and syntaxin-11 with each other and with other SNARE proteins was detected by western blotting, as in panel A and B, respectively.



## Chapter Five

### Ras Signaling in Platelets of Noonan Syndrome Patients

#### Introduction

Platelet activation is essential for hemostasis and thrombosis. Platelets sense the vascular damage and adhere to the exposed collagens. This binding to collagen and other agonists at the site triggers several signaling transduction events that lead to platelet activation, cytoskeleton rearrangements, and release of granule contents. Among the proteins that are thought to play a role in platelet function is the Ras GTPases superfamily. This chapter we will focus on Ras. Ras becomes activated by the conversion from GDP- to GTP-bound form. This is mediated by the Guanine Exchange Factor (GEF) protein, SOS. SOS binds to the effector protein, GRB2. The SH2 domain of GRB2 binds the phosphorylated tyrosine residues on tyrosine kinase receptors and that leads to the localization of SOS-GRB2 complex to the plasma membrane and activation of Ras (Buday and Downward 1993; Rozakis-Adcock, Fernley et al. 1993). Activated Ras initiates several downstream signaling cascades such as Ras/MAPK pathway; Raf, MEK, and ERK1-2. Platelet stimulation by collagen or thrombin leads to activation of MEK and ERK signaling pathways (Borsch-Haubold, Kramer et al. 1995). ERK is known to play a role in platelet function and integrin activation (Flevaris et al. 2009), and it was suggested to be downstream of Ras in human platelets (Rosado and Sage 2001; Li, Zhang et al. 2006). Watson *et al* showed that Ras is activated in platelets in response to thrombin, and glycoprotein VI (GPVI) agonists (Tulasne, Bori et al. 2002). It was suggested that Ras might play a role in integrin activation (Shock, He et al. 1997). Despite these reports, little is known about the function of Ras in platelets.

Noonan syndrome (NS) is a genetic defect in Ras/MAPK signaling pathways of PTPN11, K and N Ras, SOS, and Raf (Tartaglia, Martinelli et al. 2006; Zenker, Horn et al. 2007; Zenker, Lehmann et al. 2007; Tartaglia, Gelb et al. 2011). Thus it provides an *in vivo* tool to understand the role of Ras signaling in platelets. Platelets from NS patients with different mutations in Ras signaling pathway were investigated. Three of the four patients with mutations in K-Ras and PTPN11 showed a platelet aggregation defect. This study suggests several things: 1- NS serves as a model to study Ras signaling and its role

in platelet function, 2- Platelet function testing may be useful as a diagnostic approach to diagnose patients with NS.

## **Results**

### **Patient Genotyping**

Noonan syndrome (NS) is an autosomal genetic disorder that involves several proteins in the Ras/MAPK signaling pathway. These patients might present with bleeding problems that could be related to either a defect in coagulation pathway, such as deficiency in Factor XI or a defect in platelet function. There is insufficient data in the literature about platelets function in NS; thus, our goal is to understand the role of Ras signaling in those patients. Mutations in PTPN-11 are the most common, followed by SOS mutations. In addition, K-Ras, and N-Ras, Raf, MEK, and ERK are found to be mutated in NS patients. The purpose of this section is to characterize the function of Ras in patients with NS. Table 5-1, presents the characteristics of the four patients that were involved in our study. Patient 2 has a mutation in Raf1, patient 3 and 4 have a K-Ras mutation, while patient 5 has a PTPN11 mutation. The coagulation tests (PT and PTT) were normal (data was provided by Dr. Vlad Calin Radulescu, Pediatric Hematology Oncology University of Kentucky, KY, USA), and there were no overt bleeding abnormalities.

### **Adjusting the Experimental Conditions**

Only a few studies have examined the role of Ras in platelet function (Shock, He et al. 1997; Tulasne, Bori et al. 2002). Thus we aimed to study Ras activation in platelets from NS patients. The Ras-Effector domain pull-down assay technique was utilized as described in Chapter Two. Since we were dealing with a small quantity of patient blood (around 20-30 ml), we needed to standardize the conditions for both platelet function and Effector domain pull-down assay. Figure 5-1 shows the dose-dependent aggregation of platelets (Figure 5-1A) when stimulated with increasing concentrations of thrombin (0.02, 0.05, and 0.1) U/mL. The ATP release from dense granules (Figure 5-1B) was also dose-dependent. The Ras pull-down assay was performed using different concentrations of the recombinant protein Raf-Ras Binding Domain (Ras-RBD): 10, 20, 50, 100, 200, and 300  $\mu$ g (Figure 5-2A and B) to determine the appropriate concentration for binding of

the recombinant protein to the Ras-GTP in platelets. As indicated in Figure 5-2A, the binding was accomplished at concentrations between 100-300  $\mu\text{g}$  of Raf-RBD recombinant protein, and the 200  $\mu\text{g}$  of protein was chosen for the experiments. Fresh human platelets, stimulated with 0.1 U/mL thrombin were subjected to Ras Effector domain pull-down assay and immune blotting (Figure 5-2). Ras-GTP was found in resting but its levels increased in stimulated platelets, with a peak between 30 and 60 seconds (Figure 5-2B). Total platelet input used as a control (Figure 5-2C) showed total Ras protein.

### **Characterization of Platelet Function in Noonan Syndrome (NS) Patients**

Bleeding abnormalities have been reported in the literature as one of the most serious, yet under-studied complications associated with NS. The bleeding problems could be due to a defect in coagulation factors or a defect in platelet function. However the nature of the defect in platelets is not yet known. Noonan syndrome patients having defects in the Ras/MAPK pathway provide the ideal model to study Ras activation in platelets and to understand the cause of platelets defect in these patients.

Five patients were involved in this study. The first patient was excluded from interpretation due to insufficient amount of platelets and some technical difficulties. When stimulated with different concentrations of thrombin, platelets from patient 2 (RAF1 mutation) (Figure 5-3B), showed normal aggregation traces compared to the control (Figure 5-3A). In Figure 5-3C, the percentage of aggregation for patient 2 and the control is illustrated. There was a defect in aggregation in platelets from patient 3 and patient 4 (daughter and mother with heterozygous K-Ras mutation) (Figure 5-4). There was a defect in aggregation compared to the control (Figure 5-4A) despite increasing the concentrations of thrombin (Figure 5-4B and C) in both patient 3 and patient 4. Figure 5-4D shows the percentage of aggregation for the control, patient 3 and patient 4. Patient 5 (mutation in PTPN11) showed an aggregation defect (Figure 5-5A, B, C, and D), and ATP release defect (Figure 5-5E, F, G, and H). The percentage of aggregation was plotted (Figure 5-5J). In patient 5 (Figure 5-5D), despite increasing the concentration of thrombin (0.2 U/mL), the control had a lower percentage of aggregation (50%). Platelets could have lost activity, which is expected when platelets are kept for a long time at room

temperature. A fresher preparation of platelets will be required to rule out this possibility. However, the phenotype of platelet dysfunction is still obvious in this patient.

## **Discussion**

The defect in Noonan Syndrome (NS) is associated with mutation in Ras/MAPK gene family (Turner 2011). There are several publications about the genetic and the clinical abnormalities in NS; however, the hematological abnormalities are poorly studied in the literature. There is a 15 years gap between the last review of bleeding disorders in Noonan Syndrome, by Singer *et al* (Singer, Hurst et al. 1997) and the review recently published by Briggs *et al* (Briggs et al. 2012). There are different causes of bleeding abnormalities in patients with NS, such as: defect in coagulation factors, von Willebrand disease, thrombocytopenia, and platelets dysfunction. Furthermore, Ras function in platelets has been poorly documented; only a few reports have been published (Shock, He et al. 1997; Tulasne, Bori et al. 2002). Thus, the focus of this chapter is to understand the role of Ras in platelets function using NS as an model system.

Patient's chosen for this study, fit the genetic and the clinical criteria of NS (work was performed by (Dr. Vlad Calin Radulescu, Pediatric Hematology Oncology University of Kentucky, KY, USA). The biochemical analysis using platelet aggregation and secretion were performed in our lab. It is important to notice that, despite the fact that the patients involved in this study have no overt bleeding diathesis; this does not exclude platelet dysfunction. The conditions of isolating platelets from a small amount of blood, performing platelet function tests were established. Patient 2 had a mutation in Raf1, showed no defect in platelets aggregation. One possibility could be because not all the mutations in NS are associated with platelet dysfunction; the incidence of bleeding abnormalities is between 50-89% (Briggs and Dickerman 2012). However defects in platelets aggregation and secretion were observed in patients with K-Ras (patient 3 and 4) and PTPN11 (patient 5) mutations. In conclusions, this study showed that Ras signaling pathway is important for platelet function, however extensive investigation is required to understand the mechanism causing this defect. For instance, we need to determine if the level of Ras-GTP is affected in those patients by performing Ras pull down assay. In addition, the ERK phosphorylation, downstream of Ras, could also be affected as well. ERK is known to play a role in platelets secretion and integrin activation. Flevaris *et al*.

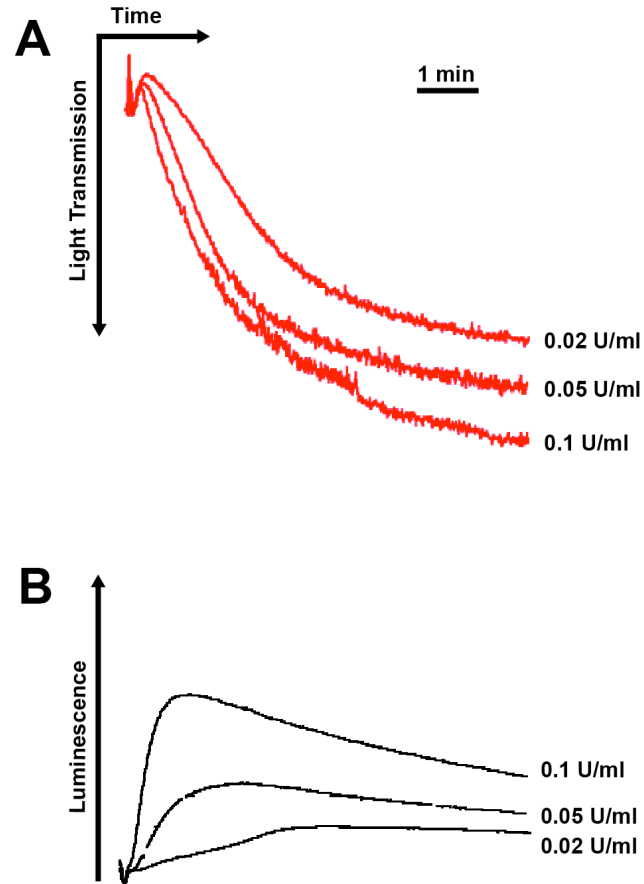
showed that ERK inhibitors affected platelet secretion (from both dense granules and  $\alpha$ -granules), aggregation, and the integrin mediated clot retraction (Flevaris, Li et al. 2009). Further analysis that involves detection of downstream signaling such as ERK phosphorylation is required. NS provides an ideal model to answer all these questions related to Ras pathway in platelets function.

<b>SUBJECT</b>	<b>Patient 2</b>	<b>Patient 3</b>	<b>Patient 4</b>	<b>Patient 5</b>
<b>Age (years)</b>	34	3	37	2
<b>Sex</b>	F	F	F	F
<b>Genetic Mutation</b>	RAF 1 P261R	K-Ras F156V	*Daughter with Noonan has K-Ras	PTPN11 D61N

**Table 5-1. Characteristics of Noonan Syndrome (NS) Patients.**

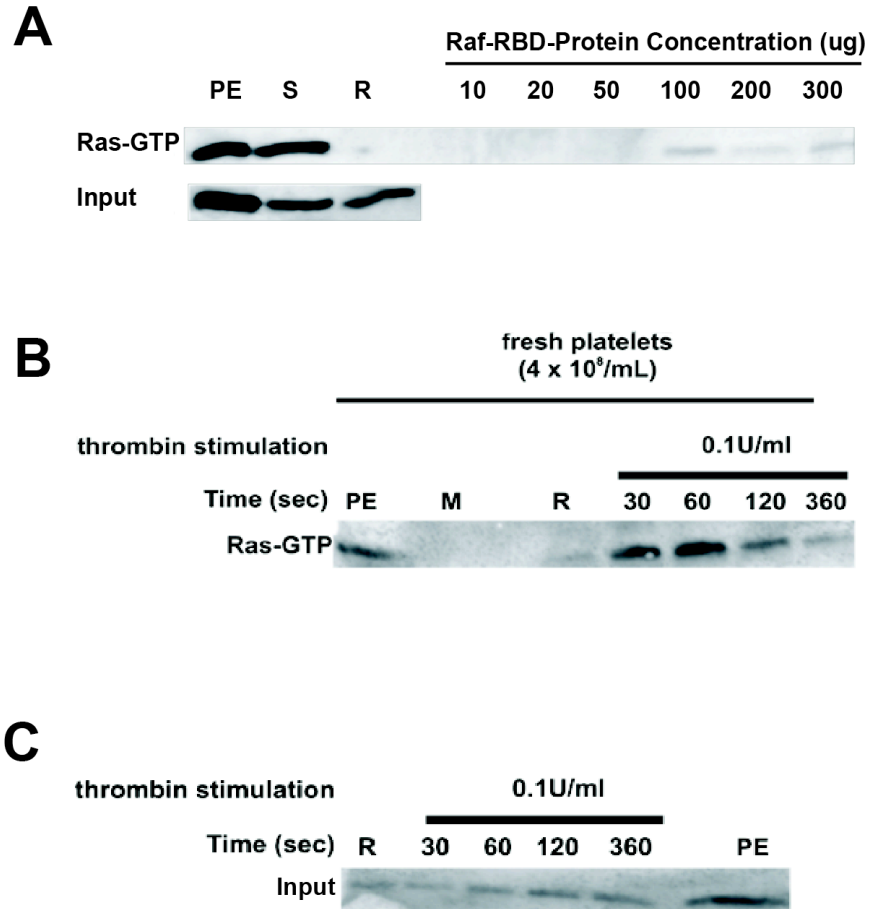
Presented in this table are the conditions of NS patients used for the study: the age, sex and the genotype of the mutations in NS patients.

\* Patient 4 is the mother of Patient 3. The genotype record is not yet identified.



**Figure 5-1. Characteristics of Platelets Aggregation and Secretion in Healthy Controls.**

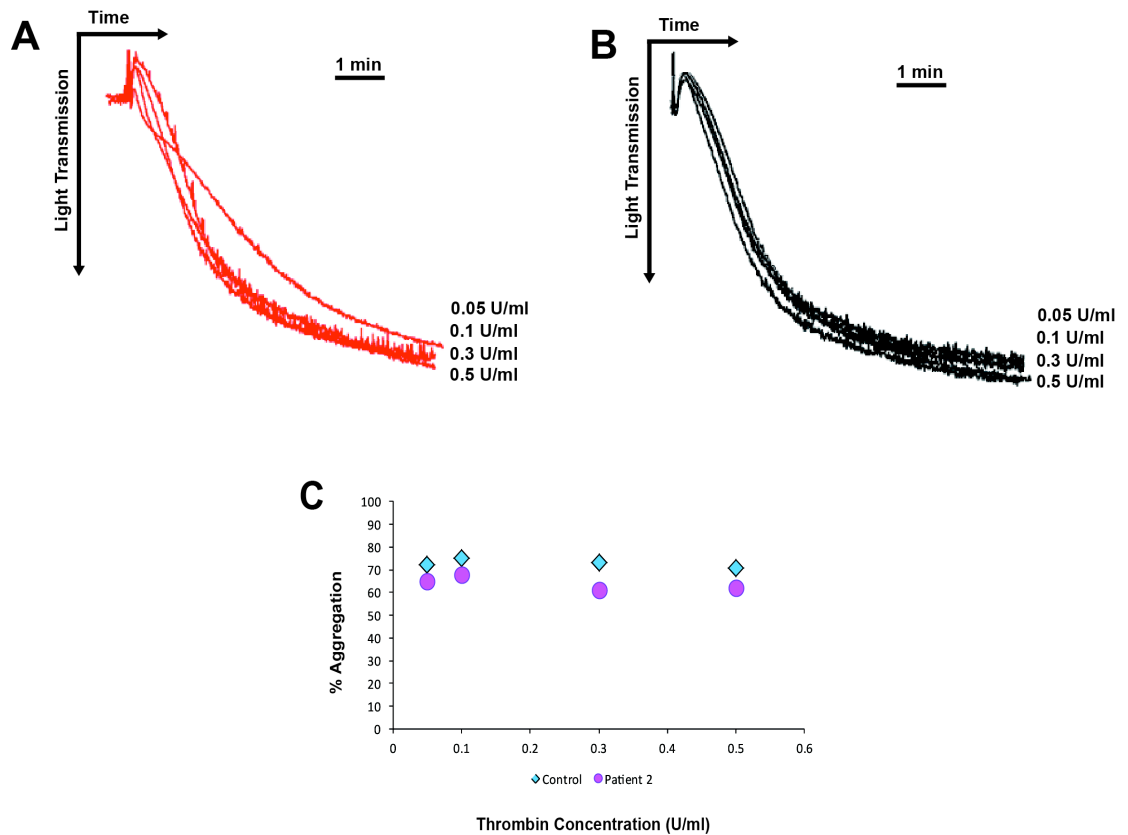
Platelets function studies including aggregation and secretion are presented. Washed human platelets from a healthy control were stimulated with different concentrations of thrombin (A and B) and incubated at 37°C with constant stirring. Platelet aggregation (A) and ATP release (B) were measured with constant stirring.



**Figure 5-2. Characteristics of Platelets Ras Pull Down Assay.**

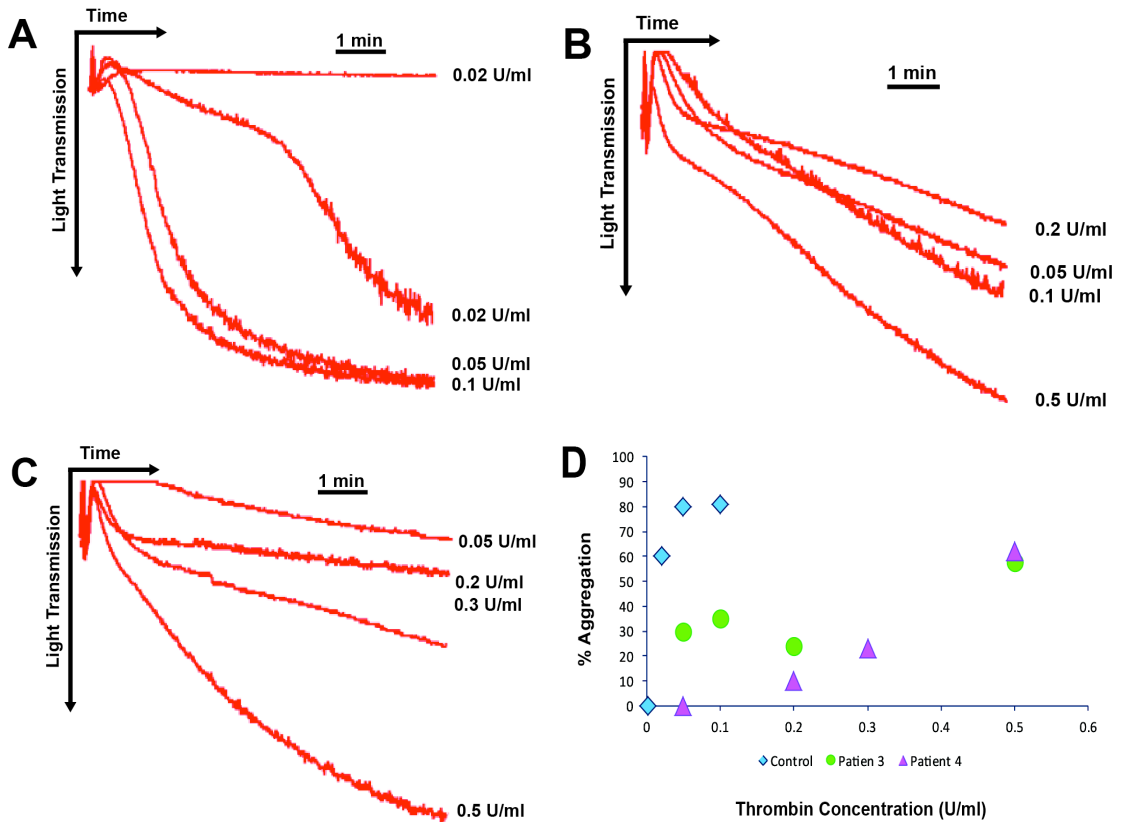
Washed human platelets ( $4 \times 10^8$ /mL) were incubated 0.1 U/mL thrombin with stirring at 37°C (A-C). Platelets were incubated with different concentrations of Ras recombinant proteins (Raf-RBD), lysed at the indicated time points and the supernatants were used to pull down the Ras-GTP (A), as described in Chapter Two. The detection of Ras was accomplished by western blot, using anti-Ras antibody. **B)** Western blot of Ras-GTP pull down in human platelets, stimulated with 0.1 U/mL thrombin, and incubated with 200  $\mu$ g of recombinant protein Raf-RBD at the designated time points. **C)** Ras-GTP in platelets input.





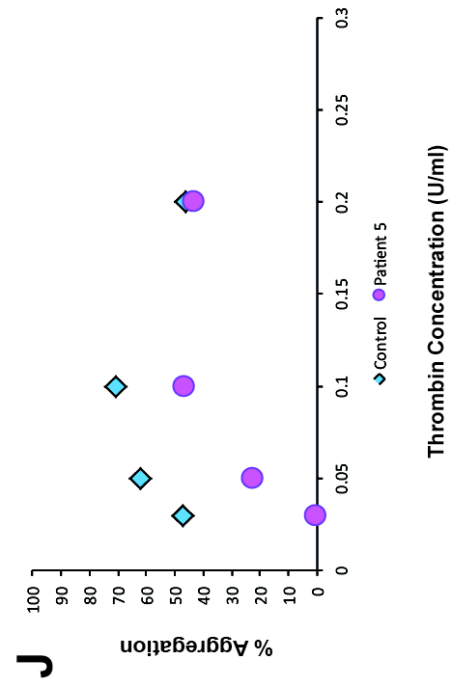
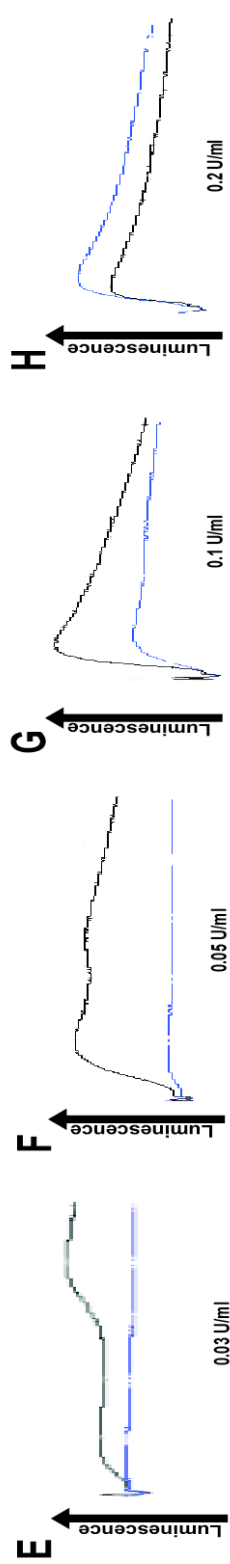
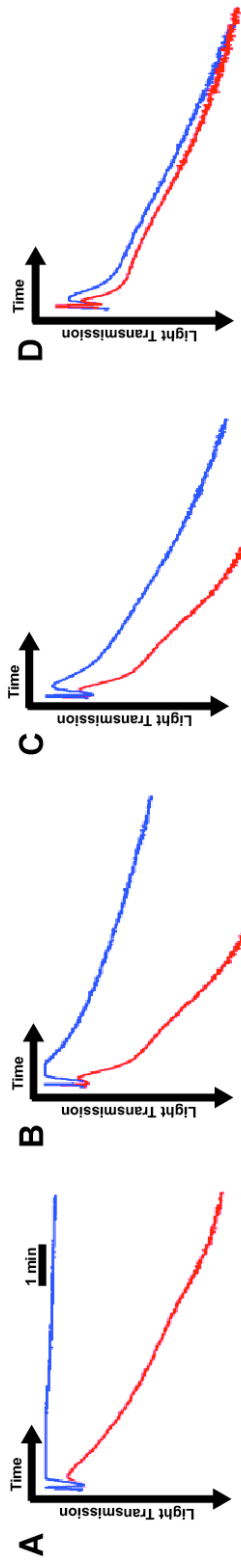
**Figure 5-3. Noonan Syndrome Patient with RAF1 Mutation has a Normal Platelet Function.**

Washed human platelets from a healthy control (**A**) and patient 2 (**B**) were stimulated with the designated concentrations of thrombin (**A and B**) and incubated at 37°C with constant stirring. Platelet aggregation was measured. **C**) Scatter graph present the percentage of aggregation for both the control (**A**, diamond shape) and patient 2 (**B**, circle shape).



**Figure 5-4. Noonan Syndrome Patients with K-Ras Mutation have Defect in Platelet Function.**

Washed human platelets from a healthy control (A), patient 3 (B), and patient 4 (C) were stimulated with the designated concentrations of thrombin and incubated at 37°C with constant stirring. Platelets traces are presented (A-C). D) Scatter graph present the percentage of aggregation for the control (diamond shape) and patient 3 (circle shape), and patient 4 (triangle shape).



**Figure 5-5. Noonan Syndrome Patients with PTPN11 Mutation have Defect in Platelet Function.**

Washed human platelets from a healthy control (red line) and patient 5 (blue line) were stimulated with the designated concentrations of thrombin and incubated at 37°C with constant stirring. Platelets aggregation traces (**A-D**, control: red line, patient5: blue line), and ATP release (**E-H**, control: black line, patient5: blue line). **J**) Scatter graph present the percentage of aggregation for the control (diamond shape) and patient 5 (circle shape).

## Chapter Six

### Conclusions and Future Directions

The data in this thesis represents a body of work that focuses on various, yet related, aspects of platelet secretion. Platelets, the small cellular fragments, circulating in the blood stream, respond to various stimuli, such as vascular injury, wound healing, and inflammation; in a very complex and strictly regulated manner. Platelets provide a unique system to study the regulation of SNARE machinery in exocytosis. Thus we asked several questions related to the regulation of platelet secretion and how it influences pathological conditions. We wanted to know 1) if the v-SNARE, VAMP-8, plays a role in atherosclerosis, 2) how the process of platelet exocytosis is regulated by Munc18b, and 3) what is role of the small GTPase, Ras, in platelets. *In vivo* systems, mice and humans, provide the novel tools to answer our questions. This chapter provides a summary of the work and future implications.

#### Platelet Secretion and Atherosclerosis

This section of the thesis is related to the first question; what is the role of VAMP-8-mediated secretion in atherosclerosis. In the literature, there has been a focus on the role of platelets in the later stages of atherosclerosis, when the plaque has ruptured. However, recent data suggest that platelets might participate in early stages of atherosclerosis as an inflammatory cell in the vessel wall. Sachais *et al.* showed evidence of PF4 deposition, secreted from  $\alpha$ -granules, in the plaques of patients with coronary heart disease (Sachais, Higazi et al. 2004). Additionally, it was shown that PF4 promotes atherosclerotic lesion development in ApoE<sup>-/-</sup> mice and its deletion had a protective effect (Sachais, Turrentine et al. 2007). These data indicate that platelet secretion is important for atherosclerosis. As mentioned earlier, the process of platelet exocytosis is complex and strictly regulated. The field of platelet secretion was controversial regarding which VAMP is important for platelet exocytosis (Flaumenhaft, Croce et al. 1999; Polgar, Chung et al. 2002; Schraw, Rutledge et al. 2003). Our group identified VAMP-8 as the primary v-SNARE that is required for platelet secretion from the three granules (Ren, Barber et al. 2007). They also showed that VAMP-8 is not only important for platelet secretion but also in thrombus formation and stability (Graham, Ren et al. 2009).

Taking these data together, we aimed to determine if VAMP-8-mediated events are important for atherosclerosis. Our results indicated that the deletion of VAMP-8 decreased the lesion size in aged ApoE<sup>-/-</sup> mice, in aortic root and arch. The effect was more prominent in the aortic root. The fact that we saw a significant difference in one area of the aorta but not the other is intriguing. Differential plaque deposition has been noted previously in the literature (Goel et al. 2008). However, some causes could be due to: 1) the variations in murine studies (each mouse respond differently to the disease though they are congenic), 2) the mouse number, if it was increased in the ApoE<sup>-/-</sup>/VAMP-8<sup>-/-</sup> group perhaps statistical significance could have been reached, or 3) the interaction of local hemodynamic forces with vessel wall (Frangos et al. 1999). Further work is needed to understand the link between the intracellular stimuli and hemodynamic forces.

Our data showed a strong phenotype. This work has several potential future implications; for the first time, it links VAMP-8-mediated secretion events to the inflammatory process of atherosclerosis. Another study suggested a correlation between an SNP in the 3' untranslated region of the VAMP-8 gene and early onset of myocardial infarction (MI) (Shiffman et al. 2006). Complicating further interpretation is the fact that VAMP-8 is expressed in several cells in the body. It will be interesting to understand the role of VAMP-8 in other cell types to determine how these events contribute to the risk of cardiovascular diseases. In addition, a platelet-lineage-specific deletion of VAMP-8 will provide a unique tool to study several inflammatory processes in which platelets might play a role, such as sepsis (Yaguchi et al. 2004) and rheumatoid arthritis (Wang et al. 2007). In addition, this model gives an opportunity to uncover the role of platelet secretion in other pathological conditions such as cancer. Platelets contribution to cancer progression has been suggested in the literature (Varki et al. 2001; Borsig 2008)

Understanding the role of VAMP-8 in secretion and its effect on various pathological conditions such as atherosclerosis is very important for pharmaceutical interventions: 1) Platelet secretion is important in atherosclerosis, thus suggesting anti-platelet therapy might be valuable to individuals with risk of cardiovascular disease. 2) VAMP-8 is important in secretion events; thus it is a unique molecular target for pharmaceutical interventions. From a bird's eye view, targeting VAMP-8 might be

useful, but anti-VAMP-8 therapeutics could have several side effects. Deletion of VAMP-8 in mice does cause abnormalities in the pancreas and the salivary and lacrimal glands (Wang et al. 2007). It can also cause splenomegaly (Wang, Ng et al. 2004). Thus targeting the interaction between the complex involved in platelet secretion (VAMP-8, Syntaxin-11 and SNAP-23) might be a more specific approach. This interaction is specific for SNARE regulated platelets exocytosis. Evidence presented in this thesis suggests that VAMP-8 might play a role in inflammation. The deletion of VAMP-8 in ApoE<sup>-/-</sup> mice lessen the plaque compared to the control group, the reason could be due to the effect of VAMP-8 on the secretion of inflammatory molecules from platelets, thus targeting VAMP-8 might lead to less inflammation in the patients with high risk of cardiovascular diseases and thus reduces the risk of plaque development and thrombosis. For instance, small-molecule-targeted therapy might lead to inactivation of VAMP-8 hindering it from binding the t-SNARE and driving the fusion and secretion. However that should not lead to complete inhibition of secretion. In addition, anti-platelet therapy might be useful not only as anti-thrombotic but also as anti-inflammatory target since cytokines released by platelets (e.g. PF4) are known to present in atherosclerotic plaques in patients with cardiovascular diseases and corresponds to the severity of the disease. From the diagnostic point of view, measuring the levels of inflammatory molecules released by platelets (e.g. CD40L and P-Selectin) might be useful for early diagnosis and evaluating the severity of the disease. 4) Platelet function tests can be used to evaluate the efficacy of anti-platelet therapy (Brass 2010) such as patients on anti-platelet therapy who could present with a second episode of myocardial infarction; or it can be used as a low-cost diagnostic tool for patients at high risk of cardiovascular diseases compared to magnetic resonance imaging scans. For example aggregation studies and flow cytometry (measuring P-Selectin exposure and PAC-1 binding) could indicate platelets hyperactivity but can not exclude the risk of thrombosis in patients with normal platelet function, thus this test will be useful if combined with medical history of the patient.

Taken together the above conclusions, preventive measures and future directions might be useful in advancing the field of cardiovascular diseases.

### **Munc18b in Platelet Secretion and its association to Familial Hemophagocytic Lymphocytosis type 5 (FHL5)**

The Sec1/Munc18 family proteins are known to play a regulatory role in SNARE secretion machinery by acting as a chaperone for syntaxins. Among the three isoforms expressed in platelets, data from our laboratory and others show that Munc18a, and Munc18c, could be important for platelet secretion. (Reed, Houg et al. 1999; Houg, Polgar et al. 2003; Schraw, Lemons et al. 2003). The role of Munc18b could not be defined at the time of those studies due to limited reagents. FHL5 patients, with defects in the Munc18b gene, offer an opportunity to study the role of Munc18b in platelet exocytosis. Our data show that Munc18b is the most abundant isoform and that it might be a limiting factor in platelet secretion. There was a complete loss of Munc18b protein in the two-biallelic patients and a partial decrease in the heterozygous patient. The gene mutations in these patients might lead to instability of the Munc18b protein that leads to its degradation as suggested by the study of FHL5 in NK cells (Cote et al. 2009). Platelet secretion defect was prominent and suggests that Munc18b might be the primary Munc family member in platelets. The reasoning behind this is the secretion phenotype. Platelet secretion was severely affected from dense granules (serotonin and ADP/ATP) and  $\alpha$ -granules (PF-4 and P-Selectin exposure) in the biallelic patients and partially so in the heterozygous patient. This would indicate that neither Munc18a nor Munc18c compensate for the loss of Munc18b and that Munc18b could be a limiting factor in platelet secretion. Similar phenotype was seen by our group in heterozygous Munc13d<sup>Jinx</sup> mice with reduced level of Munc13-4 (Ren, Wimmer et al. 2010). There was no difference in platelet morphology between the FHL5 patients and the control. This suggests that platelet biogenesis and cytoskeleton rearrangements were not affected by the loss of Munc18b and the present phenotype is due to the effect that the loss of Munc18b has on membrane fusion and thus the secretion. Hennies group and de Saint Basile group suggested that Munc18b/syntaxin-11 is a functional pair in NK cells (zur Stadt et al. 2009) (Cote, Menager et al. 2009). The loss of Munc18b caused a decrease in syntaxin-11 levels in NK cells from patients with FHL5. This is consistent with our results. The loss of Munc18b caused a decrease in syntaxin-11 levels in platelets from FHL5 patients and suggests that it is a functionally relevant pair. To further confirm this observation, the immunoprecipitation experiment showed that Munc18b and syntaxin-11 interact in a complex. Analysis of platelets from FHL-4 patient with a defect in syntaxin-



11 showed similar phenotype seen in FHL-5 platelets (Ye *et al.* 2012 in revision). Munc18b not only found associated with syntaxin-11, but also with other SNARE machinery (VAMP-8 and SNAP-23) and its regulators (Munc13-4 and Rab-27a). These data together suggest a key function of Munc18b in platelet exocytosis. Thus it is important to further investigate these interactions and identify the proteins that Munc18b interacts with in the secretory pathway (Figure 6-1).

The experiments describing the FHL5 platelets in this thesis were more definitive and suffered from fewer caveats than did the previous approaches for analyzing the role of Munc18's in platelet secretion. For instance, the previous methods relied on inhibitory peptides (Munc18a/Pep3 and Munc18c/Pep3 peptide) and antibodies (Munc18a/b polyclonal antibody) that inhibit platelet secretion from permeabilized platelets (Schraw, Lemons *et al.* 2003). In addition, previous work with Munc18c<sup>+/-</sup> mice showed no platelet secretion defect (Schraw, Crawford *et al.* 2004). In permeabilized platelets, the use of monoclonal anti-Munc18c antibody and a peptide-based inhibitor affected the interaction between Munc18c and syntaxin-4 and enhanced platelet secretion (Reed, Houg *et al.* 1999; Houg, Polgar *et al.* 2003). These results are in agreement with one of the closed confirmation hypothesis, where Munc18s bind to syntaxins in closed confirmation preventing them from interacting with SNARE complex, thus function as negative regulators for syntaxins and have an inhibitory effect on secretion. However, the results presented here and previous work by our lab (Schraw, Lemons *et al.* 2003) is in better agreement with the recent hypothesis that Munc18s remain associated with SNARE proteins (syntaxin and SNAP-23) throughout the secretion process thus function as a positive regulator for membrane fusion (Carr *et al.* 2010). In addition recent work from our lab showed that syntaxin-4, the cognate t-SNARE for Munc18c, has no role in platelet secretion (Ye *et al.* 2012 submitted). Thus Munc18b results in this thesis show more definitive role among the Munc18 isoforms in platelets secretion.

The work presented here shows that Munc18b is the cognate Munc for syntaxin-11 and that a decrease in Munc18b leads to a loss of syntaxin-11. Munc18b not only binds to syntaxin-11 but also forms a complex with SNAP-23. This provided more evidence of the role that Munc18b plays in regulating SNARE machinery. However, further molecular work is required to understand the nature of the association of other

SNAREs proteins such as Munc13-4 and Rab27a, and its importance in regulating SNARE machinery. Figure 6-1 summarizes the conclusions of this part.

FHL is a life-threatening disease, caused by uncontrolled and hyperactivation of the NK cells and T-cells, which lead to ineffective immune response that might lead to death if not treated. Clinical signs and symptoms involve fever that might associate with upper respiratory tract infection, hepatosplenomegaly, pancytopenia, coagulopathy, anemia, hypertriglyceridemia, and neurological abnormalities such as seizures (Janka 2007). The treatment for FHL is immunosuppressive drugs followed by stem cell transplantation (Baker et al. 1997).

Despite the clinical symptoms and the laboratory measures that have been created to aid the diagnosis, FHL5 still could be overlooked. Some of the reasons are that the symptoms are very similar to symptoms from normal infection, the clinical examination of hepatosplenomegaly could be poorly performed, and the prolonged antibiotic treatment might even further delay the diagnosis. The lipid profile is not a routine test. Some of the symptoms, such as fever might disappear (Janka 2007). These diagnostic measures are still not completely efficient and it is still difficult to diagnose unless genetic testing is performed, but it is costly. Therefore the presented data suggest a new diagnostic approach. Using platelet function test might lead to an early diagnosis and improve the survival rate of the patients as suggested recently by Pagel and et al. (Pagel et al. 2012).

Taken together, these data provide several important considerations. 1) FHL provides a unique model to study other regulatory proteins in platelets secretion. The data showed that the defect in Munc18b affected secretion from the granule content. Further analysis of Munc18 association with SNAREs machinery (Munc13-4 and Rab27a) and how it regulates platelet secretion is required. Platelet as a secretory cell provides a unique system to study the regulation of SNAREs machinery. The outcomes of this study will assist in understanding the role of Munc18b in other secretory cells in the body. 2) As mentioned earlier, the diagnosis of FHL5 cannot rely only on the clinical symptoms of fever and fatigue, which are common symptoms of cold and can be misleading, unless genetic test is performed to assess the diagnosis. However, the platelet secretion defect, observed in FHL5 patients, raises the possibility that platelet function assays can be

advancement in the diagnosis of FHL. For instance, FACS analysis of P-Selectin exposure in platelets is considered to be a quick and efficient way to assist the diagnosis before the expensive genetic tests performed. The outcomes of these studies will provide the field with answers that facilitate understanding of the regulation of SNAREs machinery in other systems, such as secretion from natural killer (NK) cells, and improve the diagnostic methods for FHL patients.

### **Ras GTPase in Platelets and Noonan Syndrome (NS)**

The last of this thesis is related to the role of Ras GTPase in platelet secretion. In platelets, the roles of other members of Ras and Rho GTPases have been tied to the control of cytoskeletal remodeling and integrin activation. These events are important for platelet function: adhesion, aggregation, and clot remodeling (Crittenden et al. 2004; Chrzanowska-Wodnicka, Smyth et al. 2005; McCarty, Larson et al. 2005; Akbar, Kim et al. 2007; Cifuni et al. 2008; Varga-Szabo, Pleines et al. 2008). However, very little is known about the role of Ras in platelets. Parise *et al.* showed that Ras is present in platelets and its GTP level increase upon platelets stimulation (Shock, He et al. 1997). Watson *et al.* showed that Ras is activated in platelets in response to thrombin (Tulasne, Bori et al. 2002). However it is not clear yet how Ras is activated in platelets and how this activation affects platelet function.

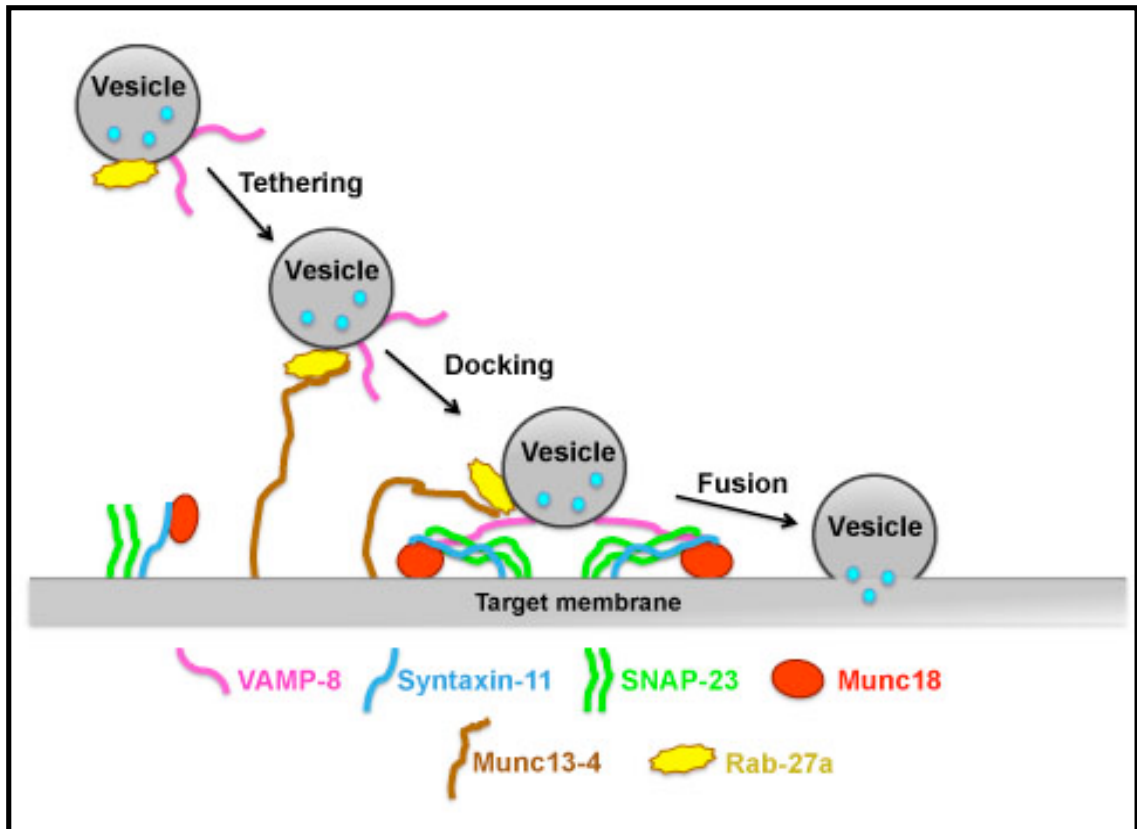
Noonan syndrome is a common inherited disorder, associated with various clinical features, such as reduced growth, distinctive facial dysmorphism and hypertrophic cardiomyopathy (Noonan 1994). There is a high incidence of bleeding disorders in patients with NS (Briggs and Dickerman 2012). Patrick *et al.* made a recommendation to the clinicians to consider the diagnosis in patients with any bleeding disorder associated with congenital heart disease or short stature. In addition she recommended hematological tests to be performed, such as blood count and coagulation studies for all patients in general, and to investigate hemostasis further in patients with bleeding or bruising (Patrick et al. 2010). Platelet function tests such as aggregation have been used previously to investigate the function of platelets in NS patients; nevertheless in general platelet function in NS was poorly studied in the literature (Flick et al. 1991; Singer, Hurst et al. 1997; Briggs and Dickerman 2012). Unlike FHL patient who have a

clear platelet secretion defect, platelet secretion defect is common but not in all the population of NS, though we have seen it in the patient with K-Ras mutation.

As mentioned above, Noonan Syndrome mutations are linked to the Ras/MAPK pathway, and bleeding problems. Therefore it is of a great benefit to use platelets from NS patients in order to dissect Ras activation and how it might influence platelet function. Noonan syndrome patients with different kinds of mutations in Ras/MAPK were included in a study to analyze their platelet function and investigate if there is a relation between the phenotype of platelet function and the genetic mutations in Ras/MAPK signaling pathway. The results showed that certain mutations in Ras/MAPK pathway, such as K-Ras mutation in the mother and the daughter, there was a defect in platelet aggregation despite the increasing concentration of thrombin. Similar phenotype was observed in platelets from the patient with PTPN11 mutation. Our data highlighted the fact of the importance of Ras in platelets aggregation and ATP release, and that a defect in Ras signaling might causes a defect in platelet function. There are still questions to be answered; how Ras activation affects platelet function and why it is important. There is a possibility that Ras might play a role in integrin activation. Platelet stimulation with agonist lead to activation of PLC followed by IP3 and an increase in cytoplasmic  $Ca^{2+}$  and induces inside-out signaling of integrin and subsequently platelet aggregation (Rink et al. 1982; Lian et al. 2005; Offermanns 2006). Activation of Ras could be upstream of PLC. It will be interesting to measure the  $Ca^{2+}$  mobilization in patients with mutation in Ras. In addition, it is also important to make sure that Ras is inactive in these patients by doing pull down assay as explained previously in Chapter Two. Another possibility of Ras function in platelets is that Ras could play a role in platelet cytoskeleton rearrangements similar to Rho GTPase.

This study has several potentials; understanding platelet biology and the signaling events that influence platelet activation, aggregation, and secretion is required to maintain hemostasis and prevent bleeding. NS is a model to study defect in Ras signaling in platelets and other cells. In addition, understanding the importance of Ras in platelet function might be a useful therapeutic target for inhibiting platelet function in several diseases.

Over all the work in this thesis provides an insight about the molecular mechanism of platelet exocytosis and its role in physiological and pathological conditions. Understanding platelet biology and the process of secretion will have a great impact on the science field, not only for detection of new therapeutic targets but also to appreciate platelet as a unique system to study exocytosis and SNARE proteins. In addition to considering platelet function assays as a quick and efficient diagnostic tool in genetic disorders that involve platelet secretion defect.



**Figure 6-1: Summary of the Role of Munc18b in Platelet Secretory Pathway.**

Membrane fusion occurs in several steps. Munc18b (red) interacts with the t-SNARE (syntaxin-11 (blue) and SNAP-23 (green)) and the v-SNARE VAMP-8 (pink) to promote membrane fusion and secretion of the granule content. Munc18b might also play an early role in regulating the secretion by its association with other regulators in the secretory pathway such as Rab-27a (yellow) and Munc13-4 (brown). Further work is needed to further identify the interaction with other proteins upstream of the fusion step.

## LIST OF ABBREVIATIONS

5-HT	Serotonin (s-hydroxytryptamine)
ADP	Adenosine di-phosphate
ALL	Acute lymphocytic leukemia
AP	Alkaline phosphatase
Arf	ADP-ribosylation factor
ATP	Adenosine tri-phosphate
BCA	Bicinchoninic acid
$\beta$ -TG	$\beta$ - Thromboglobulin
DAG	Diacylglycerol
DMS	Demarcation membrane system
ECF	Enhanced chemi-fluorescence
ECM	Extra cellular matrix
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-linked immuno sorbent assay
FACS	Fluorescent-activated cell sorting
FHL2	Familial hemophagocytic lymphohistiocytosis type 2
FHL3	Familial hemophagocytic lymphohistiocytosis type 3
FHL4	Familial hemophagocytic lymphohistiocytosis type 4
FHL5	Familial hemophagocytic lymphohistiocytosis type 5
FITC	Fluorescein isothiocyanate
FPLC	Fast protein liquid chromatography
GAP	GTPase activating protein
GDP	Guanosine di-phosphate
GEF	Guanine nucleotide exchange factor
GPVI	Glycoprotein VI
GST	Glutathione S-transferase
GTP	Guanosine tri-phosphate

HUVEC	Human umbilical vein endothelial cell
IL-1 $\beta$	Interleukin-1 $\beta$
ICAM	Intercellular adhesion molecule
<i>i.p.</i>	Intraperitoneal injection
IP	Immunoprecipitation
IP <sub>3</sub>	Inositol tri-phosphate
PI-4,5-P2	Phosphatidylinositol 4,5-bisphosphate
IPTG	Isopropyl-beta-D-thiogalactopyranoside
LAMP	Lysosomal-associated membrane protein
LDCV	Large dense-core vesicle
LDL	Low density lipoprotein
LIMP	Integral membrane protein
MCP-1	Monocyte chemotactic protein-1
MI	Myocardial infraction
MMP-9	Matrix metalloproteinase-9
NK	Natural killer
NS	Noonan syndrome
NSF	N-ethylmaleimide-sensitive factor
OCS	Open canalicular system
PAGE	Polyacrylamide gel electrophoresis
PAI-1	Plasmingoen activator inhibitor-1
PAR	Protease-activated receptor
PF4	Platelet factor 4
PGI <sub>2</sub>	Prostaglandin I <sub>2</sub>
PKC	Protein kinase C
PLC	Phospholipase C
PRP	Platelet-rich plasma
RIAM	Rap1GTP-interacting adapter molecule
SNARE	Soluble N-ethylmaleimide-sensitive factor activating protein receptor



SNAP	Synaptosome-associated protein
SNP	Single nucleotide polymorphism
TNF- $\alpha$	Tumor necrosis factor- $\alpha$
TGF- $\beta$	Transforming growth factor- $\beta$
<i>unc</i>	Uncoordinated mutants
VAMP	Secretory vesicle-associated membrane proteins
VCAM	Vascular cell adhesion molecule
vWF	von Willebrand factor

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MS Rawlins Scholarship, University of Kentucky, 2006-2008  
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King Faisal University Scholarship for the Doctoral Degree, 2006  
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**MEMBERSHIPS**

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American Society of Clinical Lab Sciences, 2009-2011  
International Society of Laboratory Hematology, 2007-2009  
American Heart Association, Heart Walk- Biochemistry group, 2007-2010

**PUBLICATIONS**

- 1- Rania Al Hawas, Qiansheng Ren, Shaojing Ye, Zubair A. Karim, Alexandra H. Filipovich, and Sidney W. Whiteheart. FHL5/STXBP2/Munc18b Is Important for Platelet Granule Secretion. (In Preparation)
- 2- Shaojing Ye, Zubair A. Karim, Rania Al Hawas, Alexandra H. Filipovich, and Sidney W. Whiteheart. Syntaxin11. Submitted (2012).
- 3- Karim, Z.A., Zhang, J., Al Hawas, R., Chicka, M., Roche, P.A., and Whiteheart, S.W. (2012) I $\kappa$ B Kinase Controls SNARE Complex Assembly and Platelet Secretion. (In preparation).

**ABSTRACTS AND PRESENTATIONS**

The Role of Munc18b in Platelet Secretion. Cardiovascular Research Day. Lexington, KY. 2011



Does the v-SNARE VAMP-8 Play a Role in Atherosclerosis? Biochemistry Department Retreat. Lexington, 2010

Does the v-SNARE VAMP-8 Play a Role in Atherosclerosis? ASCLS Conference. Chicago, 2009

Does the v-SNARE VAMP-8 Play a Role in Atherosclerosis? ISLH Conference. Vegas, 2009

Does VAMP-8 play a The Role of VAMP-8 in Atherosclerosis. Cardiovascular Research Day. Lexington, 2008

Does the v-SNARE VAMP-8 Play a Role in Atherosclerosis? Midwest Platelet Conference. Lexington, 2008

Does the v-SNARE VAMP-8 Play a Role in Atherosclerosis? University of Kentucky Graduate Student Congress, Lexington, 2008

Does the v-SNARE VAMP-8 Play a Role in Atherosclerosis? Cardiovascular Research Day. Lexington, 2007