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THE ROLE OF SYNTAXIN AND TOMOSYN IN PLATELET SECRETION

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THE ROLE OF SYNTAXIN AND TOMOSYN
IN PLATELET SECRETION

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DISSERTATION

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requirements for the degree of Doctor of Philosophy in the
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ABSTRACT OF DISERTATION

THE ROLE of SYNTAXIN AND TOMOSYN

IN PLATELET SECRETION

Platelet secretion is important for hemostasis and thrombosis. The components released are also involved in atherosclerosis, inflammation, angiogenesis, and tumor growth. Though the exact mechanism(s) of platelet secretion is still elusive, accumulating evidence demonstrates that SNAREs (Soluble N-ethylmaleimide Sensitive Factor Associated Receptor) and their regulatory partners are critical for platelet exocytosis. Formation of a *trans*-bilayer complex composed of one v-SNARE (*i.e.* VAMPs) and two t-SNAREs (*i.e.* syntaxin and SNAP-25-type) is minimally required for membrane fusion. Regulatory proteins control the rate and specificity of the complex assembly. VAMP-8 and SNAP-23 (a SNAP-25-type t-SNARE) are clearly important; however, the identity of the functional syntaxin has been controversial. Previous studies, using anti-syntaxin antibodies in permeabilized platelets, suggested roles for both syntaxin-2 and -4. These conclusions were experimentally tested using platelets from syntaxin knockout mice and from a Familial Hemophagocytic Lymphohistiocytosis type 4 (FHL4) patient that lacks syntaxin-11. Platelets from syntaxin-2 and syntaxin-4 single or double knockout mice had no significant secretion defect. However, platelets from the FHL4 patient had a robust defect, though their morphology, activation, and cargo levels appeared normal. Semi-quantitative western blotting showed that syntaxin-11 is the most abundant syntaxin in both human and murine platelets. Co-immunoprecipitation experiments showed that syntaxin-11 forms SNARE complexes with VAMP-8 and SNAP-23. These data conclusively demonstrate that syntaxin-11, but not syntaxin-2, or -4, is required for platelet exocytosis.

We also show that a syntaxin binding protein, tomosyn-1, is important for platelet exocytosis and hemostasis. Tomosyn-1 was identified from platelet extracts using affinity chromatography, RT-PCR analysis, and western blotting analysis. Tomosyn-1 was co-immunoprecipitated with syntaxin-11/SNAP-23 from both resting and activated

platelet extracts. Platelets from tomosyn-1$^{-/-}$ mice displayed a secretion defect, but their morphology and activation appeared normal. Tomosyn-1$^{-/-}$ mice showed impaired thrombus formation in two different injury models. Given the importance of platelet secretion to hemostasis, it is hoped that the insights gained from these studies in this dissertation will help to identify new and more valuable therapeutic targets to control clot formation.

KEYWORDS: PLATELET EXOCYTOSIS, SNARE, SYNTAXIN-11, TOMOSYN-1,

                      Familial Hemophagocytic Lymphohistiocytosis 4 (FHL4)

_____Shaojing Ye_____

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To my husband: **Guogen**

and children: **Cici** and **Zachari**

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Introduction

Platelet Overview:

Platelets are small, anucleated blood cells produced from megakaryocytes in bone marrow [1]. Their structures are simple: they contain plasma membrane, microtubules, mitochondria and three types of storage granules: dense granules, α-granules and lysosomes. Platelets respond to vascular damage via surface receptors that directly bind to agonists exposed or produced at the site of damage. The resulting activation leads to increases in intracellular Ca^{2+}, dramatic changes in cell shape and release of granule contents. These steps help to attract more platelets which aggregate to form a thrombus, which stops bleeding. Hypoactive platelets lead to bleeding problems [2]; hyperactive platelets cause spurious thrombosis which leads to occlusive events such as heart attack or stroke [3, 4]. Thus, regulating and controlling platelet function has significant therapeutic value for preventing cardiovascular diseases.

Secretion of the cargo from the platelet's three stores is critical for hemostasis [1, 5-7] and important for other diseases [8-10]. Hemostasis is a physiological process to stop bleeding from an injured vessel. Platelets first adhere and aggregate to the damaged site to form a platelet rich thrombus. ADP release from dense granules serves as secondary agonists to activate and recruit other circulating platelets, thus it is important for secretion dependant platelet aggregation. Followed by the coagulating cascade activation and thrombin generation, a fibrin rich clot is formed to temporarily seal the vessel wall. Fibrinogen and Factor V from α-granules participate in fibrous clot formation, clot retraction, and coagulation [1, 11, 12]. Finally, the released growth factors from α-granules initiate the wound repair. Lysosomal proteins are thought to assist in clot remodeling. Platelet secretion defects have been shown to ablate or at least delay thrombogenesis in murine models [13]. Platelet secretion is not only important for hemostasis and thrombosis but it is also associated with many pathological processes such as inflammation, atherosclerosis, angiogenesis and tumor growth. Platelets are thought to contribute to the initiation and propagation of the inflammatory process

through secretion of bioactive components such as chemokines, cytokines, and procoagulants from α-granules [7, 14]. Platelets with dense granule secretion defects show reduced formation of platelet-leukocyte aggregates and arterial [15]. Atherosclerosis is a chronic inflammatory disease on the arterial walls characterized by the infiltration of immune cells [8, 9]. Accumulating evidence demonstrates that platelets play a role in both the initiation and progression of atherosclerosis [16, 17]. Chemokines and P-selectin from α-granules are thought to be the factors. Specific knockout of platelet P-selectin reduces plaque lesion development in apoE null mice [18]. Inhibition of the interaction between platelet chemokines decreases the progression of atherosclerosis [19-21]. Upon stimulation, platelets release a variety of pro-angiogenic proteins, such as vascular endothelium growth factor (VEGF), platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), and epidermal growth factor (EGF) [5]. These angiogenic activators promote vessel wall permeability and proliferation of endothelial cells and fibroblasts, thus facilitating tumor growth [10, 22]. Understanding the mechanism of platelet secretion and secretory machinery has the potential to lead to the development of new drug targets for bleeding problems, thrombosis, atherosclerosis, heart attack, stroke, cancer, and inflammation.

Platelet Structure and Function

Platelets contain mitochondria, microtubules, granules, plasma membrane and a specialized region of the plasma membrane called the open canalicular system (OCS). On the plasma membrane, there are receptors including protease activated receptors (PAR1, 3, 4), GPVI, and purinergic receptors (P2Y$_1$ and P2Y$_{12}$) which are involved in platelet activation [1, 23]. The OCS is an invagination of the plasma membrane which is thought to be an important site for granule secretion and endocytosis [24]. There are three types of granules (Figure 1): α-granules, dense granules, and lysosomes. The most abundant granules, α-granules, contain polypeptides such as platelet derived growth factor (PDGF), cytokines, von Willebrand factor, P-selectin, fibrinogen, GPIIb/IIIa and platelet factor IV (PF4). The cargo released from α-granules are important for platelet aggregation, wound repair, and inflammation. Dense granules contain small molecules such as ADP, calcium, magnesium, and 5-hydroxytryptamine (5-HT, serotonin), which

serve as signaling molecules or ligands to recruit and activate other platelets to the lesion site. Lysosomes contain hydrolytic enzymes such as β-hexosaminidase, cathepsin D, and acid phosphatases, which are thought to be important for clot remodeling. Platelets also contain cytoskeletal structures which are necessary for maintaining the resting platelet and changing its shape during activation.

The primary function of platelets is the maintenance of pressurized vasculature integrity. When the receptors on the platelet surface detect vascular damage, such as exposed matrix collagen or von Willebrand factor (vWF) from damaged endothelial cells, platelets are activated. Initially, the activated platelet undergoes a rearrangement of its actin-based cytoskeleton sending out filopoida then lamellipodia, centralizing its granules and spreading onto the damaged area. This increases the surface contact sites. The centralized granules fuse with the OCS, resulting in the release of components from the dense granules, α-granules, and lysosomes. Once released, these components propagate thrombus formation and activate other circulating platelets to aggregate at the damaged site. Finally, the granule membrane becomes part of the exterior platelet membrane, which further increases surface contacts. Platelet activation and aggregation for hemostasis is a tightly regulated process, small changes in this process can result in diseases. Given the central role played by platelet secretion, understanding the mechanisms of the platelet secretory machinery might be useful in controlling and/or regulating platelet functions. Such knowledge might direct the development of therapeutics that could reduce spurious thrombosis and related vascular diseases.

A

100 nm

B

Alpha
Granule

Open
Canalicular
System

Dense Core
Granule

Lysosome

Cortical
F-actin

Mitochondria

Microtubules

4
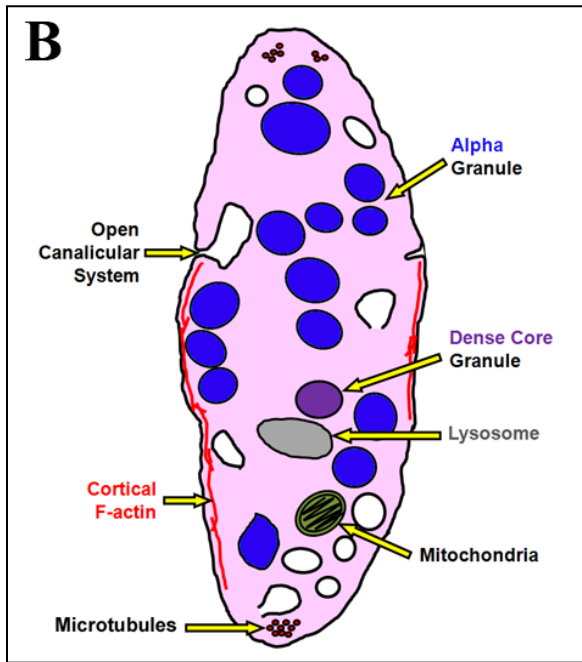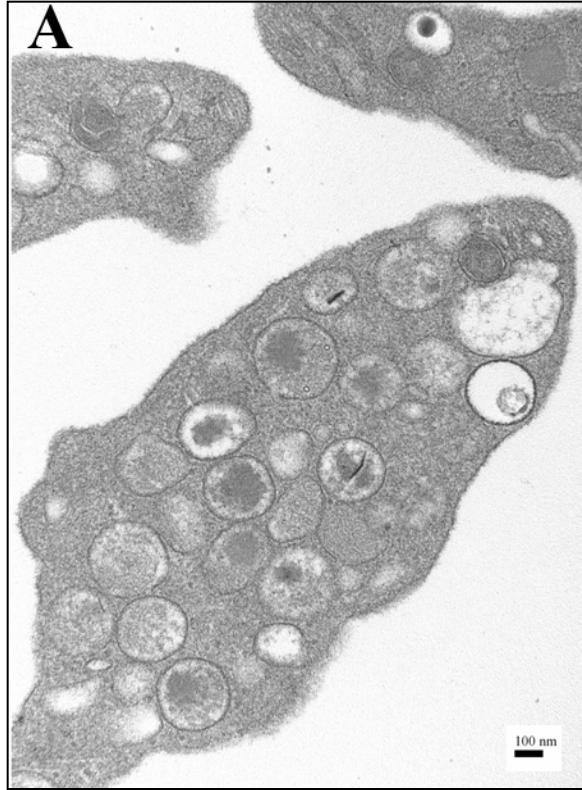
Figure 1-1. Platelet Ultrastructure. (A) A transmission electron micrograph of a washed murine platelet. (B) A cartoon image (made by Dr. Wangsun Choi) of the structural components of a platelet: α-granules (blue), dense-core granules (purple), and lysosomes (grey). Microtubules are located at each pole. The open canalicular system (OCS) is the invaginated plasma membrane structure.

Platelet Granule Cargo

To date, hundreds of proteins and small molecules have been shown to be secreted from activated platelets [25]. This "secretome" can be classified by granular source or proposed function. Dense granules are the source of small molecules, such as, ADP, serotonin, and calcium, which are critical for platelet activation. α-Granules contain proteinaceous components. Platelets also release lysosomal enzymes *i.e.* cathepsins and β-hexosaminidase, which may facilitate clot remodeling. Functionally, the "secretome" can be divided into even more categories. The most obvious class is the adhesive proteins *e.g.* fibrinogen, von Willebrand Factor and thrombospondin, which are released from α -granules and function in platelet-platelet binding. The most diverse category is the mitogens *i.e.* IGF-1, VEGF, and bFGF, which promote wound healing and vessel regrowth. Platelets also release chemokines and cytokines *i.e.* RANTES, IL-8, and MIP1α/β which activate passing leukocytes and lead to a range of inflammatory responses. This catalog suggests that platelet secretion may be pivotal in establishing the microenvironment at a vessel wound site. Understanding more about how the secretion process works may yield a unique tool to control the microenvironment at vascular lesions.

Platelet and Granules Biogenesis

Platelets are produced by precursor cells, megakaryocytes, which are descended from pluripotent stem cells in the bone marrow [1]. In the early stages of megakaryocyte development, the premegakaryocytes differentiate from pluripotent stem cells and then undergo endomitosis and maturation. Maturation includes four stages: megakayoblasts, stage I; basophilic megakaryocytes, stage II; granular megakaryocytes, stage III; and platelet-producing megakaryocytes, stage VI. At the early stages of maturation, this progenitor cell gradually acquires distinct structures, including a demarcation membrane system, dense tubular system, and granule precursors. Platelet-specific proteins such as platelet factor IV (PF4), and β-thromboglobulin (β-TG) are synthesized and packaged into α-granules at the late stages of maturation. Platelets are then produced from the matured megakaryocytes and begin to circulate [26]. Circulating platelets live about 10 days and are primarily destroyed in the liver and spleen. Blood platelets have three types

of granules with distinct ultra-structure, content, and release kinetics. The ontogeny of these granules and their relationships are unknown. Both dense core granules and α-granules originate from *trans*-Golgi network (TGN) elements in megakaryocytes [5] Little is known about the lysosome biogenesis. Depending on the source, the cargo in all three granules can be characterized as endocytic cargo, such as fibrinogen and *de novo* synthesized cargo, such as PF4, β-TG, vWF, and PDGF. Most cargo are packed into granules via one of the two routes.

SNARE Hypothesis

In 1993, Söllner *et al.* [27] suggested that the soluble N-ethylmaleimide-sensitive-factor (NSF) Attachment Protein (SNAP) receptors (SNAREs) are involved in neurotransmitter exocytosis. SNAREs are membrane proteins that contain conserved heptad repeat regions which associate to form coiled-coils. The crystal structures [28] show that the SNARE complex is a parallel four-helix bundle with SNARE motifs assembled into parallel, twisted coiled-coil four-helix bundles by burying the hydrophobic residues inside. Analysis of this structure shows that it is composed of fifteen hydrophobic layers, arranged perpendicular to the axis of the bundle, with a central hydrophilic zero-layer containing one R- and three Q-residues connected by highly polar bonds. Accordingly, the contributing SNARE motifs are classified as Qa-, Qb-, Qc- and R-SNAREs [29-31] (Figure 1-2). The endosomal SNARE complex [32], which contains syntaxin-7 (Qa), Vti1b (Vps ten interacting-1b, Qb), syntaxin-8 (Qc) and VAMP-8 (R), has a similar structure, suggesting that the hydrophobic layers that surround the "0" layer are highly conserved. The formation of SNARE complexes is thought to make membrane fusion energetically favorable.

Reconstituted proteoliposome experiments confirm that SNAREs are minimally sufficient for membrane fusion [33]. According to the original definition, SNAREs that reside on vesicles (or granules) are referred to as v-SNAREs (also called vesicle associated membrane proteins, VAMPs, R-SNAREs) and SNAREs that reside on target membranes are t-SNAREs (Q-SNAREs). VAMPs and syntaxins are generally type II integral membrane proteins with their SNARE motifs facing the cytoplasm [30]. The

core SNARE complex is comprised of one helical domain each from syntaxin (Qa) and v-SNARE (R-SNARE) and two from SNAP-25 (Qb,c). Therefore, functional SNARE complexes bridge the two fusing bilayers as hetero-oligomer, requiring one of each of the Qa-, Qb-, Qc- and R-SNAREs. There are several v-SNAREs (R-SNARE): cellubrevin, VAMP1-12, *etc* [34], and two types of t-SNAREs: syntaxin-like (Qa) and SNAP-25-like (Qbc). Numerous syntaxin-like homologues have been reported (syntaxin-1 to -19) [35], while there are only a few SNAP-25-like t-SNAREs (SNAP-23, SNAP-25, SNAP-29, Sec9p) [34].

Although most SNAREs have a standard integral-membrane protein structure, there are some exceptions. SNAP-25-like t-SNAREs lack transmembrane domains (TMD) and are generally anchored to the membrane through thiolester-linked acyl groups. A stretch of cyteines in the linker between SNARE motifs are through to be modified. A subset of VAMPs lacks TMD but have hydrophobic post-translational modifications that mediate membrane anchorage. For example, Ykt6, a *S. cerevisiae* VAMP, has a C-terminal CAAX box that is farnesylated [36]. Similarly, the mammalian syntaxin-11 does not contain a TMD, but instead has a cluster of six cysteine residues, which are predicted to anchor to the membrane through palmitoylation. Studies by using transfected NRK cells and human B lymphocytes have demonstrated that syntaxin-11 behaves like an integral membrane protein [37, 38]. Though the hydrophobicity of these TMD surrogates is critical for *in vitro* fusion of proteolipisomes [39], it not clear what degree of modification is required *in vivo*.

N-terminal domain · SNARE motif · Transmembrane domain

Qa-SNARE

Qb-SNARE

Qc-SNARE

Qbc-SNARE
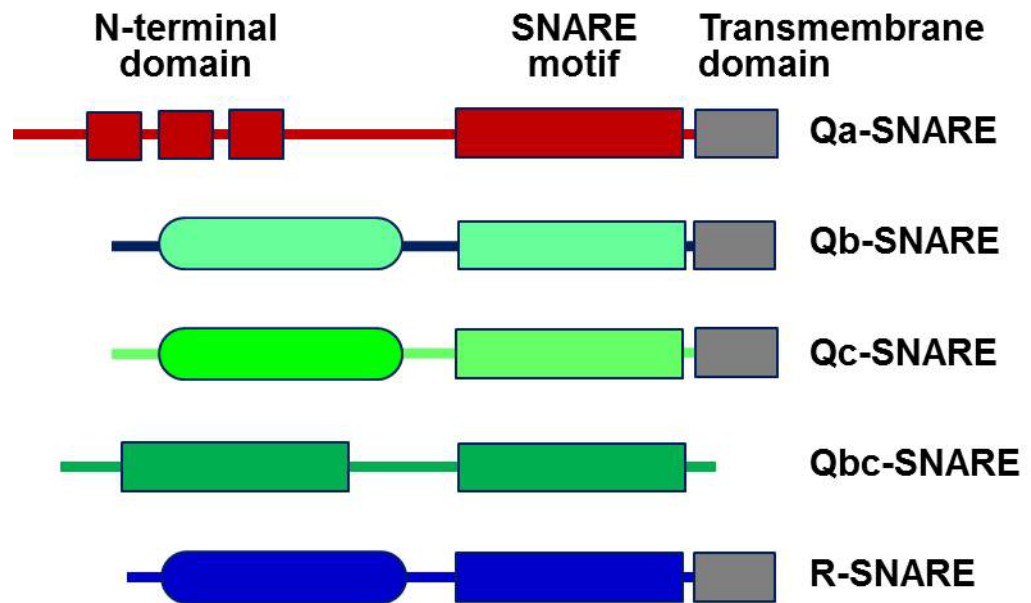
R-SNARE

Modified from Jahn *et al.* **[30]**

Figure 1-2. The Structure of SNAREs. Qa-SNAREs have N-terminal three-helix bundles (called the Habc domain). The N-terminal domains of Qb-, Qc- and R-SNAREs are represented by an oval shape. SNARE motifs are shown as rectangles. Transmembrane domains are shown as gray rectangles. Qbc-SNAREs represent a small subfamily of SNAREs (the SNAP-25-like t-SNAREs), that contain one Qb-SNARE motif and one Qc-SNARE motif.

SNARE Machinery in Platelets

To date, four VAMPs (-2, -3, -7, -8), four syntaxins (-2, -4, -7, -11), and SNAP-23, SNAP-25, and SNAP-29 have been demonstrated to be present in platelets [40-47]. Studies involving *in vitro* permeabilized platelet secretion assay, genetic manipulation and *ex vivo* platelet secretion assays, *in vivo* studies, and human genetic disorders have helped to identify many important proteins associated with platelet exocytosis. In this thesis, we show a role for syntaxin-11. Biochemical studies have also identified potential SNARE regulators that interact with t-SNAREs or t-SNARE complexes. Some of these proteins have been shown to be important (*i.e.* Munc13-4, Rab27) [48-50] and others have yet to be characterized (*i.e.* granuphilin and tomosyn), which will be described in this thesis (*i.e*. tomosyn) [Figure 4-1 and Table 4-1].

Proteins interacting with t-SNAREs or t-SNARE complexes are potential SNARE regulators. With this notion, several components have been identified, such as Munc18b, Munc13-4, tomosyn-1, and granuphilin [Figure 4-1 and Table 4-1]. Some of them have been shown to be important secretory regulators in platelets [48-50].

Platelet v-SNAREs

Four v-SNAREs: synaptobrevin/VAMP-2, cellubrevin/VAMP-3, TI-VAMP/VAMP-7, and endobrevin/VAMP-8 [43-47] have been detected in platelets. For the sake of simplicity, only the VAMP nomenclature will be used. VAMP-8 is the most abundant v-SNARE in both murine and human platelets [46]. Oddly, VAMP-2 is almost as abundant in murine platelets but is almost undetectable in human platelets. VAMP-3 and VAMP-7 are detected at approximately similar levels and a per platelet basis.

VAMP-3, a homolog of VAMP-1 and -2, was first identified in human platelets in 1999 by PCR and cloning [43] and reported to play a role in α-granule secretion [44]. It was thought that VAMP-3 was required for platelet secretion since other VAMPs had not been identified in platelets at that time and a commercial preparation of VAMP-1/2/3-cleaving Tetanus toxin inhibited secretion from permeabilized platelets [44, 51]. Consistently, studies using an anti-VAMP-3 peptide antibody [52] inhibited Ca2+-induced alpha-granule secretion by approximately 50%, and the cytoplasmic domain of VAMP-3 affected both dense and α-granule secretion [45]. Although *in vitro*

permeabilized platelet studies using antibodies, toxins, and peptides have potential drawbacks such as steric hindrance by antibodies, contaminants in the commercial preparations and cytoplasmic-domain-binding promiscuity. It was surprising when platelets from VAMP-3 null mice had no secretion defect [53]. These ex vivo studies demonstrated that VAMP-3 was not absolutely required. During the analysis of VAMP-3 null platelets, VAMP-2 was detected in both human and murine platelets. Considering that VAMP-2 is important for neurotransmitter release [54, 55], and Tetanus toxin had affected permeabilized-platelet secretion, it could play a role in platelet secretion. However, partial deletion of VAMP-2 (~50% reduction) had no defect on murine platelet secretion nor did deletion of VAMP-3 and reduction of VAMP-2. These results indicated that neither VAMP-2 nor VAMP-3 were essential for platelet secretion. VAMP-7 was found in platelets using western blot analysis, however there was no evidence, using antibodies and inhibitor peptides, to show that VAMP-7 is involved in platelet secretion. VAMP-7 was first identified in epithelial cells as a TeNT-insensitive VAMP (TI-VAMP) involved in exocytosis [56]. Its unique amino-terminal regulatory Longin domain was thought to be involved in post-Golgi and endosomal trafficking [57]. VAMP-8, was originally identified as an endosomal v-SNARE involved in fusion between early and late endosomes [58], [58], but subsequent studies suggested a role in many regulatory secretion processes [59-62]. The cytosolic domain of VAMP-8 did not have a dominant negative effect on platelet secretion [45]. More conclusive studies by Ren *at al.* [46] clearly showed that VAMP-8 is required for platelet secretion. In comparative analysis of platelets from VAMP-3$^{-/-}$, VAMP-2$^{+/-}$, VAMP-2$^{+/-}$ /VAMP-3$^{-/-}$, and VAMP-8$^{-/-}$ mice, only those lacking VAMP-8 had attenuated secretion. Permeabilized VAMP-8$^{-/-}$ mouse platelets further treated with Tetanus toxin showed no release; all three secretion events were totally abolished. These findings indicate that VAMP-8 is required for normal granule secretion though VAMP-2 and -3 do appear to compensate, in a less efficient manner, when VAMP-8 is absent. This suggested that perhaps there is a hierarchy in VAMP usage with VAMP-8 being the primary v-SNARE required for rapid and efficient release and VAMP-2 or-3 play a secondary role. This ranked redundancy of v-SNARE usage has been seen in neuroendocrine chromaffin cells from the studies of VAMP-2 KO mice [55]. Alternatively, this differential usage could imply distinct fusion steps leading

to release: primary granule-plasma membrane *vs.* granule-granule (compound) fusion

Studies by Jonnalagadda and co-workers (Jonnalagadda *et al*, in preparation) suggest that granule cargo release is best described as a mixture of single and compound fusion processes that have distinct rates. Clearly more experimentation will be required to understand the precise kinetics of VAMP usage and granule secretion. That said, it is clear that among the four VAMPs (-2, -3, -7, -8), VAMP-8 is the primary v-SNARE required for platelet secretion and VAMP-2 and -3 play some less efficient compensatory role. It is possible that such ranked redundancy in SNARE usage might be common for many important physiological processes.

Platelet t-SNAREs

Fusogenic SNARE complex in platelets is thought to be formed by one v-SNARE and two different t-SNAREs, which include a SNAP-25-type and a syntaxin-type. Although reports indicate that all three SNAPs (SNAP-23, -25, -29) [5, 63] are present in platelets, we have only detected SNAP-23 in significant quantities. This family of t-SNAREs does not contain classical TMD's but instead is anchored in the membrane *via* thioester linked palmitates attached to a series of cysteines between the two SNARE motifs. Perhaps between of the type of membrane anchor, SNAP-23 rapidly relocalizes to lipid rafts upon platelet activation (Karim *et al*, in preparation). Studies using inhibitory antibodies and peptides in permeabilized platelets suggest that SNAP-23 is required for all three granule secretion events [40, 41, 44, 45, 63-65]. Co-immunoprecipitation experiments indicate that SNAP-23, in platelet extracts, can associate into complexes with most of the platelet syntaxin (-2, -4, -11) and with at least VAMP-3 and -8. SNAP-23 is phosphorylated upon activation, suggesting SNAP-23 might be an important link between the platelet activation signaling event and the regulatory secretion process [45, 66]. A study by Polgar *et al* [45], revealed that human platelet SNAP-23 is phosphorylated at Ser23/Thr24 and Ser61 after platelet activation by thrombin. In murine mast cells and platelets, SNAP-23 is phosphorylated at Ser95 or Ser120 [66]. SNAP-23 is also a substrate for calpain in activated platelets. Calpains are calcium-dependent, non-lysosomal, neutral, cysteine proteases present in most mammalian tissues. Several inhibitors, *i.e.* calpeptin, calpastatin, and E64d, block

SNAP-23 cleavage in heavily activated platelets [67, 68]. It is not clear what role SNAP-23 cleavage plays since calpain inhibitors had limited effects on release of soluble cargo. Given the caveats of the described experiments, it seems reasonable to conclude that SNAP-23 is important for platelet secretion. Studies with knockout murine strains or humans with SNAP-23 defects will be required to confirm this conclusion. However, global deletion of SNAP-23 is embryonic lethal and attempts to make condition deletion strains have been unsuccessful [69] (Dr. Roche, NCI, personal communication with Dr. Whiteheart).

In addition to the SNARE motif, syntaxins contain an additional N-terminal helical domain (the Habc domain), which is thought to serve a regulatory and/or auto-inhibitory function. Initial studies focused on syntaxin that had predominantly plasma membrane localization in nucleated cells. Syntaxin-2 and -4 were first detected in platelets but syntaxin-1, -3, and -5 were not [5, 64]. Both syntaxin-2 and -4 had been shown to be involved in other secretion events [38, 70-72] and studies using permeabilized platelets suggested that syntaxin-2 was important for all three granule release events while syntaxin-4 played a role in only alpha granule and lysosome release [40, 41, 44]. These early data suggested that syntaxin-2 and -4 was important for platelet secretion. Strikingly, platelets from mice lacking either syntaxin-2 and -4 alone or together showed no secretion defect [Chapter 3, this thesis]. As with the studies with VAMPs, these conflicting data showed that syntaxin-2 and -4 are not required for secretion from mouse platelets. As shown for the VAMPs, using antibodies as inhibitors can lead to ambiguous results. Considering that the syntaxin-2 polyclonal antibody used as an inhibitor in the permeabilized platelets [40, 41] was generated against full length recombinant protein, it is possible that this antibody was not as isoform-specific as was thought. The experiments in this thesis directly address the question of which syntaxin isoform(s) is/are required for platelet secretion.

Syntaxin-7 is localized to an intracellular compartment instead of the plasma membrane in nucleated cells [38] and thus was not considered a likely candidate to mediate platelet exocytosis. Although syntaxin-7 was found in platelets over 10 years ago [40], the role of syntaxin-7 is still unclear. Introducing specific syntaxin-7 antibodies into permeabilized platelets had no effect on any of the three granule secretion events

[42]. This is the only data to suggest that syntaxin-7 is not required for platelet exocytosis.

Finally, proteomics studies were the first to show that syntaxin-11 is present in platelets [73]. Syntaxin-11 is a unique member of syntaxin family which has the Habc and SNARE motifs but lacks a classical TMD. It appears to be anchored to membranes *via,* yet to be defined modifications of its C-terminal cysteine residues [74] [37]. Syntaxin-11 is associated with late endosomes and the *trans*-Golgi network in Hela cells [37]. Interestingly syntaxin-11 mutations are associated with Familial Hemophagocytic Lymphohistiocytosis type 4 (FHL4). FHL4 is a rare autosomal recessive disorder of immune dysregulation associated with uncontrolled T cell and macrophage activation. Patients usually present with fever, hepatosplenomegaly, and cytopenias. Syntaxin-11 deficient NK-cells exhibit a defect in cytotoxic granules degranulation .ndicating that syntaxin-11 could be involved in fusion to the plasma membrane. However, other studies show that activation of syntaxin-11 deficient T-cells with IL-2 does restore degranulation [75, 76]. Comparative analysis of wild type and syntaxin-11 deficient CD8^{+} T-cells suggested that syntaxin-11 affects the granule-granule fusion step before final fusion with the plasma membrane [76]. These data suggest that syntaxin-11 is important for T-cell degranulation although its mode of action is not clear. Moreover, syntaxin-11 also has been shown to play a negative regulatory role in macrophage [77]. Syntaxin-11 regulates Vti1b-containing SNARE complex formation by directly binding to Vti1b (Qb-SNARE), thus controlling late endosome to lysosome fusion. Considering that cytotoxic T-lymphocyte (CTL) and natural killer (NK) cell require syntaxin-11 for membrane fusion and degranulation, this negative role for syntaxin-11 in macrophage suggests that the function of syntaxin-11 may be tissue- or SNARE-specific. Further studies are required to demonstrate the different roles of syntaxin-11 in these immune cells. Work in this thesis shows that syntaxin-11 is important for platelet exocytosis (Chapter 3).

SNARE Regulators in Platelets

Regulated platelet secretion happens only in respond to external signals, but how these signaling pathways are coupled to the SNARE machinery is still not clear. Based on the number of binding partners identified and the presence of the Habc domain, it has

been thought that most regulation of SNAREs is through the syntaxins. Very few VAMP and SNAP-23/25 regulators have been identified. In addition, post-translational modifications, such as phosphorylation of SNAREs and SNARE regulators could provide links from signaling to secretion. Several potential SNARE regulators have been identified in platelets and most are associated with t-SNARE heterodimers or t-SNARE containing complexes. We will discuss what is known about the following: Munc18b, Munc13, granuphilin, and tomosyn.

Munc18s

Munc18s belong to the evolutionarily conserved Sec1/Munc18 (SM) family, which is a critical component of the secretory machinery [30]. There are three mammalian Munc18 isoforms and all (Munc18a, b, and c) have been detected in human and murine platelets by immunoblotting and/or PCR [47, 63, 78]. It has been thought that different SM proteins are required for specific secretion steps through their interactions with specific syntaxins. Munc18a is almost exclusively expressed in neurons where it binding to syntaxin-1 while Munc18c is ubiquitous expressed in other tissues and appears to interact with syntaxin-4 [79]. In platelets, Munc18a was shown to interact with syntaxin-2, Munc18c with syntaxin-4, and Munc18b was found associated with SNARE complexes [47] and with syntaxin-11 (Al Hawas *et al*. in preparation). Early studies suggested that Munc18c phosphorylation and Munc18c/syntaxin-4 complex formation could be important for platelet secretion [63, 78]. Monoclonal antibodies that disrupted Munc18c/syntaxin-4 complexes had a positive effect on release from permeabilized platelets [63]. Further, introduction of peptides, based on conserved regulatory domain of Munc18s, peptides into permeabilized platelets inhibited Ca^{2+}-stimulated release from all three granule release events [47]. These *in vitro* studies suggest that Munc18s was important for platelet secretion. Surprisingly, platelets from Munc18c heterozygous mice (with a ~50% reduction in Munc18c) exhibited normal stimulus-induced release [80]. Double deletion of Munc18c died either in utero or within 6 hours after birth [81]. Based on that the syntaxin-4 level is reduced ~50% in Munc18c heterozygous mouse platelets, it is consistent with Munc18c being the chaperone for syntaxin-4. The association between Munc18c and syntaxin-4 is required for the stability of both proteins [47, 80]. Although

in vitro studies [47] suggest Munc18c plays a role in platelet secretion, the lack of secretion defect could be attributed to functional compensation by other Munc18s. Munc18a levels are low in human and murine platelets and though it is phosphorylated and binds syntaxin-2 [47, 63], it is not clear if it plays any role in exocytosis. The most promising candidate for a functional Munc18 in platelets is Munc18b. Munc18b is the most abundant isoform and it was found to interact with syntaxin-11. Munc18b was also detected in pull-down experiments using SNARE complexes [47] and t-SNARE heterodimers (Figure 4-1A and Table 4-1). Most importantly, Sandrock *et al* [50] and our unpublished data (Al Hawas *et al*. in preparation) showed that Munc18b deficient platelets from Familial Hemophagocytic Lymphohistiocytosis type 5 (FHL5) patients have a robust secretion defect suggest that Munc18b is required for platelet secretion. The fact that heterozygous patients have a partial secretion defect might suggest that Munc18b is a limiting factor in the secretion process remains to be tested. Syntaxin-11 levels were affected in these patients.

Rab Proteins

The Rab subfamily of small Ras-like GTPase includes over 60 members in mammals and regulates intracellular membrane traffic [82]. Several Rab proteins have been identified in platelets, such as Rab3B, Rab4, Rab6, Rab8, and Rab27 [83-86]. It is generally thought that different Rabs regulate different secretion routes through associations with specific effectors. It has been shown that Rab6 and Rab8 are preferentially targeted to the plasma membrane and α-granules [83]. Studies using a dominant negative mutant of Rab4 suggested that Rab4 regulates α-granule but not dense granule secretion [85]. Biochemical studies have shown that the majority of Rab27 is associated with dense granules while a small portion is located on the plasma membrane [49]. Rab27ab are involved in both dense granule biogenesis in megakaryocytes and in platelet secretion. Deficiency of Rab27a in *ashen* mice exhibits "ghost" dense granules (with normal number but defective contents) [84] while loss of Rab27b shows around 50% reduction in both dense granule number and serotonin content [86] with normal α-granule contents and secretion. This data suggest that the Rab27 family is critical for dense granule biogenesis, but not important for α-granule and lysosome biogenesis.

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Further Rab27 double KO murine platelets display more severe defect in serotonin secretion than single Rab27b KO, indicating that Rab27a can partially compensate for dense granule secretion [86]. The exact role of Rab27 in dense granules remains unclear. Presumably the distinct functions of Rab27 require different effectors. The fact that one of Rab27b effectors, Munc13-4, is required for platelet secretion [48, 49] could explain why Rab27b plays a role in exocytosis. It is also possible that Rab27s interacts with unknown factors, which are important for dense granule package and trafficking. On the other hand, Munc13-4 is not required for granule biogenesis since there is no granule defects observed in Munc13-4 depleted murine platelets [48]. Further exploration of the mechanisms for Rab27 function in platelet secretion will clarify whether different granule secretion requires specific sets of Rab proteins, effectors, or even secretory machinery components in platelets.

Munc13s

The Munc13 family has four members: Munc13-1, -2 and -3 are expressed in neuron tissue; Munc13-4 is ubiquitously expressed. Munc13s are widely believed to be key vesicle priming factors. The strongest evidence is from genetic ablation of both Munc13-1 and Munc13-2 in neurons. This completely abolishes synaptic vesicle priming and release [87, 88]. Munc13-4 was first identified in platelets as a Rab27a binding partner [49] and is essential for platelet secretion. Mice lacking Munc13-4 have a severe bleeding diathesis and the release from dense granules is totally abolished while release from α-granules and lysosomes is severely impaired [48]. This shows that Munc13-4 mediates at least one step that is uniquely required for rapid dense granule release, a step that is also critical for hemostasis *in vivo*. The residual secretion from α-granules and lysosomes could be due to compensation by another Munc13 family member considering that both Munc13-1 and Munc13-4 can interact with Munc18a/syntaxin-2 complex in platelets (Figure 4-8). Compared to other Munc13s, Munc13-4 does not contain a diacylglycerol-binding C1 domain, but does have two calcium/phosphatidylserine-binding C2 domain (C2A and C2B) and Munc Homology Domains (MHD). Mutations in the MHD domain or deletion of C2B result in FHL3 in humans [89]. Recombinant Munc13-4 lacking C2B potently inhibits platelet secretion from permeabilized cells [48]

suggests that both the MHD domain and C2B domain are important for its function. It is possible that the MHD domain is required for interactions with important secretory machinery components while C2B is important for anchoring to the plasma membrane or regulating membrane fusion in response to Ca^{2+} signaling. Identification of key Munc13-4 interacting partners will be an important step in understanding the SNARE complex formation and membrane fusion upon stimulation. Clearly Rab27 is a possible candidates as is another Rab27 effector called granuphilin since they are present in platelets.. Another possible candidate Munc13-4 effector is DOC2α because it have been shown to interact with other Munc13 isoforms. In summary, Munc13-4 is an essential component in dense granule secretion pathway. Further exploration of its mechanism will not only identify new elements of the secretory machinery, but also clarify whether secretion of different granules requires specific sets of secretory machinery in platelets.

Synaptotagmin-Like Proteins (Slps):

Slps possess a C-terminal synaptotagmin-like domain with C-terminal tandem C2 domains (putative Ca^{2+}-dependent phospholipid binding sites) and an N-terminal Rab27-binding domain. There are five Slp isoforms (Slp1 to 5) in mammals [90]. Slp1 and Slp4 (or granuphilin) are present in platelets [91]. Studies by Neumuller *et al* [91] demonstrated that purified Slp1 can partially inhibit dense granule secretion from permeabilized platelets. Endogenous Slp1 forms a trimetric complex with Rap1GAP2 and Rab27, suggesting Slp1 is a Rab27 effector that regulates dense granule secretion. Though little is known about granuphilin in platelets, it has been well studied in other secretory cells. Granuphilin is abundantly expressed in beta cells and considered to be essential for the stable attachment of insulin granules to the plasma membrane where it acts by linking Rab27a on the granule membrane with the syntaxin-1a/Munc18a complex on the plasma membrane [92]. Loss of granuphilin causes a decrease in docked granules in these cells [93]. Granuphilin can bind to both syntaxin-1a and Munc18a [94-96]. The enhanced insulin secretion from granuphilin deficient beta cells [97], despite the docking defect, argues for it being a negative regulator. Granuphilin could have a negative effect on a step just prior to the final fusion reaction. It is possible that a local signal such as increased $[Ca^{2+}]_i$ after stimulation may cause dislocation of granuphilin from the

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Munc18a-syntaxin-1a complex and allow SNARE assembly. This is consistent with the ability of both Slp and Munc13 families binding Rab27 and containing C-terminal Ca^{2+} binding C2 domains.

Tomosyn-1

Tomosyn-1 belongs to a family of WD-40 repeat containing proteins associated with exocytosis including Sro7/Sro77 in yeast, Tom1 in *c.elegans*, amisyn and lethal giant lavea (lgl) in mammalian [98-101]. Two genes, tomosyn-1 and tomosyn-2, were found in mammalian but alternative splicing can produce a total of seven isoforms [102, 103]. Little is known about the exact role of tomosyn-2 in secretion [102] while tomosyn-1 is well studied in neuron and endocrine cell exocytosis. Tomosyn-1 has three isoforms (b-, m-, s-) and has several predicted phosphorylation sites (Figure 1-3). m-tomosyn-1 was originally identified as a syntaxin-1 binding partners from rat brain tissue cell [104]. The s-tomosyn-1 and m-tomosyn-1 isoforms are enriched in synapse-specific domains while b-tomosyn-1 is ubiquitously expressed [105]. Structurally, mammalian tomosyn-1 contains an N-terminal WD40 repeat, a small highly variable linker region, and a C-terminal R-SNARE (v-SNARE) motif [103]. Both the R-SNARE motif and N-terminal WD40 repeat have been shown to inhibit secretion from PC12 cells [106] and neurons [107]. Thus tomosyn-1 has been thought as a negative regulator for neurotransmitter release events. Consistently, deletion of tomosyn-1 enhances secretion [107]. Over-expression of tomosyn-1, in PC12 cells, reduces the ready-release pool and resident time of secretory vesicles [108]. This observation is consistent with the hypothesis that tomosyn-1 functions by disrupting vesicle priming. It has also been suggested that the N-terminal WD-40 domain may catalyze the formation of nonfunctional SNARE complexes [109] or through its loop region by interactions with unknown factors [110]. Alternatively, the SNARE domain could block v-SNARE binding to t-SNAREs, thus inhibiting membrane fusion and exocytosis. Post-translational modifications such as phosphorylation have been suggested to regulate this process [111]. Despite these data, the exact mechanism of tomosyn-1 as negative regulator is still unclear.
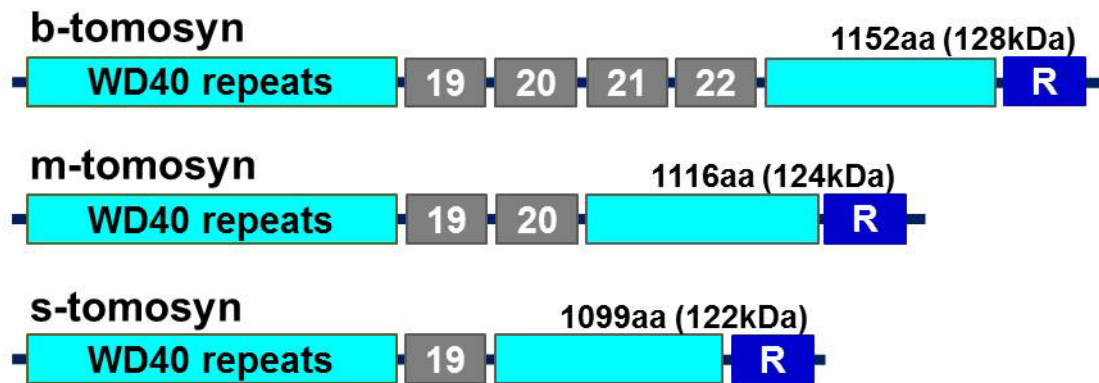
Figure 1-3. Tomosyn-1 Isoforms in Rat and Human. The domain structure of the three isoforms of Tomosyn-1. b-tomosyn-1 is a full length isoform. m-tomosyn-1 skips exon 20 and 21, and s-tomosyn-1 skips exon 20-22. The WD40 repeats are in cyan, R-SNARE coiled-coil motif is in blue, and variable region is in gray. The bottom panel shows the predicted phosphorylation sites in the variable region. The labels represent sites of phosphorylation by PKA as A, PKC as C, PKG as G, casein kinase II as K, GSD3 and DNAPK as X.

Although data supports a negative role for tomosyn-1, it has also been shown to function permissively in exocytosis. Over-expression of tomosyn-1 in SCG neurons enhances release [111]. The long term potential (LTP) of mossy fiber without tomosyn-1 is not inhibited. Moreover, depletion of both Sro7/Sro77 [100] inhibits secretion while a deletion of tomosyn-1 in β-cell causes a secretion defect [112]. In light of our observation that tomosyn-1 can form a complex with the t-SNARE heterodimers, Munc18C [113], and Munc18b [Figure 4-1], tomosyn-1 could control secretion by activating t-SNARE complex and thus facilitating t-/v-SNARE complex formation. Experiments in this thesis address the role of tomosyn in platelets.

Cytoskeletal Polymerization and Platelet Secretion

Upon vascular damage, human platelets become activated and dramatically change their shape through cytoskeleton rearrangement. This precedes granule centralization and content release mediated by membrane fusion [114]. Disruption of the actin cytoskeleton by latrunculin A or inhibition of actin polymerization with cytochalasin E [115] inhibits alpha-granule secretion induced by different agonists. This connects actin polymerization with platelet secretion. In a recent paper, Woronowicz *et al*. [115] show that the actin cytoskeleton can associate with t-SNAREs and actin polymerization is important for alpha-granule secretion. Further interactions between the platelet cytoskeleton and the secretory machinery are yet to be identified.

Platelet Activation and Secretion

Platelet activation occurs through signaling cascades [116]. Different agonists bind to the diverse platelet surface receptors, ultimately leading to common intracellular signaling events that activate a phospholipase C (PLC). Activated PLC catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate to release inositol trisphosphate (IP_3) and diacyglycerol (DAG), which activate calcium mobilization and protein kinase C (PKC), respectively. The hallmark of platelet activation is activation of integrin $\alpha_{IIb}\beta_3$ to mediate aggreagation [116-118]. Platelet secretion is also one of the end steps of these signaling cascades, but how they are connected and how secretion is regulated is still not clear. One hypothesis is that post-translational modification plays an important role.

Phosphorylation, S-nitrosylation, or sumoylation of SNARE proteins or their regulatory proteins has been suggested to be important. Another possibility is that secondary signal molecules, such as calcium, may remove inhibitory proteins or activate SNAREs. Calcium is clearly an important factor in the activation of the secretory machinery, and it alone can initiate release from permeabilized platelets. Calcium influx can also regulate actin-binding proteins and caused dissolution of the cortical F-actin, which can affect granule localization and and give granules freer access to exocytic sites on the plasma membrane. Depolymerization of cortical F-actin or polymerization of F-actin, or myosin Va might mediate the recruitment of secretory granules near the plasma membrane (OCS) since granule centralization is observed.

Thesis Overview

Understanding the molecular mechanism of platelet exocytosis requires the identification and characterization of the important core SNARE proteins and their regulatory molecules. The demonstration that VAMP-8 is the primary v-SNARE required for platelet secretion and that platelet secretion is mediated by SNARE complexes, led to the central hypothesis of this thesis; platelet secretion and hemostasis are controlled by primary t-SNAREs and t-SNAREs regulators. The focus of this thesis is to determine the primary t-SNARE(s) required for platelet secretion and the t-SNARE regulating proteins. The answer to this question will not only define the core SNARE components required for platelet secretion, but also expand the possible ways to regulate platelet function and hemostasis.

The original anti-syntaxin-2 polyclonal antibody was found to cross react with syntaxin-11 and it also immunoprecipitated syntaxin-11 from platelet extracts. This partially explains why syntaxin-2 antibodies inhibited permeabilized platelet secretion while syntaxin-2/4 double KO mice have no secretion defect. The syntaxin-11 deficient platelets from a FHL4 patient showed significant defects in dense granule and α-granule secretion while lysosome secretion was partially. The aggregation defect from these same platelets is likely caused by an ADP release defect, which is required for a second wave of aggregation through activation of integrin $\alpha_{IIb}\beta_3$. Taken as a whole these data show that syntaxin-11 is required for platelet secretion.

A specific role for tomosyn-1 in platelets has also been studied. Tomosyn-1 was first identified in platelets by mass spectrometry as a specific binding partner of recombinant t-SNARE complexes [Chapter 4]. Additionally, we showed that tomosyn-1 is phosphorylated and associated with SNAP-23 in both resting and activated platelets. The role of endogenous tomosyn-1 has been investigated using tomosyn-1 KO mice. Tomosyn-1 deficient platelets have a normal aggregation phenotype, but a partial secretion defect. The partial nature of the defect suggests that other members from tomosyn-1 family may play a compensatory role when tomosyn-1 is missing. Most interestingly, tomosyn-1 deficient mice display a robust bleeding phenotype in two *in vivo* thrombosis models: prolonged bleeding times and impaired thrombosis tail bleeding and $FeCl_3$ carotid injury. These data demonstrate tomosyn-1 is important for hemostasis and thrombosis.

Chapter Two
Materials and Methods

Materials

Antibodies

Anti-syntaxin-2, -4, -7, and SNAP-23 polyclonal antibodies were generated in the Whiteheart laboratory as described previously [40, 41]. Anti-syntaxin-11 rabbit polyclonal antibody (110113) was purchased from Synaptic System GmbH (Gottingen, Germany) while murine monoclonal antibody (611282) was obtained from BD Transduction Laboratories (San Jose, CA). Anti-Munc18a (clone131.1) was from Synaptic System (Goettingen, Germany). Anti-Munc18b antibody (sc-14563) was from Santa Cruz Inc. (Santa Cruz, CA). Anti-Munc18c polyclonal antibody was produced in the Whiteheart laboratory as described previously [47]. Anti-synaptobrevin/VAMP-2 monoclonal antibody (CL69.1), anti-endobrevin/VAMP-8 polyclonal antibody, anti-rat-Munc13-1 monoclonal antibody (CL266B1), and anti-cellubrevin/VAMP-3 polyclonal antibody were generous gifts from Dr. Jeffery Pessin (Albert Einstein College of Medicine). Anti-TI-VAMP/VAMP-7 monoclonal antibody (TG158.2) was a generous gift from Dr. Thierry Galli (Institut Pasteur, Paris, France). Polyclonal rabbit-anti-human VAMP-8 antibody were generated in our laboratory using recombinant versions of the cytoplasmic domain of human VAMP-8 (1-73 aa) and the Longin domain of human VAMP-7 (1-120 aa) as antigen, respectively. Anti-RabGDI polyclonal antibody was generated in our laboratory by Dr. Tara Rutledge using recombinant RabGDIα as antigen. Anti-tomosyn-1 monoclonal antibody (611296) was purchased from BD Transduction Laboratories (San Jose, CA) while anti tomosyn-1 polyclonal antibody (H-55, sc-98350) was from Santa Cruz Inc. (Santa Cruz, CA). Anti-Munc13-4 monoclonal antibody (designated as Munc13-4 mAb C) was also generated using the domain C as antigen as generous gift from Dr. Christian Wimmer (Basel Institute for Immunology, Basel, Switzerland). Anti-human PF4 monoclonal antibody was obtained from R&D Systems (R&D Systems, Minneapolis, MN). Anti-human LAMP-1 monoclonal antibody was obtained from the Developmental Studies Hybridoma Bank (University of Iowa, Iowa City, IA). Anti-fibrinogen monoclonal antibody was purchased from Innovative

Research (Southfield, MI). The monoclonal anti-phospho-tyrosine (pY) antibody (4G10) was from Upstate (Lake Placid, NY). Fluorescein isothiocyanate (FITC)-conjugated PAC-1 (recognizes activated αIIb/β3 complex) and phycoerythrin (PE)-conjugated anti-human CD41a (integrin αIIb) monoclonal antibody were purchased from BD Biosciences (San Jose, CA). FITC-labeled anti-human P-selectin monoclonal antibody (AK-4), FITC-conjugated PECAM1 (553372), and FITC-conjugated anti-murine P-selectin (553744) were from BD Pharmingen[TM] (BD Bioscience, San Jose, CA). FITC-conjugated anti-murine GPVI (M011-1), FITC-conjugated GPIbbeta (M050-1), PE-conjugated GPIIb/IIIa (M023-2, active form) were purchased from Emfret Analytics (Germany). Anti-phosphotyrosine (clone 4G10) monoclonal antibodies were purchased from Upstate Biotechnology (Lake Placid, NY). Anti-integrin β3 rabbit polyclonal antibody was obtained from Cell Signaling (Danvers, MA). For the sandwich Enzyme-Linked ImmunoSorbent Assay (ELISA) of human and murine PF4, two sets of paired antibodies were used: for human PF4, a monoclonal anti-human PF4 antibody was used as the capture antibody (R&D systems) while a biotin conjugated rabbit-anti-human PF4 polyclonal antibody was used as the detection antibody (Leinco Technologies Inc., St. Louis, MO); for murine PF4, a monoclonal, anti-murine PF4 antibody (R&D systems) was used as the capture antibody to bind murine PF4 while a biotin conjugated rabbit-anti-murine PF4 antibody was used as the detection antibody (R&D systems). Alkaline phosphatase conjugated secondary anti-murine, anti-rabbit, anti-sheep, and anti-goat IgGs were from Sigma. FITC or TexasRed conjugated anti-murine, anti-rat and anti-rabbit IgGs were purchased from Vector Laboratories (Burlingame, CA). HRP conjugated streptoavidin was from R&D systems.

General Reagents

Apyrase, benzamidine, poly-D-lysine, type I bovine plasma fibrinogen, glutathione-conjugated agarose, 2-methyl-2-butanol, 2,2,2, tribromoethanol, tetramethyl rhodamine isothiocyanate (TRITC)-conjugated phalloidin, and Ferric chloride (FeCl$_3$) were obtained from Sigma (St Louis, MO). *Ortho*-[^{32}P]-phosphoric acid was purchased from MP Biochemicals Inc. (Irvine, CA). HisProbe[TM]-HRP and BCA[TM] Protein Assay kit were purchased from Pierce (Rockford, IL). Complete, EDTA-free protease inhibitor

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cocktail was obtained from Roche (Indianapolis, IL). Acid citrate dextrose (ACD) blood collection tubes (BD Vacutainer®) were purchased from BD Diagnostics (Sparks, MD). Formaldehyde (37%) and glutaraldehyde (70%) were purchased from Electron Microscopy Sciences (Hatfield, PA). Type I collagen (equine tendon), human plasma thrombin, and ADP were purchased from Chrono-log (Havertown, PA). Convulxin, a snake venom toxin, was purchased from Centerchem Inc. (Norwalk, CT). A23187, Ca^{2+} ionophore, was obtained from Calbiochem (San Diego, CA). Thrombin-activated receptor peptide (known as PAR4 peptide, AYPGKF) was purchased from Anaspec (San Jose, CA). Whatman filter paper (VWR, San Francisco, CA), 1 mm disc shape was used.

Methods

Murine Strains, Genotyping, and FHL4 Patient

Epimorphin/Syntaxin-2$^{-/-}$ Mice (Syntaxin-2 KO Mice)

Epimorphin/Syntaxin-2$^{+/-}$ mice on a C57/BL6 background were kindly provided and generated by Dr. Deborah C. Rubin (Washington Univ. School of Medicine) [119]. The mutant mice were maintained by crossing with C57/BL6 mice. Murine genomic DNA was extracted from murine tail. Genotyping was performed using primers:

For the wild type gene:

forward primer: 5'-GACAGTGTGTTAACTAGTCAAGC-3'

reverse primer: 5'TTAGATTCCCTCCTAGAGACGC-3'

For the mutated gene:

forward primer: 5'-GACAGTGTGTTAACTAGTCAAGC-3'

reverse primer: 5'-GGACACGCTGAACTTGTGGC-3'

PCR conditions were as follows: 1 cycle at 94 °C for 7 min, then 35 cycles at (94 °C for 1 min, 60 °C for 0.5 min, 72 °C for 3.5 min) and 1 cycle at 72 °C for 10 min.

Syntaxin-4$^{flox/flox}$/PF4-Cre$^+$ Mice (Syntaxin-4 KO Mice)

Syntaxin-4$^{flox/flox}$ mice on a C57/BL6 background were kindly provided by Dr. Jeffery Pessin (Albert Einstein College of Medicine). Genotyping was carried out with two sets of reactions.

For the LoxP site:

forward primer, 22F: 5'-GTTGAGGCAGGTGAGAAACC-3'

reverse primer, 25R: 5'-ATCACCAAGAGGGCTGAAGA-3'.

PCR conditions were as follows: 1 cycle at 94 °C for 7 min, then 30 cycles at (94 °C for 1 min, 60 °C for 1.0 min, 72 °C for 1.5 min) and 1 cycle at 72 °C for 10 min. For the neo cassette:

forward primer, 58F: 5'-AGCCCAGTTGCTGGTGTATC-3'

reverse primer, 55R: 5'-AGGAGGAAGAGGTGGAGGAG-3'.

PCR conditions were as follows: 1 cycle at 94 °C for 7 min, then 30 cycles at (94 °C for 1 min, 60 °C for 1.0 min, 72 °C for 1.5 min) and 1 cycle at 72 °C for 10 min.

PF4-Cre[+] mice were kindly provided by Dr. Radek Skoda (University Hospital Basel, Switzerland). Genotyping was performed using the following primers. For the wild type gene:

forward primer, 5'-CCCATACAGCATACCTTTTG-3'

reverse primer, 5'-GAAACAACAGGCCCAGAAGC-3'

For the Cre transgene:

forward primer, 5'-CCCATACAGCATACCTTTTG-3'

reverse primer, 5'- TGCACAGTCAGCAGGTT-3').

PCR conditions were as follows: 1 cycle at 94 °C for 7 min, then 30 cycles at (94 °C for 1 min, 50 °C for 1.0 min, 72 °C for 1.5 min) and 1 cycle at 72 °C for 10 min.

Syntaxin-4 platelet specific syntaxin-4 deletion mice (syntaxin-4 KO mice) were generated by a cross of the above two murine strains.

Syntaxin-2^{-/-}/Syntaxin-4[flox/flox]/Cre[+] (Syntaxin-2/4 Double KO Mice)

Syntaxin-2/4 double KO mice were generated by crossing above three strains: syntaxin-2 KO mice, Syntaxin-4[flox/flox] mice, and PF4-Cre[+] mice.

Tomosyn-1^{-/-} Mice

Heterozygous Embryos of tomosyn-1 were kindly provided by Dr. Yoshimi Takai (Kobe University Graduate School of Medicine, Japan) and Dr. Jun Miyoshi (Osaka Medical Center, Japan). The genetic background of tomosyn-1 knockouts is the mixture of 129 50%, C57BL6 25% and DBA2 25%. The Embryos were

recovered into live mice by the Jackson Laboratory. Genotyping of tomosyn-1 KO mice was performed by PCR using primers: for KO mice:

forward primer, 5'-GGGCGCCCGGTTCTTTTTGTC-3'

reverse primer, 5'-GCCATGATGGATACTTTCTCG-3'

for wild type mice:

forward primer, 5'-TTCTGCTCCCCGCTGCTCCTT-3'

reverse primer, 5'-TCCCCGCTCCCTTCACCTTGC-3'.

PCR conditions were as follows: 1 cycle at 94 °C for 7 min, then 40 cycles at (94 °C for 1 min, 57 °C for 1 min, 72 °C for 1.5 min) and 1 cycle at 72 °C for 10 min. The expected PCR products are 224 bp for tomosyn-1 KO and 300 bp for the wild type.

All procedures and usage of mice were approved by the DLAR at the University of Kentucky.

Syntaxin-11 Deficient Platelets from FHL4 Patient

15 mL of whole blood was collected from a FHL4 patient who displayed bleeding problems and a profound defect of NK cell degranulation (Dr. Filipovich Cincinnati Children's' Hospital, personal communication with Dr. Whiteheart). The platelets from the patient were isolated using standard procedures. The genotype of the patient was determined by standard sequencing methods: Allele 1 - 173T>C (D58R), Allele 2 - 173T>C (D58R). All procedures were approved by the IRBs at the Cincinnati Children's Hospital and at the University of Kentucky.

Platelet Preparation from Mice and Human Blood

Mice were euthanatized by CO_2 inhalation. After the heart was exposed, a 1 mL syringe filled with ~100 μL of 3.8% sodium citrate and attached to a 26 G needle was used to collect blood from the right ventricle.

In order to accurately measure the individual murine platelets and erythrocytes, whole blood without dilution from syntaxin KO mice and wild type mice were counted with Hemvet (DREW Scientific Inc., Oxford, Connecticut) for all three blood cells at the same time.

The citrated blood was then mixed with an equal volume of PBS and adjusted to 0.38% sodium citrate final concentration. Human blood were collected into Acid citrate dextrose (ACD) blood collection tubes and then diluted with an equal volume of PBS. Both murine and human blood was first centrifuged at $250 \times g$ for 10 minutes (min) at RT. The Platelet Rich Plasma (PRP) was carefully removed (avoiding the buffy coat between the PRP and the red blood cell layer). After adding 100 ng/mL prostaglandin I_2 (PGI_2, Sigma) for 5 min at RT, the platelets were harvested by centrifugation at $900 \times g$ for 15 min. The pellet was gently resuspended in HEPES/Tyrode's Buffer (10 mM HEPES/NaOH, pH 7.4, 5.56 mM glucose, 137 mM NaCl, 12 mM $NaHCO_3$, 2.7 mM KCl, 0.36 mM KH_2PO_4, 1 mM $MgCl_2$) containing 0.3 U/mL apyrase (Sigma).

Measurement of Secretion from Intact Murine Platelet

Washed platelets were labeled with 2 μCi/mL [^{3}H]-5-HT (Perkin-Elmer Cetus Life Sciences, Boston, MA) for 45 min at 30°C. After washing twice with HEPES/Tyrode's Buffer (pH 7.4) in the presence of 0.3 U/mL apyrase, the platelets were resuspended with HEPES/Tyrode's Buffer (pH 7.4). Platelet concentration was determined by Z2 Coulter particle counter and size analyzer (Beckman Coulter, Fullerton, CA) and adjusted to 2.5×10^8 platelets/mL. A final concentration of 0.7 mM $CaCl_2$ was added to the platelet suspension before stimulation.

For thrombin titrations, a concentration series of thrombin (Chrono-Log, Havertown, PA) was added to stimulate platelets (50 μL of resuspension) at RT. Reactions were stopped by adding a twofold excess of hirudin (Sigma) and then placed on ice. For the time course experiments, 0.05 U/mL of thrombin was added for the indicated time periods, and 0.1 U/mL of hirudin was added to stop the reaction. When all reactions were finished, the samples were subjected to centrifugation at $13,000 \times g$ for 1 min. The supernatant was recovered to another tube. The pellet was lysed with an equal volume of lysis buffer (PBS, pH 7.4, 1% Triton X-100) for 1 hr on ice. An equal volume of the supernatant and the pellet was assayed for the three granule cargo markers: [^{3}H]-5-HT for dense core granules, PF4 for α-granules. and β-hexoaminidase for lysosomes, as described previously [53]. For the dense core granule release assay, 25 μL of the supernatant or the pellet was added to 3 mL of scintillation cocktail solution. The

samples were analyzed using a Tri-Carb 2100TR liquid scintillation analyzer (Beckman, Fullerton, CA). For α-granule secretion, a sandwich ELISA method was used to detect the specific α-granule marker, PF4. This ELISA assay was performed according to the manufacturer's instruction (R&D systems). To assay for lysosomal secretion, the enzymatic activity of β-hexosaminidase was measured. Six microliters of supernatant and 3 μL of the pellet samples was added to 100 μL of citrate-phosphate buffer (53.4 mM citric acid, 93.2 mM Na_2HPO_4, pH 4.5) containing 10 mM p-nitrophenyl-N-acetyl-β-D-glucosaminide. The reactions were incubated at 37°C in a sealed, 96 well plate for 18 hrs and stopped with 100 μL of 0.08 N NaOH. The optical density of each well was measured at 405 nm using a Biotek Elx808 plate reader (BioTek Instruments Inc., Winooski, VT). The percent release for each marker at each data point was calculated by using the supernatant fraction divided by the supernatant plus the pellet fraction (total).

Platelet Aggregometry

Washed platelets or PRP were centrifuged at 13,000 rpm for 1 minute in a tabletop centrifuge to obtain platelet-poor plasma (PPP) which was used to set the baseline. Platelet suspension (500 μL) was warmed at 37°C in a siliconized glass cuvette (Chrono-log, Havertown, PA) containing a metal stir bar (Chrono-log) with stirring (800 rpm) using a Model 460Vs Lumi-Dual aggregometer (Chrono-log) for 2-3 minutes. Agonists were added to initiate platelet activation. Aggregation traces were monitored using a Model 810 Aggro/Link computer interface and Aggro/Link software (Chrono-log).

Platelet ATP Release

ATP release from platelet dense-core granules was monitored by the luciferin/ATP-mediated luminescence using an aggregometer. Washed platelets (475 μL) were preincubated with 25 μL of Chrono-Lume® reagent (Chrono-log) containing luciferin and luciferase for 2 minutes at 37°C. Addition of thrombin, collagen (Chrono-Log), or A23187 (Calbiochem, La Jolla, CA), initiated ATP release from platelets, ATP-driven activation of luciferase, followed by production of luciferin which were monitored by absorption at 560 nm. Luminescence traces along with aggregation were using the same interface and software.

Flow Cytometry Analysis

Washed murine or human resting or thrombin activated platelets (20 μL) at a concentration of 1×10^8 platelets/mL were incubated with 2.5 μL FITC-conjugated or PE-conjugated antibodies for 15 minutes at RT. Platelet samples were then fixed with 700 μL of 0.5% formaldehyde in phosphate-buffer saline (PBS) at RT for 30 minutes. Samples for flow cytometry were moved in a polystyrene Falcon™ tube (BD Biosciences, San Jose, CA). Fluorescent intensity was measured using FACScan™ flow cytometer (BD Biosciences). Platelet population was detected by adjusting the voltages for forward light scattering (FSC; E-01/linear) and side light scattering (SSC; 450/linear). Fluorescent intensity was optimized by adjusting the voltages for excitation of blue (FITC) and red (PE) channels. Platelet fluorescent intensities were monitored using an acquisition and analysis application, CellQuest™ (BD Biosciences). A total of 10,000 platelets were analyzed and fluorescent intensities were plotted as a histogram with statistical values.

Western Blotting and Quantification

Protein samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) [120, 121]. The separated proteins were transferred electrophoretically [122] to Immobilon-P polyvinylidene fluoride membrane (PVDF, Milipore, Bedford, MA) with constant voltage (100 V) for 1 hour. Blotted membranes were then blocked with blocking solution containing 5% nonfat milk in TBS-T (Tris-buffered saline: 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Tween-20 (Sigma)) containing 0.02% sodium azide (Thermo Fisher Scientific Inc., Waltham, MA) for 1 hour at room temperature. The blocking solution was replaced with primary antibody diluted into blocking buffer and membranes were incubated at room temperature for 1-2 hours or at 4°C overnight. Membranes were washed with TBS-T three times and incubated with AP-conjugated secondary antibody diluted into blocking buffer for 1 hour at room temperature. Following three washes with TBS-T, membranes were incubated with enhanced chemofluorescence (ECF) substrate (GE Healthcare, Piscataway, NJ) for 5 minutes. Membranes were covered with a piece of transparency film (3M, St. Paul, MN) and substrate fluorescences were detected using Typhoon™ 9400 Phosphoimager (GE

Healthcare). Quantification of bands was performed using ImageQuant 5.2 software (GE Healthcare).

Metabolic Labeling of Human Platelets

To metabolically label proteins in human platelets with o-$[^{32}P]$-phosphoric acid, PRP from banked human platelets was centrifuged at $900 \times g$ for 10 minutes at room temperature, followed by resuspension with phosphate-free HEPES-Tyrode's buffer (20 mM HEPES/KOH, pH 6.5, 128 mM NaCl, 2.8 mM KCl, 1 mM MgCl$_2$, 5 mM D-glucose, 12 mM NaHCO$_3$) containing 0.37 units/mL apyrase, 100 ng/mL PGI$_2$, and 1 mM EGTA in a final platelet concentration of 8×10^8 platelets/mL. The platelet suspension was labeled with 0.5 mCi/mL o-$[^{32}P]$-phosphate at 37°C for 1 hour. Labeled platelets were recovered by centrifugation at $900 \times g$ for 7 minutes and resuspended in an equal volume of HEPES-Tyrode's buffer (pH 7.4), the estimated final platelet concentration is approximately 4×10^8 platelets/mL (50% recovery of resuspension).

Immunoprecipitation of Platelet Proteins

Washed unlabeled or $[^{32}P]$-labeled platelets (500 µL) were warmed at 37°C for 5 minutes in either a heat block or in the aggregometer, followed by stimulation with the agonist, thrombin (0.1 U/mL) for 5 minutes. The reaction was stopped by adding equal volume of $2 \times$ ice-cold IP-lysis buffer (20 mM HEPES/KOH, pH 7.4, 128 mM NaCl, 9 mM MgCl$_2$, 2% Tx-100, 20% glycerol, 2 mM sodium orthovanadate, 2 mM benzamidine, 2 mM EGTA, 2× EDTA-free protease inhibitor cocktail) and the lysates were incubated for 30 minutes to 1 hr on ice. The insoluble cytoskeleton was removed by centrifugation at $13,000 \times g$ for 5 minutes and the supernatant (500 µL) was precleared with 10 µL Protein A or Protein G-Sepharose beads at 4°C for 30 minutes on the rotator. The beads were spun down at $500 \times g$ for 10 seconds and the clarified supernatant was incubated with 5 µg of antibody bound to 10 µL Protein A or Protein G-Sepharose beads at 4°C for 2 hours on the rotator. Non-specific binding was eliminated by three washes with 1 mL of 1× ice-cold IP-lysis buffer without protease inhibitor cocktail at $500 \times g$ for 10 seconds and the bound proteins were eluted with 2× SDS

sample buffer by boiling for 10 minutes. Proteins were resolved by SDS-PAGE, transferred to PVDF membrane, and then analyzed with a specific antibody by western blotting. For storage phosphor detection of [^{32}P]-labeled proteins, the resolved PVDF membrane was covered and then exposed to a storage phosphor screen (GE Healthcare) overnight. The screen was excited at 600 nm and detected the emission intensity peaked at 390 nm using Typhoon^{TM} 9400 Phosphoimager and analysis of bands was performed using ImageQuant 5.2 software. For detection of coimmunoprepitates, western blotting was performed using an antibody for the protein of interest.

Electron Microscopy of Platelets

Washed human or murine platelets were warmed in a 37°C metal block for 5 minutes. Platelets were either kept resting or stimulated with 0.1 U/mL of thrombin for 5 minutes. The platelets were then processed for electron microscopy as described previously [42, 46] with slightly modification. An equal volume of 0.1% glutaraldehyde in White's saline (120 mM NaCl, 5 mM KCl, 2.3 mM MgSO$_4$, 3.2 mM Ca(NO$_3$), 6.5 mM NaHCO$_3$, 0.42 mM Na$_2$HPO$_4$, 0.19 mM KH$_2$PO$_4$, 0.0005% phenol red) was added to the platelet suspension for 15 minutes at 37°C. The platelets were centrifuged at 3,000 × 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, the platelets were osmicated in 1% OsO$_4$ in White's solution. This modification was known to generate a stable fixation of granule contents and provides a better identification of dense-core granules osmicated platelets were washed twice with 0.1 M Sorenson's buffer (16.2 mg/mL KH$_2$PO$_4$, 3.76 mg/mL Na$_2$HPO$_4$, pH 8.0) and dehydrated in a serial ethanol washes (50%, 70%, 80%, 90%, 100%, and previously unopened absolute ethanol) for 5 minutes. The platelets were rinsed twice with propylene oxide and infiltrated overnight in a 1:1 mixture of propylene oxide and Spurr's resin (10g vinyl cyclohexane dioxide (VCD), 6g DER epoxy resin, 26g nonenyl succine anhydride (NSA) with final addition of 0.4g dimethylaminoethanol (DMAE)). After one wash in Spurr's resin for 1 hour, samples were embedded in 200 μL Spurr's resin and polymerized in a 60°C incubator for 48 hours. Polymerized blocks were sectioned and mounted on copper grids. Following counterstaining with uranyl acetate, samples were examined using a Philips Tecnai 12 transmission electron

microscope (FEI, Hillsboro, OR) and images were obtained with Gatan Digitalmicrograph software (Pleasanton, CA).

Platelet Membrane Fractionation

Platelet membrane fractions were isolated using sonication. Platelets were resuspended in sonication buffer (50 mM Tris/HCl, pH 7.4, 250 mM sucrose, 1× protease inhibitor cocktail) and the concentration was adjusted to 4×10^8/mL. Resting and stimulated platelets were placed in an ultrasonic dismembrator Model 100 (Thermo Fisher Scientific) and disrupted by three rounds of 10 seconds pulses with 50 W output. The samples were chilled on ice for 10 minutes and the membrane and cytosolic fractions were separated by ultracentrifugation at $186,000 \times g$ for 1 hour. The membrane fraction was washed three times with sonication buffer in order to remove cytosol components. The final membrane pellet (membrane fraction) and cytosol fraction were dissolved in SDS sample loading buffer. The equivalent amount of platelets for membrane fraction and cytosol were loaded

Isolation of Platelet Lipid Rafts

Resting and stimulated platelets (1×10^9/mL) were lysed in 2× Raft lysis buffer (50 mM MES, pH 6.5, 150 mM NaCl, 1% Tx-100, 2× protease inhibitor cocktail), and mixed with 80% sucrose solution in MES saline (50 mM MES, pH 6.5, 150 mM NaCl). Final 40% sucrose/platelet lysates (2 mL) were layered into 15 mL ultracentrifuge tubes (Beckman Coulter), with 30% (6 mL) and 5% (2 mL) sucrose layers added on top. Samples were centrifuged at $200,000 \times g$ for 18 hours without a break and the tubes were snap frozen in an ethanol-dry ice chamber for 10 minutes. Nine fractions (1 mL each) were collected as a frozen entity using a graduated platform for cutting with a hacksaw (named 'Raftolator') and tube debris were removed by centrifugation at $1,000 \times g$ for 2 minutes. Protein concentration was measured in each fraction using BCA™ assay and proteins were concentrated using trichloroacetic acid (TCA) precipitation, followed by boiling with 2× SDS sample buffer.

Identification of Unknown Proteins by Mass Spectrometry

Stained specific protein bands were visualized on a UV light box and the gel image was captured using Molecular Imager Gel Doc XR System and Quantity One 4.6 image analysis software (Bio-Rad). Bands of interest were excised either using the Gel Doc System or a ProteomeWorks™ automated spot cutter with PDQuest 7.2 software (Bio-Rad). Digestion with trypsin and peptide analysis by mass spectrometry using Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) method were performed at the University of Kentucky, Center for Structural Biology Protein Core Facility (Lexington, KY). Peptide fragments were analyzed for identification of protein using the web-based search engine MASCOT (Matrix Science Inc., Boston, MA).

Streptolysin-*O* (SLO) Permeabilized Human Platelet Secretion

This assay was established in the laboratory [123]. Human PRP was obtained from the Kentucky Blood Center as units (Lexington, KY). Twenty five milliliters of human PRP were first labeled with 0.4 μCi/mL [^{3}H] 5-HT for 45 min at 37°C. After centrifugation at $150 \times g$ for 10 min, the supernatant was moved to another conical tube and centrifuged at $500 \times g$ for 15 min. The pellet was washed twice with Ca^{2+}-free Tyrode's Buffer (154 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 5.6 mM D-glucose, 7 mM $NaHCO_3$, 0.6 mM NaH_2PO_4, 5 mM sodium PIPES, pH 6.5, 5 mM EGTA, 0.35% BSA) containing 3 μg/mL apyrase and finally resuspended in Buffer A (120 mM sodium glutamate, 5 mM potassium glutamate, 20 mM HEPES, pH 7.4, 2.5 mM EGTA, 2.5 mM EDTA, 3.15 mM $MgCl_2$, 1 mM DTT). The platelet concentration was determined by Z2 Coulter particle counter and size analyzer and adjusted to 5×10^8/mL. Fifty microliters of platelet suspension were mixed with 50 μL of Buffer A containing 8 mM ATP, 1.6 U/mL SLO (Corgenix, Peterborough, United Kingdom) and the effectors (recombinant proteins, antibodies, *etc.*). The reaction was first incubated at RT for 10 min and then 30 min on ice. The samples were then warmed to 25°C using a RT water bath for 5 min and stimulated with calcium for 5 min. The reaction was stopped by centrifugation at 13,800 $\times g$ for 1 min to separate the supernatant from the pellet. The pellet samples were lysed with 100 μL lysis buffer (PBS, pH 7.4, 1% Triton X-100) for 1 hr on ice. The assay for secretion from all three granules is similar to the assay used to analyze mice platelet secretion (see above). The only difference is the usage of an ELISA assay, specific for

human PF4. This assay was developed in the laboratory by Dr. Todd Schraw and described in detail in [124].

Cloning of DNAs and Recombinant Protein Production

Proteins Tagged with His$_6$ or GST

The coding region of each protein was amplified by polymerase chain reaction (PCR) using forward and reverse primers. PCR products and prokaryotic expression plasmids (pProEX-HTb (Invitrogen, Carlsbad, CA) for His$_6$-tagged protein and pGEX-KG for GST-tagged protein) were digested with corresponding endonuclease restriction enzymes and separated on a 1% low-melting agarose gel (ISC BioExpress, Kaysville, UT) in TBE buffer (45 mM Tris, 45 mM boric acid, 1 mM EDTA). DNA bands were excised and eluted in sterile water using a Gel Extraction kit (Qiagen, Valencia, CA). Inserts (PCR products) and vectors (pProEX-HTb or pGEX-KG) were ligated in a 3:1 molar ratio using T4 DNA ligase (Invitrogen) and the ligates were transformed into DH5α *E.coli* strain by 45 seconds heat shock at 42°C. Transformed bacteria were recovered by incubating in 1 mL Luria-Bertani (LB) media at 37°C for 1 hour and grown on LB/agar plate containing 100 μg/mL ampicillin overnight at 37°C. Each colony was screened with cloning PCR and double digestion testing with the corresponding enzymes after purification of plasmid DNA Miniprep kit (Qiagen). The clones were further confirmed by DNA sequencing at Davis Sequencing (Davis, CA).

For production of recombinant proteins, purified plasmid DNAs were transformed into the Rosetta^{TM}(DE) *E coli* strain which harbors a plasmid (pRARE) containing rare tRNA sequences (Novagen, Gibbstown, NJ). The colonies were amplified by culturing in 100 mL LB media containing 100 μg/mL ampicillin and 35 μg/mL chloroamphenicol (LB/amp/cam) and the culture was further scaled to 2 L. Recombinant protein production was induced by adding 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 4 hours at 37°C or overnight at 16°C. The cells were harvested by centrifugation at 6,000 × *g* for 5 minutes and washed once in ice-cold PBS. The bacterial pellet was weighed and resuspended in breaking buffer. The cells were broken by three passages using French Press cell disruptor at 12,000 psi and centrifuged at 100,000 × *g* for 1 hour. The supernatant was filtered using 0.2 μm nylon filter (Fisher Scientific) and applied to

column purification using either Ni^{2+}-NTA agarose or glutathione agarose. The purified recombinant proteins were dialyzed in HEPES buffer (20 mM HEPES/KOH, pH 7.4, 100 mM NaCl, 2 mM $MgCl_2$, 1 mM DTT, 5% glycerol) overnight at 4°C and the aliquots were stored at -80°C.

Syntaxin-Containing Complexes Production

Dual expression plasmids for full-length SNAP-23, Munc18a, and Munc18c, syntaxin-2 (residues 1-251, representing the cytoplasmic region), and syntaxin-4 (residues 1-265, representing the cytoplasmic region) were constructed using pRSFDuet-1. Amino acid substitutions were introduced using the site-directed mutagenesis (Strategene). All syntaxin-containing complexes were overexpressed in the Rosetta Escherichia coli cells (Novagen) and purified from cell lysates by both Ni^{2+}-affinity chromatography and S-protein affinity chromatograph.

Production of His$_6$-Tomosyn-1 from Sf9 Cells

His$_6$-tomosyn-1 full length and fragments were generated in Sf9 (*Spodoptera frugiperda*) cells according to the manufacturer's instruction. Full-length tomosyn-1 cDNA was inserted into the pFastBac-HTB and the sequence was confirmed by commercial dideoxy sequencing. The pFastBac-HTB-tomosyn-1 plasmid was then transformed into DH10Bac competent cells (Invitrogen) and tomosyn-1-Bacmid was purified according to the manufacturer's instructions.

Tomosyn-1-Bacmid was used to transfect Sf9 cells which had been previously adapted to serum-free suspension culture (SF-900 II SFM medium, Invitrogen). After two days, the supernatant of the transfected Sf9 cells was collected (containing secreted baculovirus, P1 virus) and used to directly infect a new culture of Sf9 cells to produce P2 virus. P2 virus titer was determined by a virus plaque assay according to the manufacturer's instructions. P2 virus was then used to infect 200 mL mid log-phased Sf9 cells ($1\sim2 \times 10^6$/mL) at the indicated multiplicity of infection (1 to 5) for 3 days. The whole cell pellet was collected, washed twice with cold PBS, and resuspended in 20 mL cold Lysis Buffer (50 mM Tris, pH 8.0) in the presence of 1 mM phenylmethanesulphonylfluoride (PMSF) and protease inhibitor cocktail (Roche Applied

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Science, Indianapolis, IN). The cell suspension was sonicated 10 times, 30 sec each on ice. The lysate was first centrifuged at $500 \times g$ for 10 min to remove unbroken cells and disrupted cell debris, then at $13,800 \times g$ for 20 min at 4°C. The supernatant was loaded to a 1 mL Ni^{2+}-NTA column pre-equilibrated with 50 mM Tris (pH 8.0). The column was washed sequentially with 10 volumes of Buffer I (50 mM Tris, 500 mM NaCl, pH 8.0) and of Buffer II (50 mM Tris, 500 mM NaCl, 5 mM imidazole, pH 8.0), then eluted with 5 mL of Elution Buffer (50 mM Tris, 500 mM NaCl, 0.5 M imidazole, pH 8.0).

Generation of the Tomosyn-1 and Syntaxin-11 Antibodies

DNAs encoding the rat tomosyn-1 and murine syntaxin-11were inserted into the pProEX-HTB vector and used to transform Rosetta DE3 competent *E. coli*. The His_6-tagged insoluble proteins were dissolved in 8 M urea then purified under denaturing conditions. The eluted proteins were dialyzed extensively with PBS in order to remove residue imidazole and urea. The protein concentration was analyzed using SDS-PAGE with BSA as a standard.

To generate antibodies, 1 mg of protein was emulsified in 1 mL Freund's complete adjuvant (Sigma) and injected subcutaneously into a total of ten sites along the back of a New Zealand Rabbit (100 μL/site). Two weeks later, the rabbit was boosted with 0.25 mg of protein emulsified with Freund's incomplete adjuvant (Sigma) using a similar pattern of injection sites. Two weeks following the boost, 35 mL of blood was collected from the rabbit ear vein. Following the first bleed, the rabbit was boosted then bled every two weeks for 15 cycles. Sera were prepared, and stored as aliquots at -20°C.

To affinity purify the antibodies, purified protein was coupled to 2 mL GSH-Agarose beads. The column was washed with 20 volumes of PBS to remove unbound protein, then 20 volumes of 200 mM HEPES (pH 8.5). The column was incubated with 10 mL of 20 mM dimethyl pimelimidate (DMP) in 200 mM HEPES (pH 8.5) at RT for 1 hr with gently rocking. After removing the crosslinking solution, 10 mL of 200 mM ethanolamine (pH 8.2) was added and incubated for 30 min at RT with gently rocking to terminate any residual crosslinker. After washing the column with 10 volumes of Elution Buffer (150 mM NaCl, 200 mM Glycine/HCl, pH 2.0), the column was washed with TBS (25 mM Tris, 150 mM NaCl, pH 7.5). Ten milliliters of sera were diluted, 1:1, with TBS

and filtered through a 0.2 μm filter. The filtered sera was loaded onto the column and incubated overnight at 4°C. The column was sequentially washed with 10 volumes of TBS, 10 volumes of Wash Buffer (500 mM NaCl, 20 mM Tris/pH 7.4, 0.1% Triton X-100), and 10 volumes of TBS. The column was eluted with 10 volumes of Elution Buffer. One milliliter fractions were collected into 1.5 mL microtubes containing 100 μL the Neutralizing Buffer (2 M Tris, pH 8.5). Antibody was collected, concentrated, dialyzed against PBS, and stored as aliquots at -20°C. The antibody concentration was determined by Bicinchoninic acid (BCA) assay (Pierce, Rockford, IL). The affinity-purified anti-tomosyn-1 antibodies immunoprecipitate endogenous tomosyn-1 from platelet extracts (Figure 2-1).

A

Marker (kDa) | Purified Tomosyn Proteins
B-fragment F-fragment

203 -
119 -
100-
52 -
37-
29 -
19 -
7 -

B

beads alone | rabbit IgG | B of Tomosyn Ab 20 ul 5 ul | F of Tomsyn Ab 20 ul 5 ul | Input 0.5%

220-

114 -

C

Tomo-PAb 40 ug	-	+	+	+	+	+	+	+	+
R7285-IgG 40 ug	+	-	-	-	-	-	-	-	-
Thro-30 sec	-	-	-	-	-	-	-	+	+
Thro-2 min	+	-	-	-	-	+	+	-	-
PGI₂-5 min	-	-	-	+	+	-	-	-	-
Protein A beads	+	+	+	+	+	+	+	+	+

Blot for Tomosyn

114-
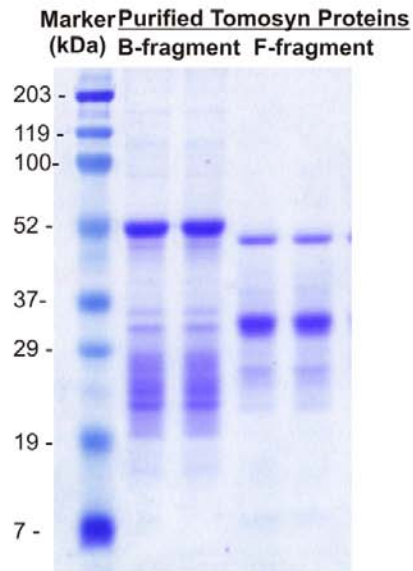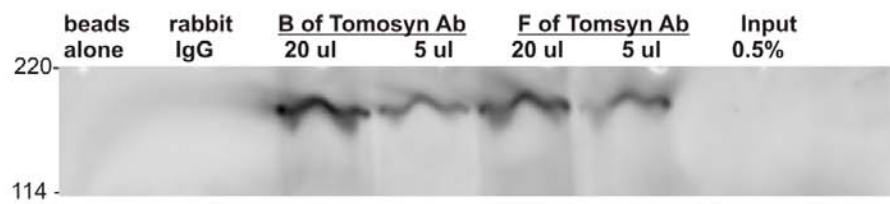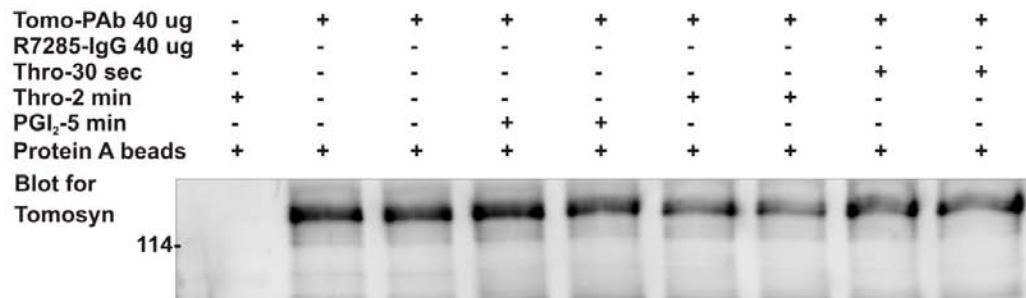
Figure 2-1. Generation of Tomosyn-1 Antibodies. Tomosyn-1 antibodies were generated by immunizing rabbit with purified recombinant tomosyn-1 fragments. (**A**) Denaturing purified recombinant His-tagged B-fragment and F-fragment of tomosyn-1 were separated on a 10% SDS-PAGE gel and visualized by Commassie Brilliant Blue R-250 staining. These two recombinant protein preps were pure as determined by western blotting for tomosyn-1 monoclonal antibody and mass spectrometry (the lower bands are fragments of corresponding proteins) and used as antigen for antibody production. Two fragments of rat tomosyn-1 are indicated as B and F of tomosyn-1. B-fragment was cloned from N-teriminal sequence (1-390 aa) of rat tomosyn-1, which is predicated to form the first β-propeller structure, while the F-fragment is from the C-terminal region (670-1056 aa) and has a flexible predicated structure. (**B**) Antibodies generated against both B and F-fragments of tomosyn-1 are able to immunoprecipitate endogenous tomosyn-1 protein from platelet extracts. (**C**) Anti-tomosyn-1 polyclonal antibodies also recognize the endogenous tomosyn-1 protein from resting, PGI2 treated, and thrombin activated platelet extracts.

Tail Vein Bleeding Time

Tail vein bleeding time was determined as described by Broze *et al.* [125]. Briefly, mice 5-6 weeks of age were anesthetized with ketamine 75 mg/kg *i.p.* A transverse incision was made with a scalpel at a position around 3 mm from the tip of murine tail. Then the tail was immediately immersed into a 15 mL tube filled with 37°C normal saline. The time from incision to bleeding cessation was recorded as the bleeding time. Animals with prolonged bleeding times greater than 10 min were stopped manually.

FeCl$_3$-Induced Arterial Thrombosis *in vivo* Model

FeCl$_3$-induced arterial thrombosis is a method to analyze platelet functional defects *in vivo* [126, 127]. This model is believed to be one of the better models to mimic thrombotic disease *in vivo* because it generates a controlled and localized vascular damage that the platelets respond to. Mice 8-12 weeks of age were anesthetized with Avertin (0.2 g/kg, *i.p.*) in a supine position on a 37°C heating pad. The toe-pinch reflex was used to monitor the anesthesia state. A segment of the left carotid artery was exposed using blunt dissection under a dissecting microscope. A miniature Doppler flow probe (0.5VB, Transonic system Inc., Ithaca, NY, USA) was placed on the carotid artery to monitor blood flow. Thrombus formation was induced by placing a small piece of filter paper saturated with 5% FeCl$_3$ solution in saline on the top of the vessel for 3 min and then removed. Time from application to cessation of flow, defined as flow stopped for more than 1 min, was measured. Experiments were terminated at 30 min.

Statistics Analysis

All statistical analysis was performed using ANOVA (GraphPad Prism 5). *P* values less than 0.05 were considered significant.

Chapter THREE
The Role of Syntaxins in Platelet Secretion

Introduction

It is generally accepted that secretion is mediated by formation of *trans*-membrane SNARE complexes which drive two lipid bilayers to fuse together, leading to the release of granule contents. The minimal functional SNARE complex is formed by one v-SNARE and two t-SNAREs. According to the original SNARE hypothesis, specificity for different membrane fusion events is provided by specific interaction of t-SNARE/v-SNARE pairs [33, 128]. This has been challenged in recent years. A more modern version of the hypothesis posits that only certain SNARE complex combinations are fusogenic [30, 79]. In platelets, it has been demonstrated that VAMP-8 is required for platelet secretion. VAMP-8 is the primary VAMP among the four known platelet VAMPs (VAMP-2, -3, -7, -8); only VAMP-8 null mouse platelets have a secretion defect. Since this defect is not complete, unless Tetanus toxin is added, VAMP-2 and -3 must serve some compensatory, secondary role with lower efficacy [46]. It has been demonstrated that SNAP-23 is required for platelet secretion [40, 41]. The role of the syntaxins has been more equivocal and has not been determined definitively.

Several syntaxins (-2, -4, -7, -11, and Vti1b) are present in platelets. Of the known syntaxins in platelets, syntaxin-2 and -4 were the only two that were found on the plasma membrane [38]. Thus, they were the most likely candidates to mediate exocytosis. Consistently, introduction of a syntaxin-2 polyclonal antibody into SLO-permeabilized human platelets inhibited release from α-granules, dense granules, and lysosomes [40, 41]. *In vitro* studies have also shown that syntaxin-2 can interact with SNAP-23 and VAMP-8 [129], suggesting that syntaxin-2 can form SNARE complexes. In parallel, studies of syntaxin-4 suggested that it also played a role in platelet exocytosis. Introduction of a syntaxin-4 monoclonal antibody into SLO-permeabilized human platelets inhibited release from α-granules and lysosomes and co-immunoprecipitation experiments showed that syntaxin-4 could form complexes with VAMP-8 and SNAP-23 [41, 44]. These results were consistent with other studies showing a role for syntaxin-4

in exocytosis; syntaxin-4 is required for insulin secretion from pancreatic beta-cells [70, 130]. However, there were inconsistencies. A syntaxin-4-specific polyclonal antibody had no effect on platelet secretion (Lemons *et al* unpublished) and reduction of the syntaxin-4 chaperone Munc18c and concomitant reduction in syntaxin-4 had no effect on release from murine platelets [80]. Given the possibility that the antibodies used may not be completely isoform-specific or that steric hindrance could account for the inhibitory effects of the antibodies, we used transgenic mice to reexamine the roles of syntaxin-2 and-4 in murine platelets.

Syntaxin-2 KO mice are viable but the null males are sterile due to abnormal testicular development and impaired spermatogenesis [119]. Syntaxin-4 is important for embryo development and its global deletion causes early embryonic lethality before day 7.5 (E7.5). Syntaxin-4 is probably required for glucose transporter 8 transports in embryoblasts [131]. These knockout mouse systems make ideal tools to re-examine the roles of syntaxin-2 and -4 in platelet exocytosis because that are not subject to the same ambiguities as are the permeabilized platelet assays. Surprisingly, in our analysis (this Chapter), platelets lacking both syntaxin-2 and -4 had no secretion defect, suggesting that other syntaxins are required for platelet exocytosis.

Considering that syntaxin-7 is not located on the plasma membrane and introduction of syntaxin-7 antibody into platelets did not affect platelet secretion [42], we reasoned that that syntaxin-7 may not be critical for platelet secretion. The syntaxin, Vti1b, was found in platelets by western blotting analysis during my thesis preparation. Vti1b has been shown to be important in other hematopoietic cells. Vti1b is necessary for the release of secretory lysosomes in cytotoxic lymphocytes [131, 132]. However no evidence is available to show that it plays an important role in platelet secretion. Based on the close sequence homology with prototypical t-SNAREs, the mammalian syntaxin family can be further divided into Qa (syntaxin-1, -2, -3, -4, -5, -7, -10, -11, -16, *etc*.), Qb (Vti1a, Vti1b, *etc*.), Qc (syntaxin-6, syntaxin-8, *etc*.). SNAP-23/25 t-SNAREs are considered Qb/Qc SNAREs. Since SNAP-23 appears to be required, any candidate syntaxin in platelet should be a Qa SNARE. Since Vti1b is a Qb t-SNARE it is not likely to be critical for platelet secretion.

Among the five known syntaxins in platelets, syntaxin-11, by default, appears to be the most important candidate for a role in platelet secretion [40, 41, 73]. Most mammalian syntaxins are type II integral membrane proteins anchored by their C-terminal TMD [74]. Syntaxin-11 is a non-conventional syntaxin without a TMD. Syntaxin-11 has been shown to be associated with late endosomes and the *trans*-Golgi network in Hela cells [37]. Interestingly, syntaxin-11 mutations are associated with Familial Hemophagocytic Lymphohistiocytosis Type 4 (FHL4). FHL4 is a rare autosomal recessive disorder of immune dysregulation associated with uncontrolled T cell and macrophage activation. Syntaxin-11 deficient NK-cells exhibit a cytotoxic granule degranulation defect. Thus it has been thought that syntaxin-11 is involved in granule-plasma membrane fusion. However, other studies suggest that syntaxin-11 might be involved in granule-granule fusion instead [75, 76]. Moreover, syntaxin-11 has been shown to play a negative, regulatory role in macrophages by controlling access to Vti1b and thus affecting late endosome to lysosome fusion [77]. Although the exact role of syntaxin-11 in immune cell exocytosis is still not clear, the genetic association of syntaxin-11 mutations with FHL4 is clear. We took advantage of this in our analysis of platelets from an FHL4 patient that lacked syntaxin-11.

The goal of the studies described in this Chapter was to determine which syntaxin is required for platelet secretion. We used syntaxin KO mouse models and syntaxin-11 deficient human platelets. Deletion of syntaxin-2 or -4 alone or in combination had no effect on secretion from murine platelets. Platelet secretion from patients with FHL4, which lack syntaxin-11, was severely defective. Thus, syntaxin-11, but not syntaxin-2 or -4, is critical for platelet exocytosis.

Results:

The Effect on Murine Platelets of Deleting Syntaxin-2:

The syntaxin-2 KO mice were on a C57Bl/6J background. Compared to wild type littermates, the syntaxin-2 null mice had normal size, body weight, and fur color. The males were sterile and for unknown reasons most females had some problems with

delivering; thus, producing syntaxin-2 null mice was difficult. These data are consistent with syntaxin-2 being important for certain physiological processes.

To investigate the specific role of syntaxin-2 in platelets, first we analyzed the morphology of platelets from the knockout mice. The number and size of the circulating platelets were in the same range as wild type (Figure 3-7). The ultrastructure was normal with similar shape, granules, OCS, and mitochondria (Figure 3-1), compared to the wild type platelets from littermate controls. There were no overt, morphological differences between platelets from wild type and syntaxin-2 null animals.

To study the specific role of syntaxin-2 in platelet secretion, we first examined whether deletion of syntaxin-2 affected the levels of other secretory machinery components (Figure 3-2). There was no obvious effect on VAMP-2, VAMP-3, or VAMP-8. There was no significant effect on other t-SNAREs: syntaxin-4, syntaxin-7, syntaxin-11, or SNAP-23. We confirmed the deletion of syntaxin-2. The protein level of syntaxin chaperone proteins, Munc18 (a, b, c), appeared normal when probing with Munc18a/b and Munc18c polyclonal antibodies.

Because a syntaxin-2 polyclonal antibody inhibited secretion in *in vitro* permeabilized platelets, syntaxin-2 was hypothesized to be required for platelet secretion. If this hypothesis was correct, a secretion defect should be observed when using syntaxin-2 KO mouse system. Surprisingly the depletion of syntaxin-2 has no secretion defect (Figure 3-3) from all three granules (α-granules, dense granules, and lysosomes). For analysis, we performed dose response and a time course of release experiments using thrombin as agonist [46, 133]. Intact platelets were first loaded with [^{3}H]-5HT, washed with HEPES buffer, and then stimulated with increasing concentrations of thrombin for 1 min (Figure 3-3, left panel). The platelets were pelleted by centrifugation, and the supernatants were assayed for the presence of granule cargo markers: [^{3}H]-5HT from dense granules, PF4 from alpha granules, and β-hexosaminidase from lysosomes. As expected, agonist-induced release of dense core and α-granule cargo from wild type platelets (black square symbols) was greater than the release from lysosomes. Comparison of platelets from wild type and null mice (red circle symbols) showed that each responded equally to thrombin and released granular cargo to similar extents. To assess the efficiency of release, the time course of granule release was analyzed by

stimulating platelets with 0.05 U/mL thrombin (Figure 3-3, right panel). Thrombin stimulation initiated dense granule release rapidly and appeared to be complete by 45 seconds. This same response also was seen for α-granule and lysosome release, although the extent of release was not as great as dense granule. Comparison of release kinetics between wild type and syntaxin-2 null platelets showed no significant difference in the time course of their granule release.

Additionally, there was no observable defect in platelet aggregation (Figure 3-11) for syntaxin-2 null platelets. The washed platelets were used after recalcifying with 1 mM $CaCl_2$. Under these conditions platelets from wild type (black color) and syntaxin-2 KO mice (green color) were responsive to thrombin. The platelets showed an initial, agonist-induced shape-change response followed by aggregation as indicated by the increase in light transmission. The kinetics and extent of aggregation to 0.1 U/mL of thrombin were essentially identical (Figure 3-11). The aggregometer measurements are consistent with the normal secretion (Figures 3-3). Thus, the absence of this t-SNARE does not result in any gross defect in platelet function.

Even though the *in vitro* SLO-permeabilized platelet assay had suggested that syntaxin-2 is important for platelet secretion, deletion of syntaxin-2 in mouse platelets had no effect on platelet secretion, suggesting that syntaxin-2 is not absolutely required for platelet secretion. It is possible that syntaxin-2 is not required for platelet secretion or other syntaxins are compensating for the secretion function when syntaxin-2 is missing. In order to exclude the possibility, we used the platelet–specific, syntaxin-4 KO mouse model.

The Effect on Murine Platelets of Deleting Syntaxin-4:

To study the specific role of syntaxin-4 in platelet secretion we used a platelet-specific knockout mouse model. Mice carrying a floxed version of the syntaxin-4 gene were mated to a Platelet Factor 4 promoter-driven Cre recombinase expressing transgenic mouse strain. Using platelets from the progeny of that cross, first we analyzed their morphology. The number and size of circulating platelets were in the same range as for wild type platelets (Figure 3-7). The ultrastructure was normal with similar shape, granules, OCS, and mitochondria (Figure 3-4), compared to the wild type platelets from

littermates. There was no overt difference between the number of granules per platelets between wild type and null platelets.

Western blotting analysis was performed to determine whether deletion of syntaxin-4 affects other secretory components. There were no obvious effects on v-SNAREs in platelets; VAMP-2, VAMP-7, and VAMP-8 levels were normal (Figure 3-5). Similarly there were no significant effects on other t-SNAREs, such as syntaxin-2, -7, -11, or SNAP-23. We confirmed the loss of syntaxin-4. The protein levels of the syntaxin chaperones, Munc18a and Munc18b, appeared normal while Munc18c levels were reduced. This is consistent with the notion that Munc18c is a syntaxin-4-specific chaperone and the stable complex they form is mutually regulated.

If the assumption is that syntaxin-4 is required for platelet secretion, a secretion defect should be observed when syntaxin-4 is deleted. Surprisingly the depletion of syntaxin-4 caused no secretion defect (Figure 3-6) from all three granules (α-granules, dense granules, and lysosomes). In Figure 3-6 left panel, a thrombin titration experiment was performed using 1 min as the incubation time. Secretion from each of the three granules showed the expected dose dependence. Comparison of platelets from wild type (black square symbols) and null mice (red circle symbols) showed that each responded equally to thrombin and released granular cargo to similar extents. To assess the efficiency of release, the time course of granule release was analyzed. When stimulated with 0.05 U/mL of thrombin, release from all three granules is clearly observed (Figure 3-6 right panel). Dense granule release was the fastest secretion event, followed by release from α-granules and lysosomes. Dense granule secretion was observed at 15 seconds when stimulated with 0.05 U/mL of thrombin and completed by 30 sec. α-granule secretion and lysosomal secretion were slightly delayed. They were detectable at 15 sec and completed by 60 second. The extent of secretion also differed among these three granule secretion events. Over eighty percent of dense granule cargo was released while ~40% of α-granule cargo and lysosome marker were released when platelets were stimulated with 0.05 U/mL of thrombin for 1 min. However, in summary, there was no significant defect in the kinetics or extent of secretion from all three granules in the syntaxin-4 deficient platelets.

Additionally, there was no observable defect of platelet aggregation (Figure 3-11) of syntaxin-4 deficient platelets. Washed platelets were used after recalcification with 1 mM $CaCl_2$. Under these conditions platelets from wild type (black color) and syntaxin-4 KO mice (blue color) were responsive to thrombin. The platelets showed an initial, agonist-induced shape-change response followed by aggregation as indicated by the increase in light transmission. The kinetics and extent of aggregation in response to 0.1 U/mL of thrombin were essentially identical (Figure 3-11). The aggregometer measurements were consistent with normal secretion (Figures 3-6). Thus, the absence of this t-SNARE does not result in any gross defect in platelet function.

Even though the *in vitro* SLO-permeabilized platelet assay has suggested that syntaxin-2 and -4 are important for platelet secretion, syntaxin-2 and syntaxin-4 single KO mouse platelets have no secretion defect, arguing that neither syntaxin-2 nor syntaxin-4 alone is required for platelet secretion. At the time of these experiments, only three syntaxins (syntaxin-2, -4, -7) were known in mouse platelets and it has been discussed syntaxin-7 was not felt to be important for platelet secretion [40]. One possibility is that syntaxin-2 and -4 could be equally and inter-changeably used. To exclude this possibility, we then generated syntaxin-2/4 double KO platelets by crossing syntaxin-2$^{-/-}$ mice and syntaxin-4$^{flox/flox}$/PF4-Cre^{+} mice. If both syntaxin-2 and -4 are the primary syntaxins required for platelet secretion, depletion of both should inhibit granule secretion.

The Effect on Murine Platelets of Deleting both Syntaxin-2 and -4:

To investigate whether syntaxin-2 and -4 together are important for platelet secretion, we utilized a syntaxin-2/4 double KO murine model. Platelets morphology was first analyzed. The size of circulating platelets is normal while the number is significantly reduced compared to wild type littermate (Figure 3-7). The ultrastructure is normal (Figure 3-8), compared to platelets from wild type littermates. There was no significant difference between the number of granules per platelets between wild type and null platelets. Despite the thrombocytopenia, there was no overt morphological difference in the double KO platelets.

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To study the specific role of syntaxin-2/4 in platelet secretion, we first analyzed the levels of known elements of the platelets secretory machinery and found that loss of both syntaxin had no effect on the v-SNAREs (VAMP-2, -3, -8), other t-SNAREs (syntaxin-7 and -11), or the syntaxin chaperone proteins, Munc18a and Munc18b. There was a loss of Munc18s since syntaxin-4 was missing (Figure 3-9). Loss of syntaxin-2/4 also did not affect the expression of several cell surface membrane protein: GPIIb/IIIa, GP1bβ, PECAM-1, and GPVI (Figure 3-10), suggesting depletion of syntaxin-2/4 in platelets does not overtly affect membrane protein trafficking.

To determine whether both syntaxin-2 and syntaxin-4 are important for platelet aggregation, we tested platelets from the syntaxin-2/4 double KO mice. Washed and recalcified platelets were analyzed for their aggregation activity. Under these conditions platelets from wild type (black color) and syntaxin-2/4 KO mice (red color) were responsive to thrombin. The platelets showed an initial, agonist-induced shape-change response followed by aggregation as indicated by the increase in light transmission. The kinetics and extent of aggregation to 0.1 U/mL of thrombin showed no significant defect compared to syntaxin-2 KO, syntaxin-4 KO, and wild type platelets from the corresponding littermates. (Figure 3-11).

Considering the syntaxin-2 polyclonal antibody and syntaxin-4 monoclonal antibody could inhibit the *in vitro* permeabilized platelet secretion, a secretion defect should be observed when double deletion of syntaxin-2/4 was used, surprisingly the double depletion of syntaxin-2/4 resulted in no secretion defect (Figure 3-12). As shown in Figure 3-12 left panel, in a thrombin titration experiment, using 1 min as the incubation time, secretion from each of the three granules showed the expected dose dependence. Comparison of platelets from wild type (black square symbols) and null mice (red circle symbols) showed that each responded about equally to thrombin and released granular cargo to similar extents. Interestingly, the release from α-granule was significantly enhanced when both syntaxin-2 and -4 were missing, suggesting that syntaxin-2 and -4 might compete with the important syntaxin for secretion. To assess the efficiency of secretion, the time course of granule release was analyzed. When stimulated with 0.05 U/mL of thrombin, release from all three granules is clearly observed (Figure 3-12 right panel). Dense granule release is the fastest, followed by

release from α-granules and lysosomes. Dense granule secretion was observed at 15 second when stimulated with 0.05 U/mL of thrombin and completed by 30 sec. α - Granule secretion and lysosomal secretion are slightly delayed. They were detectable at 15 sec and completed by 60 second. The extent of secretion also differs among these three granule secretion events. Over eighty percent of dense core granule cargo was released while ~60% of α-granule cargo and lysosome marker were released when platelets were stimulated with 0.05 U/mL of thrombin for 1 min. There is no defect in the kinetics or extent of secretion from all three granules in the syntaxin-2/4 double deficient platelets, suggesting that neither syntaxin-2 nor -4 are required for platelet secretion. Compared to the wild type platelets, deletion of both syntaxin-2 and -4 seems to enhance the secretion from α-granules, indicating the syntaxin-2 and -4 might be competing with the real functional syntaxin for formation of functional SNARE complex, thus membrane fusion. A similar enhancement, though subtle, was noticed when examining the secretion from VAMP-3 null platelets [53]

Syntaxin-11 is the Most Abundant t-SNARE in Human and Murine Platelets:

From the data above, it is clear that previous analysis of syntaxin function in permeabilized platelets may be flawed. How previous experiments could have reached such different conclusions is an open question that we attempt to resolve in this section. Initial experiments sought to determine the relative levels of the different platelet SNAREs. As was shown for VAMP-8, and assumed for SNAP-23, the most abundant SNARE appears to be the most functionally relevant. Additionally, it seems possible that the antibodies originally used in the permeabilized platelet could have some cross-reactivities that were initially undetected (since syntaxin-11 was not know that the time).

The sequence in each mammalian t-SNARE is highly conserved, for example, the sequence identity for syntaxin-11 is 84.67 % between mouse and human. In terms of detectable t-SNAREs, a total of five syntaxins (syntaxin-2, -4, -7, -11 and Vti1b) and SNAP-23 have been identified in platelets (notes: Vti1b was found in platelets during the preparation of this manuscript, so data presented here do not included Vti1b). To characterize antibody specificity and understand the syntaxin usage in mouse and human platelets, we utilized fluorescence-based, quantitative, western blotting of both human

and mouse platelet extracts. The antibodies used for syntaxin-2, -4, -7, and -11 and SNAP-23 recognized both human and mouse platelet syntaxins (Figure 3-14). To determine the relative abundance of the five t-SNAREs, recombinant proteins were used to generate a standard curve for quantification. The platelet extracts from known numbers of human and mouse platelets were loaded as indicated (Figure 3-15). The protein level of syntaxin-11 was the most abundant t-SNAREs in both human and mouse and a molecule per platelet level (Table 3-1). Syntaxin-11 was ~6 fold more abundant than syntaxin-4 and 3 fold more abundant than SNAP-23 in human platelets while in mouse platelets syntaxin-11 was 9 fold higher than other t-SNAREs. Comparing human and mouse t-SNAREs, syntaxin-2, -7, and -11, there were ~2000 molecules of syntaxin-2 per platelet, twice that for syntaxin-7 and 25 fold that syntaxin-11. However the amount of human syntaxin-4 and SNAP-23 were much higher than in mouse. The specificity of each antibody was also assessed by comparing their immuno-reactivities to the various recombinant syntaxins (Figure 3-16A). Among the four syntaxin antibodies, the syntaxin-2 polyclonal antibody, which was generated by our group and used for previously studies [40, 41], showed some cross reactivity with both syntaxin-4 and syntaxin-11. However, the small amount of cross reactivity does affect the fact that syntaxin-11 is the most abundant t-SNARE in both human and mouse platelets. From these data two points are clear, the originally used anti-syntaxin-2 antibody used in permeabilized platelets does cross react with syntaxin-11 (also see below) and syntaxin-11 is the major syntaxin by mass in platelets. These data would support the hypothesis that syntaxin-11 is required for platelet secretion.

Syntaxin-11 is Required for Platelet Secretion:

Among the four known syntaxins in platelets, all except syntaxin-11 have been ruled out as the most important syntaxin required for platelet secretion. Quantification of t-SNAREs (Table 3-1) in platelets shows that syntaxin-11 is the most abundant t-SNARE in both human and mouse platelets, suggesting that syntaxin-11 could be the most important t-SNARE in platelets. The fact that syntaxin-2 deficient platelets have no secretion defect argues that the inhibition phenotype by the syntaxin-2 polyclonal antibody [40, 41] may be caused by a steric effect or more likely by cross reactivity.

Indeed, the original syntaxin-2 polyclonal antibody showed cross reactivity to syntaxin-11 (Figure 3-16A). Additionally, the antibody also recognized endogenous syntaxin-11 since syntaxin-11 could be co-immunoprecipitated by the syntaxin-2 antibody (Figure 3-16B). The syntaxin-2 antibody cross reactivity not only explains the inconsistency between permeabilized platelet secretion data and knockout, intact platelet secretion data, but also suggests a role for syntaxin-11 in platelet secretion. To address this question, we collaborated with Dr. Alexandra H. Filipovich at Cincinnati Children's Hospital Medical Center to study platelets from a FHL4 patient. The patient, whose platelets were analyzed, had bleeding problems and a profound defect of NK cell degranulation (personal communication). Genetic analysis demonstrated that the patient was homozygous for 173T>C mutation in the syntaxin-11 gene. This point mutation causes a reduction in syntaxin-11 protein levels, below the limits of detection. The levels of 15 other secretory machinery components are normal (Figure 3-17A). As perhaps expected since Munc18b is a potential chaperone for syntaxin-11, Munc18b levels were negatively affected. The mutation causes a change in D58 to R on the Habc region. It is possible that this point mutation may disrupt the protein folding and/or stability of syntaxin-11. To address whether syntaxin-11 is required for platelet secretion, we examined release from all three granules in both thrombin-titration and time course experiments. A thrombin titration experiment was performed using 1 min as the incubation time. Secretion from each of the three granules from normal control platelets and lysosome from FHL4 patient showed the expected dose-dependence (Figure 3-17B). Secretion from dense granule and α-granule release was almost abolished in the FHL4 patient's platelets. To assess the efficiency of release, the time course of granule release was analyzed. The time course of [^{3}H]-serotonin from dense granule and PF4 release from α-granule was nearly abolished in the syntaxin-11 deficient platelets from the FHL4 patient (Figure 3-17C). Release of β-hexosaminidase from lysosomes was only modestly decreased, suggesting that syntaxin-11 is essential for dense and α-granule release while lysosome release is not fully dependent on syntaxin-11.

Platelet Aggregation, ATP Release, and P-selectin Exposure are Defective in Syntaxin-11 Deficient Platelets from a FHL4 Patient:

To confirm that syntaxin-11 is required for platelet exocytosis, we further analyzed syntaxin-11 null platelets from the same FHL4 patient. Figure 3-18A, i-iii shows that the aggregation of washed platelets (control, black traces; patient, gray traces) stimulated with 0.1 U/mL thrombin, 10 µg/mL collagen, or 100 µM A23187, was significantly defective for the syntaxin-11 deficient platelets. Concomitantly, ATP release, as measured with luciferin/luciferase luminescence, from dense granules was totally abolished (Figure 4A, iv-vi). Secretion-dependent aggregation was defective when stimulated with thrombin or A23187 (Figure 3-18A, i and iii) by comparison with collagen induced aggregation (Figure 3-18A, ii) since collagen induced aggregation is less dependent on secondary wave of ADP release [134]. A23187 is an ionphore, which increases intracellular calcium and circumvents the need for signaling cascade activation [117, 118]. Secretion in the presence of A23187 is impaired, suggesting that the secretion defect seen on syntaxin-11 deficient platelets is not in a signaling step but is indeed at the fusion step required for secretion (see EM analysis discussion).

To further confirm the secretion defect in the syntaxin-11 deficient platelets, we also performed flow cytometry analysis. P-selectin is an integral membrane protein in α-granules and its exposure at the platelet surface is widely used as a metric for α-granule exocytosis. Compared to normal control and resting platelets, the geometric mean fluorescence intensity (GMFI) of staining for P-selectin on syntaxin-11 deficient platelets only slightly increased upon stimulation with 0.1 U/mL thrombin (Figure 3-18B). When quantified, the P-selectin staining on FHL4 patient's platelets was 10 fold less than that of control, suggesting that α-granule secretion, as measured by P-selection exposure, is almost abolished.

To exclude the possibility that the observed secretion defects could be caused by a defect in granule biogenesis or platelet activation, we examined platelet cargo levels and other parameters of platelet activation. The level of PF4, an α-granule-specific cargo protein, was similar to wild type (Figure 3-17A). Based on the secretion experiments discussed about, the platelet levels of β-hexosaminidase and PF4 were similar: for PF4 from α-granules, there were ~2331 $\pm$ 184 pg/2.5×10^8 control platelets and 2220 $\pm$ 86 pg/2.5×10^8 in the syntaxin-11 deficient patient platelets; for β-hexosaminidase there were ~37 $\pm$ 5 U/2.5×10^8 control platelets (n=24) and 30 $\pm$ 5 U/2.5×10^8 in the syntaxin-11

deficient patient platelets. We also examined two important platelet activation bench marks: platelet cytoskeletal reorganization and integrin $\alpha_{IIb}\beta_3$ activation. A hallmark of platelet activation is integrin $\alpha_{IIb}\beta_3$ activation [116-118]. The activated $\alpha_{IIb}\beta_3$ can be measured with the conformation-specific, PAC-1 antibody. Flow cytometry analysis was performed. The traces of PAC-1 labeling in thrombin activated platelets (grey trace) from control and FHL4 patient shift to the right, suggesting that both platelet preparations are activated upon thrombin stimulation (Figure 3-18C). Compared with the control, the mean fluorescence intensity (GMFI) of staining for activated $\alpha_{IIb}\beta_3$ of syntaxin-11 deficient platelets from FHL4 patient was slightly decreased but not significantly, suggesting that loss of ADP release might contribute to affect integrin activation. From these data it appears that syntaxin-11 depletion does not significantly impair platelet $\alpha_{IIb}\beta_3$ inside-out signaling.

Activated platelets undergo dramatic cytoskeletal rearrangement, forming filopodia and laminipodia and causing granule centralization, which is necessary for platelet exocytosis [135]. If syntaxin-11 deletion only affects the final fusion step, we would expect that syntaxin-11 deficient platelets would form filopodia and centralize their granules, but the granule membrane would not fuse with the plasma membrane because the relevant SNARE is not present. The expected phenotype would be similar to that of VAMP-8 null [46] or Munc13-4 null mouse platelets: visible granules centralized and surround by an actin cytoskeleton [48]. Resting and thrombin-activated platelets from a control donor and the FHL4 patient were examined by transmission electron microscopy. As shown in Figure 3-18D, i and ii, resting controls and resting FHL4 platelets have normal shapes with similar arrays of granules, mitochondria, microtubular networks, and OCS. When stimulated with thrombin (0.1 U/mL; 5 min), both platelets showed a similar irregular appearance with protruding filopodia. This is consistent with thrombin-induced cytoskeletal rearrangements (Figure 3-18D, iii and iv). Control platelets showed granules depletion while patient platelets showed centralization of the granules. The presence of granules in the middle of the cells suggests that their fusion with plasma membrane did not occur. This is consistent with a lack of secretion seen previously [48] and also indicates that the thrombin induced signaling required for cytoskeletal rearrangements is still intact in the syntaxin-11 deficient platelets.

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Syntaxin-11 Has Forms SNARE Complexes with SNAP-23 and VAMP-8:

It is clear that VAMP-8 is the primary v-SNARE required for platelet secretion and SNAP-23 is one of two t-SNAREs to form the core SNARE complex in platelets. If syntaxin-11 is the primary t-SNARE required for platelet secretion, syntaxin-11 should be present in SNARE complexes with SNAP-23 and VAMP-8. Studies by Valdez *at al*. [37] have demonstrated that syntaxin-11 is capable of binding to VAMP-2 and SNAP-23 *in vitro* and SNAP-23 in lymphocytes *in vivo*. To address this question in platelets, experiments were designed to determine if endogenous syntaxin-11 associates with endogenous SNAP-23 in platelets *in vivo*. Both resting and thrombin-activated human platelets were lysed with Triton X-100-containing buffer and immunoprecipitations were performed using control IgG and anti-syntaxin-11 polyclonal antibody. Western blotting using a syntaxin-11-specific antibody confirmed that syntaxin-11 was present in both the anti syntaxin-11 (Figure 3-19A) and the anti-SNAP-23 immunoprecipitates [Karim *et al* in preparation]. This demonstrates that syntaxin-11 is associated with SNAP-23 in platelets. Moreover, syntaxin-11 co-immunoprecipitated with VAMP-8 in both resting and thrombin-activated platelet extracts (Figure 3-19, panel A). Syntaxin-11 is also associated with VAMP-3 in resting platelets but not in activated platelets. There was no association between syntaxin-11 and syntaxin-4, further confirming that the syntaxin-11 antibody is specific (Figure 3-16). The observation that syntaxin-11 co-immunoprecipitated with syntaxin-2 from both resting and activated platelets may be caused by the cross-reactivity of syntaxin-2 antibody used for the western blotting. However, it is possible that syntaxin-2 was present in a different complex considering syntaxin-2 can form complex with SNAP-23 and VAMP-8. Taken together, syntaxin-11 is associated with both core SNARE complex components: SNAP-23 and VAMP-8 in activated platelets, demonstrating syntaxin-11 can form SNARE complexes with SNAP-23 and VAMP-8 *in vivo*.

Discussion:

It is generally believed that any regulated biological exocytosis is a SNARE–mediated, membrane fusion process. We hypothesized that there is a syntaxin required for platelet secretion. The potential candidates were syntaxin-2 and -4 considering they

are important for permeabilized platelet exocytosis [40, 41, 44]. The fact that syntaxin-2/4 double deletion murine platelets had no secretion defect suggests that syntaxin-2 and -4 are not the absolutely required for platelet secretion. But mild secretion enhancement from α-granules and lysosomes (Figure 3-12) argues that syntaxin-2/4 may be involved somehow. The similar observation was found with the VAMPs. A secretion enhancement in VAMP-3 KO mice platelets was observed [46]. Perhaps syntaxin-2/4 may have the similar role to VAMP-3: serving as secondary t-SNARE. Further experiments will we required to make this assessment. However, it should be noted that the defect in secretion in the syntaxin-11 deficient platelets was more severe than that in the VAMP-8 null platelets. This may imply that there are no secondary syntaxins in platelets.

Data presented in this Chapter demonstrated a required role for syntaxin-11 in platelet secretion. Depletion of syntaxin-11 in platelets from a FHL4 patient almost totally abolished dense granules and α-granules secretion (Figure 3-17 and Figure 3-18B) and severely impaired lysosome secretion (Figure 3-17B). Loss of syntaxin-11 did not affect granule biogenesis (Figure 3-17), other SNARE proteins (Figure 3-17A) or platelet activation (Figure 3-18C, 4D) indicating that the secretion defect is due a deficit in the final fusion step due to a loss of syntaxin-11. Washed platelets from the syntaxin-11 deficient patient also displayed a profound aggregation defect in response to stimulation with thrombin and A23187 while showing mild defect upon collagen stimulation, suggesting the secretion-dependent aggregation is ablated since full aggregation requires the secondary agonists (such as ADP from dense granule) additional stimulation and more binding surface (such as fibrinogen from α-granules). Moreover, syntaxin-11 forms SNARE complex with SNAP-23 and VAMP-8 [37]. In summary, these data support the conclusion that syntaxin-11, but not syntaxin-2 and -4, is the most important t-SNARE required for platelet secretion and thus for hemostasis.

Syntaxin-2 and -4 may not be required for granule biogenesis, based on the platelets levels of β-hexosaminidase and PF4, which were similar between wild type and syntaxin-2/4 KO mice (Figure 3-12). Similarly, the level of cell surface integrin $\alpha_{IIb}\beta_3$, PECAM, GP1bβ, and GPVI (Figure 3-10) from resting platelets were similar, suggesting that syntaxin-2/4 may not be required for membrane protein transport through ordinary

secretory pathway. However, whether syntaxin-2 and -4 are important for endocytosis in platelets is not clear and will required further analysis. A preliminary experiment showed that platelets from syntaxin-2/4 double KO mice displayed a fibrinogen uptake defect by western blotting analysis of the total platelet extracts (Figure 3-13), suggesting that syntaxin-2/4 may be involved in the granule biogenesis through the endocytic pathway. Western blotting and flow cytometry analysis of other uptake molecules, such as VEGF and albumin, using syntaxin-2/4 double KO mice will be a good way to explore this point further.

Syntaxin-2/4 may be important for platelet biogenesis. Compared to normal wild type and single syntaxin-2 or -4 KO, the syntaxin-2/4 double KO mice have lower circulating platelet numbers; however, the size and general morphology of platelets was unchanged. Analysis of megakaryocytes from these mice might yield interesting results. In sum, syntaxin-2 and -4 are not the most important syntaxins required for platelet secretion, but they might be important for other platelet functions or for biogenesis.

Table 3-1. t-SNAREs in Human and Murine Platelets

t-SNAREs	Molecules/Platelet*	
	Human (n=4)	**Murine** (n=4)
Syntaxin-2	1,579 ± 429	1,766 ± 121
Syntaxin-4	9,633 ± 75	1,625 ± 184
Syntaxin-7	3,861 ± 564	3,707 ± 372
Syntaxin-11	51,717 ± 5459	46,966 ± 5516
SNAP-23	19,374 ± 5384	5,363 ± 798

*Number of molecules per platelet was calculated using the molecular weight for Syntaxin-2 (33 kDa), Syntaxin-4 (34 kDa), Syntaxin-7 (30 kDa), Syntaxin-11 (33 kDa), SNAP-23 (23 kDa).

Certain amounts of human or murine platelet extracts were subjected to western blotting with the four different syntaxin antibodies and SNAP-23 antibody. Recombinant human syntaxin-2, -4, -7, or -11 were used to generate a corresponding standard curve for quantification of the four known syntaxins and SNAP-23.
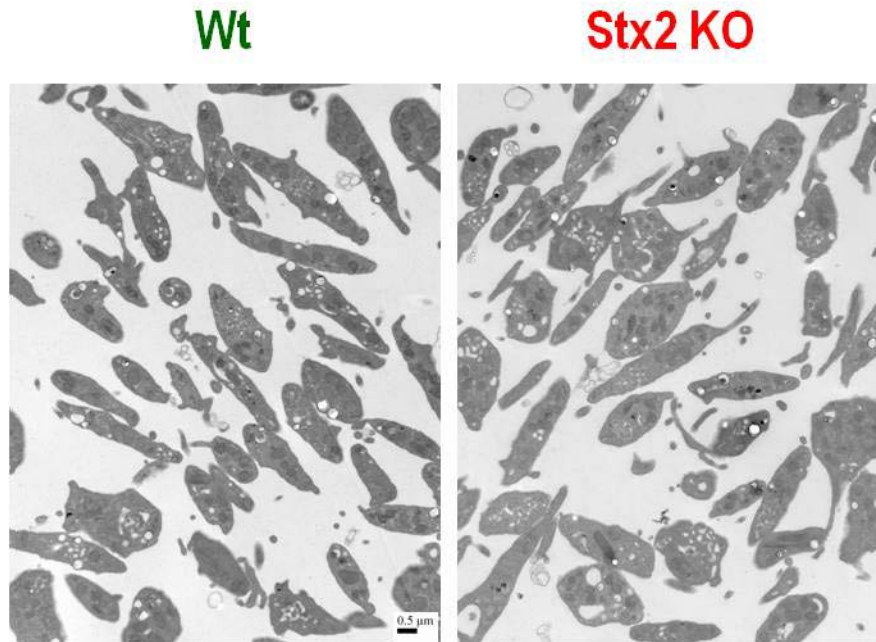
Figure 3-1. Deletion of Syntaxin-2 Does not Affect Resting Platelet Ultrastructure. Washed platelets (4×10^8/mL) from wild type (Wt) and syntaxin-2 platelet specific KO mice (Stx2 KO) were kept resting by adding PGI2. The platelets were fixed in 0.1% glutaraldehyde for 15 min at 37°C and 3% glutaraldehyde for 1 hour at 4°C. After osmication, the fixed platelets were dehydrated then polymerized in Spurr's resin. The thin sections of platelets were counterstained with uranyl acetate and the samples were analyzed using transmission electron microscope. The images were obtained using Gatan software and only the brightness and contrast were adjusted in Photoshop (the scale bar is indicated in left panel).
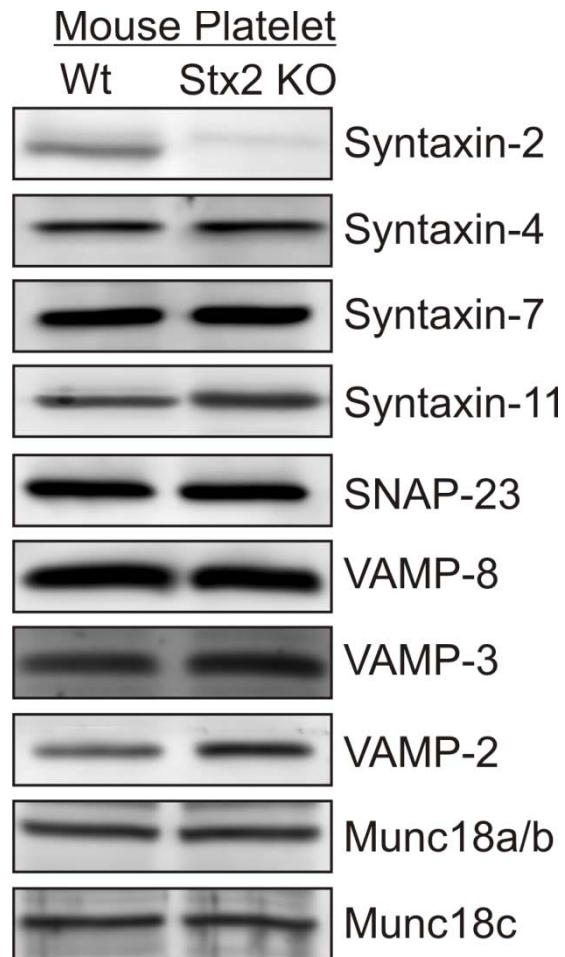
Figure 3-2. Deletion of Syntaxin-2 Does not Affect Secretory Machinery Proteins.
Platelet extracts (5×10^7 platelets/lane) from wild type and syntaxin-2 KO (Stx2 KO)
were probed by western blotting with the indicated antibodies.
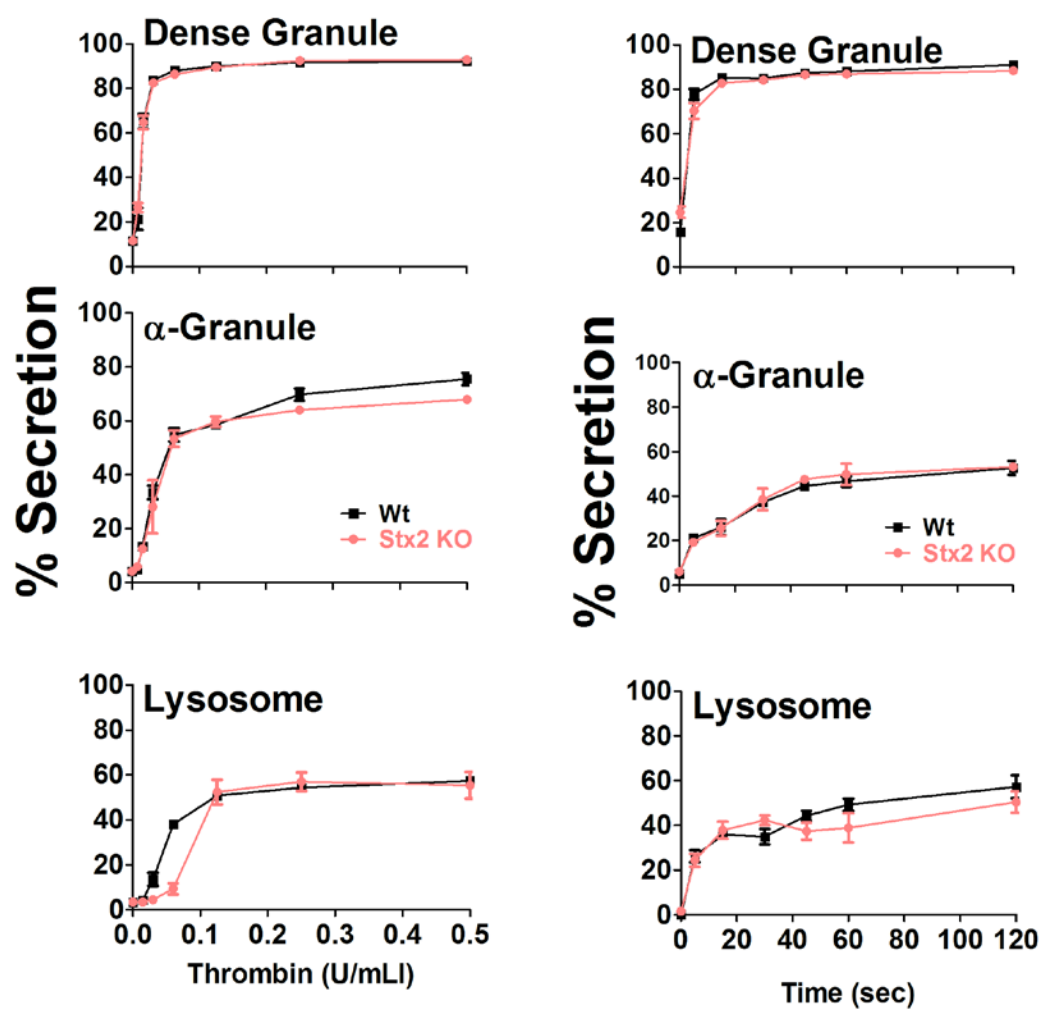
Figure 3-3. Deletion of Syntaxin-2 Does not Affect Platelet Secretion. Platelets from syntaxin-2 KO mice (red circle symbols) and wild type control (black quare symbols) were prepared as described in "Materials and methods". Aliquots of intact washed platelets (2.5×10^8 /mL) were incubated with 0.7 mM $CaCl_2$ at RT for 5 min and then incubated with the indicated thrombin concentration at RT for 1 mins (left panel) or 0.05 U/mL thrombin for the indicated time points (right panel). Release of [^{3}H]-5HT from dense granules, PF4 from α-granules, and β-hexosaminidase from lysosomes was measured, and percent secretion was calculated. The points represent the average of triplicate measurements and the standard deviations are indicated.

Figure 3-4. Deletion of Syntaxin-4 Does not Affect Resting Platelet Ultrastructure.
Washed platelets (4×10^8/mL) from wild type (Wt) and syntaxin-4 platelet specific KO
mice (Stx4 KO) were kept resting by adding PGI2. The platelets were fixed in 0.1%
glutaraldehyde for 15 min at 37°C and 3% glutaraldehyde for 1 hour at 4°C. After
osmication, the fixed platelets were dehydrated then polymerized in Spurr's resin. The
thin sections of platelets were counterstained with uranyl acetate and the samples were
analyzed using transmission electron microscope. The images were obtained using Gatan
software and only the brightness and contrast were adjusted in Photoshop (the scale bar is
indicated in left panel).
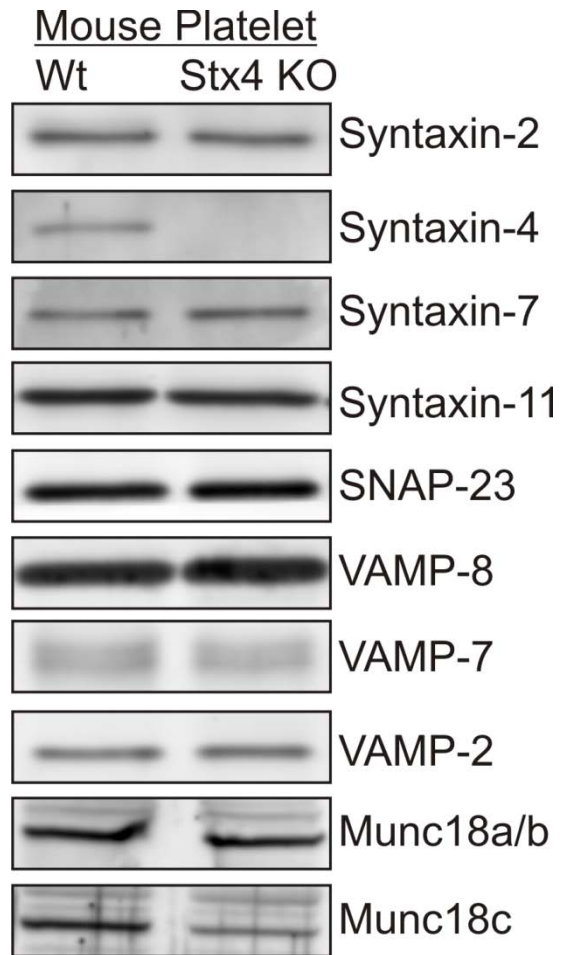
Figure 3-5. Deletion of Syntaxin-4 Does not Affect Other SNARE Proteins. Platelet extracts (5×10^7 platelets/lane) from wild type and syntaxin-4 platelet specific knockout (Stx4 KO) were probed by western blotting with the indicated antibodies.
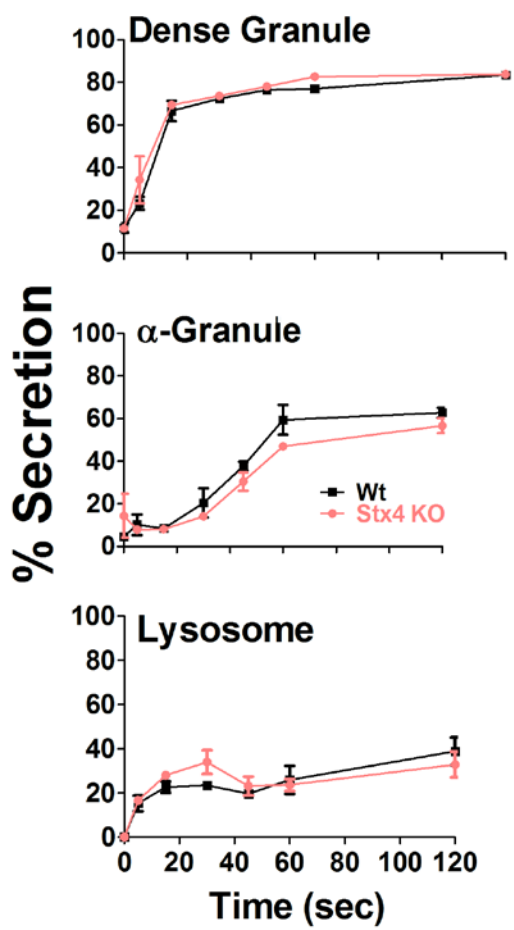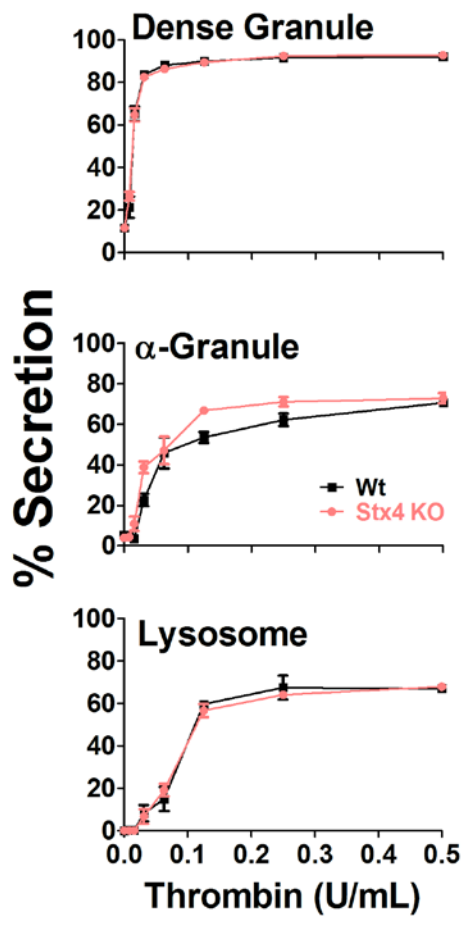
Figure 3-6. Deletion of Syntaxin-4 Does not Inhibit Platelet Secretion. Platelets from syntaxin-4 KO mice (red circle symbols) and wild type control (black square symbols) were prepared as described in "Materials and methods". Aliquots of intact washed platelets (2.5×10^8 /mL) were incubated with 0.7 mM $CaCl_2$ at RT for 5 min and then incubated with the indicated thrombin concentration at RT for 1 min (left panel) or 0.05 U/mL thrombin for the indicated time points (right panel). Release of [^{3}H]-5HT from dense granules, PF4 from α-granules, and β-hexosaminidase from lysosomes was measured, and percent secretion was calculated. The points represent the average of triplicate measurements and the standard deviations were indicated.
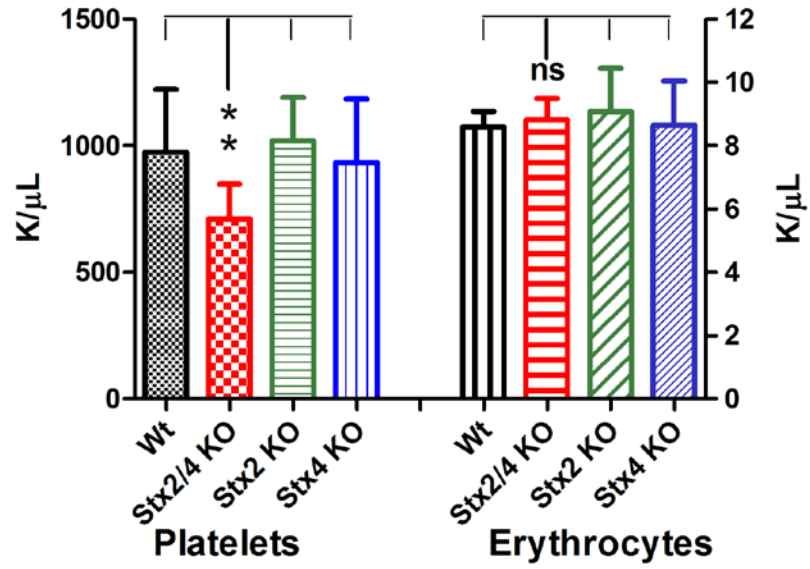
Figure 3-7. Syntaxin-2/4 Double Deficient Mice Have Lower Circulating Platelets.
Whole blood from syntaxin-2 (n=9), syntaxin-4 (n=8), syntaxin-2/4 double KO mice (n=28) or wild type littermate (n=20) was collected. The number of platelets and erythrocytes were counted with Hemvet.
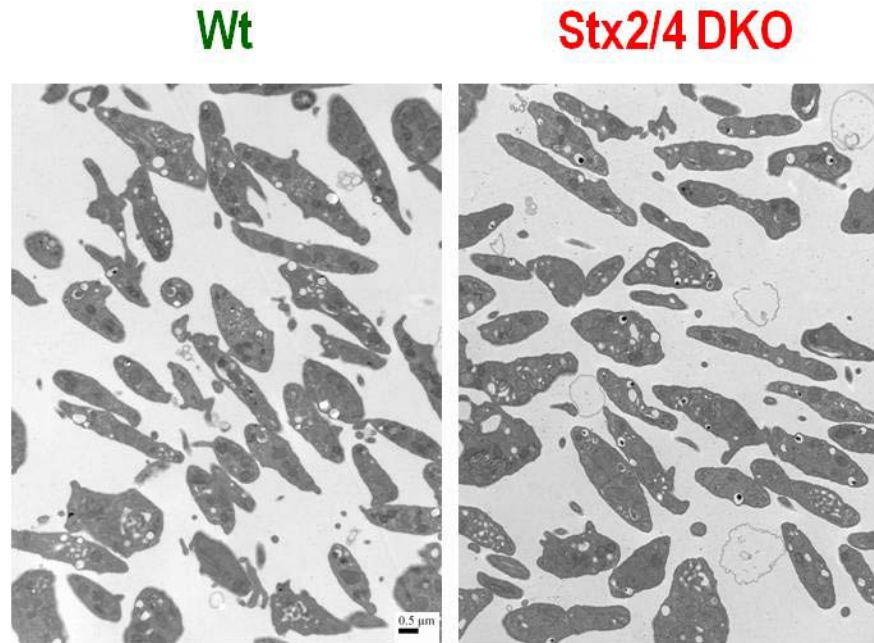
Figure 3-8. Syntaxin-2/4 Double Deficient Mice Have Normal Ultrastructure. Washed platelets (4×10^8/mL) from syntaxin-2/4 double KO mice and wild type litter mate were kept resting by adding PGI2. The platelets were fixed in 0.1% glutaraldehyde for 15 min at 37°C and 3% glutaraldehyde for 1 hour at 4°C. After osmication, the fixed platelets were dehydrated then polymerized in Spurr's resin. The thin sections of platelets were counterstained with uranyl acetate and the samples were analyzed using transmission electron microscope. The images were obtained using Gatan software and only the brightness and contrast were adjusted in Photoshop (the scale bar is indicated in left panel).
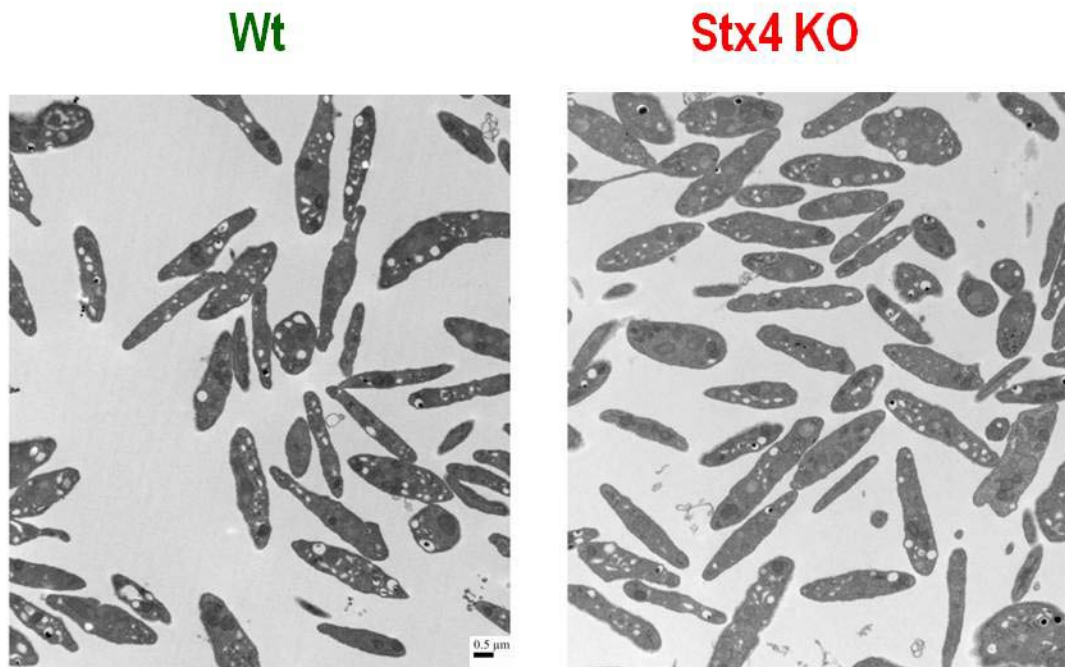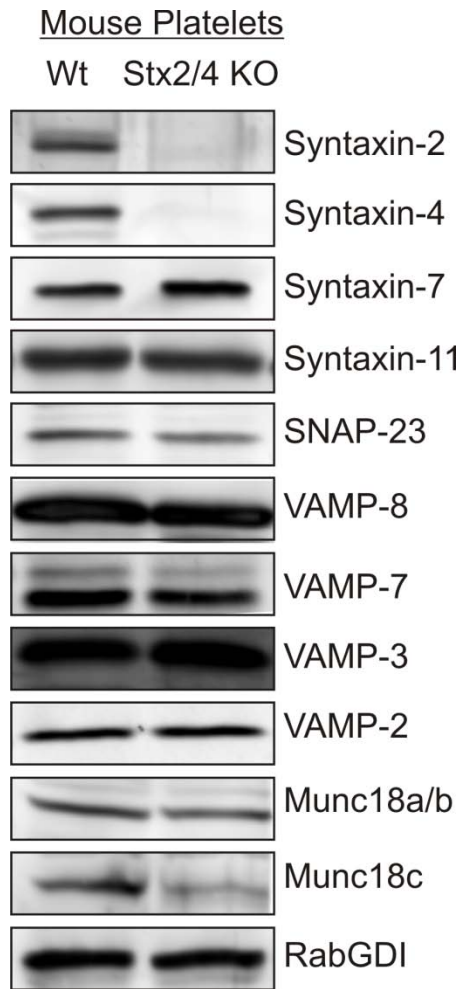
Figure 3-9. Double Deletion of Syntaxin-2/4 Does not Affect Other SNARE Proteins.
Platelet extracts (5×10^7 platelets/lane) from wild type and syntaxin-2/4 double KO (Stx2/4 KO) were probed by western blotting with the indicated antibodies.
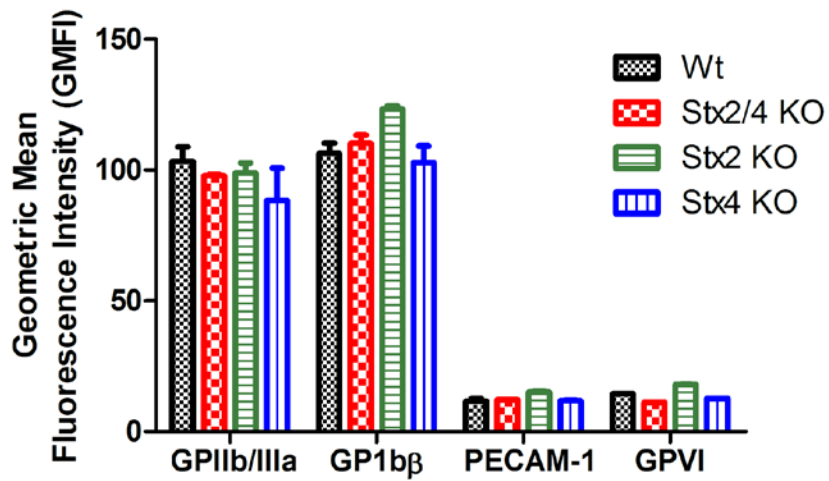
Figure 3-10. Depletion of Syntaxin-2/4 Mice Platelets Has No Effect on Surface Membrane Expression. Washed platelets (1×10^8 platelets/mL) from wild type (Wt), syntaxin-2/4 double KO (Stx2/4 KO), syntaxin-2 KO (Stx2 KO), and syntaxin-4 KO (Stx4 KO) were stimulated with or without 0.1 U/mL thrombin for 1 min and then incubated with FITC-conjugated anti-GPIIb/IIIa, GP1bβ, PECAM-1, and GPVI antibodies for 15 min at RT. The reactions were stopped by adding 10 volumes of 1% formaldehyde for 30 min and the fluorescent intensities were measured by flow cytometry.
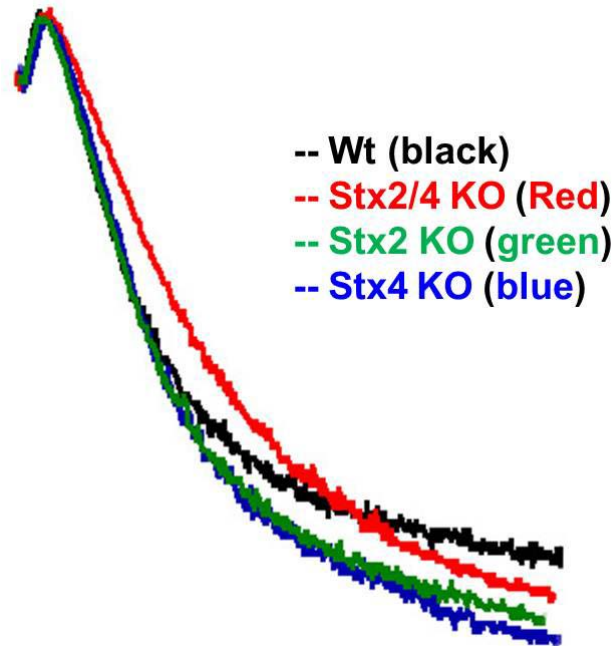
Figure 3-11. Syntaxin-2/4 Double Deficient Platelets Have Normal Aggregation Function. Platelets from wild type control (Wt, in black), syntaxin-2/4 double KO (Stx2/4 KO, in red) mice, syntaxin-2 KO (Stx2 KO, in green) mice, syntaxin-4 KO (Stx4 KO, in blue) mice, were prepared as described in Chapter 2. Platelets were stimulated with 0.1 U/mL of thrombin and aggregation was measured in the aggregometer.
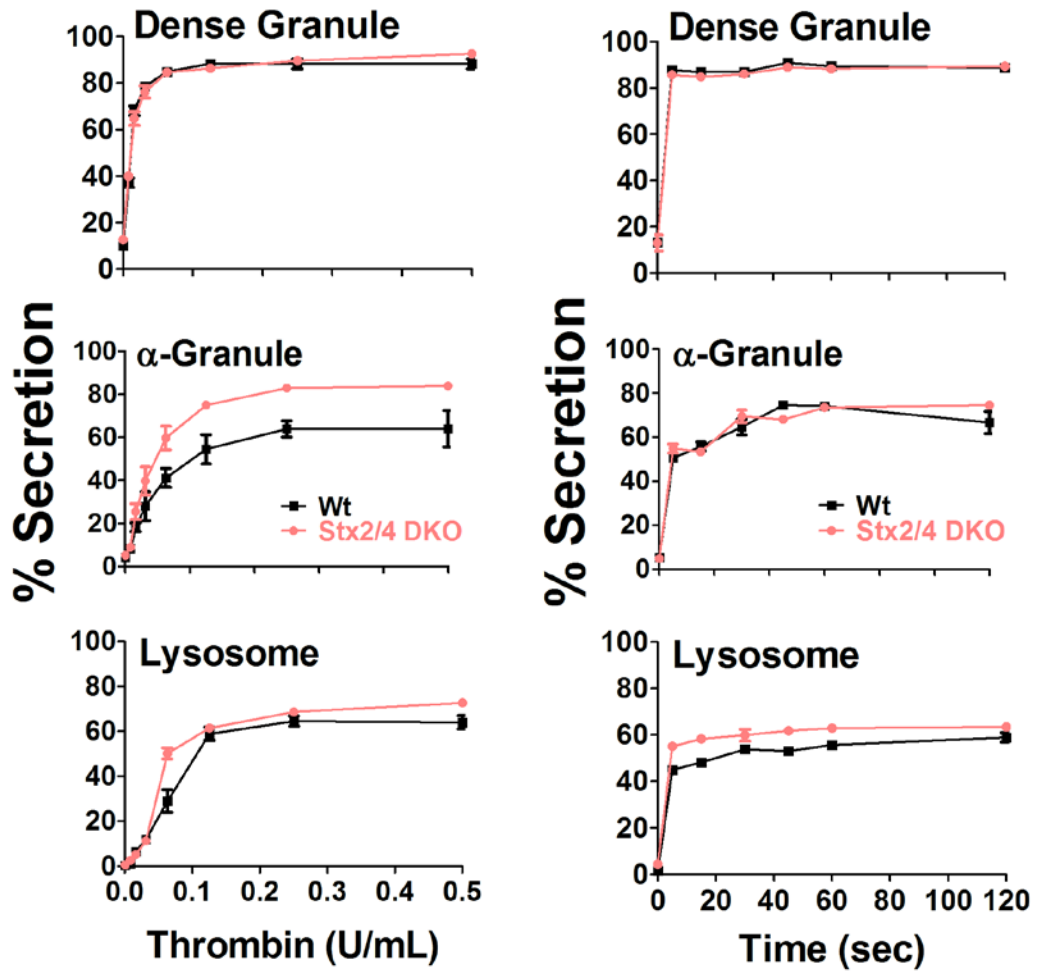
Figure 3-12. Double Deletion of Syntaxin-2/4 Does not Inhibit Platelet Secretion

Platelets from syntaxin-2/4 double KO (Stx2/4 DKO) mice (red circle symbols) and wild type control (black square symbols) were prepared as described in "Materials and methods". Aliquots of intact washed platelets (2.5×10^8 /mL) were incubated with 0.7 mM CaCl$_2$ at RT for 5 min and then incubated with the indicated thrombin concentration at RT for 1 min (left panel) or 0.05 U/mL thrombin for the indicated time points (right panel). Release of [^{3}H]-5HT from dense granules, PF4 from α-granules, and β-hexosaminidase from lysosomes was measured, and percent secretion was calculated. The points represent the average of triplicate measurements and the standard deviation is indicated.
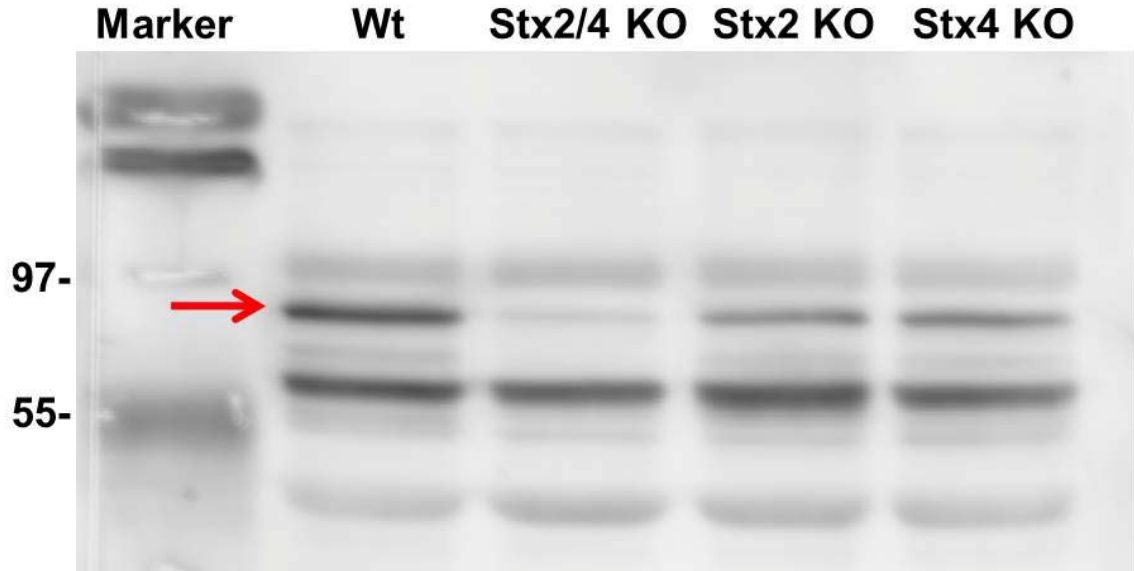
Figure 3-13. Double Deletion of Syntaxin-2/4 Affects Platelet Fibrinogen Storage.
Platelet extracts (5×10^7 platelets/lane) from wild type (Wt), syntaxin-2/4 double KO
(Stx2/4 KO), syntaxin-2 KO (Stx2 KO), and syntaxin-4 KO (Stx4 KO) were probed by
western blotting with anti-fibrinogen antibody. The red arrow indicates the position of
the alpha chain of fibrinogen.
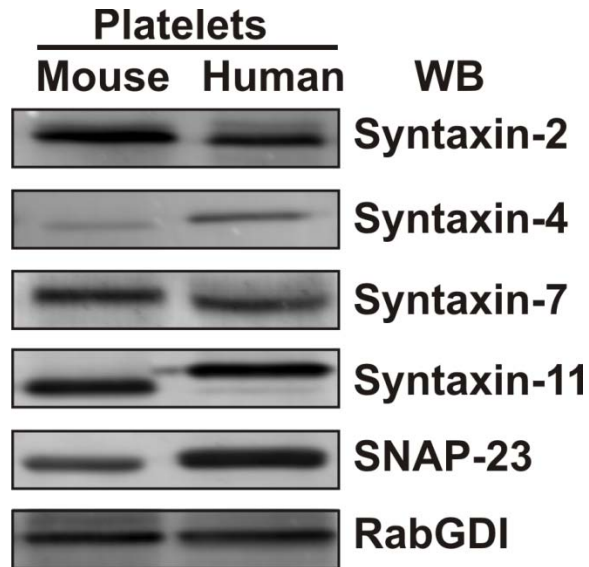
Figure 3-14. Anti-Human Syntaxin Antibodies Recognize Both Human and Murine Platelet Syntaxins. Equal amounts of human or murine platelet extracts (equivalent to 5.0×10^7 platelets/lane) were subjected to western blotting with the indicated antibodies.

His₆-Stx2 (ng) 5 × 10⁷ Platelets

His$_6$-Stx2 (ng) 5×10^7 Platelets

| 10 | 5 | 2.5 | 1.25 | m | m | m | m | h |

Syntaxin-2

His$_6$-Stx2 (ng) 5×10^7 Platelets

| 10 | 5 | 2.5 | 1.25 | m | m | m | m | h |

Syntaxin-4

His$_6$-Stx7 (ng) 5×10^7 Platelets

| 20 | 10 | 5 | 2.5 | 1.2 | m | m | m | m |

Syntaxin-7

His$_6$-Stx7 (ng) 5×10^7 Platelets

| 20 | 10 | 5 | 2.5 | 1.2 | h | h | h | h |

Syntaxin-7

GST-Stx11 (ng) 5×10^6 Platelets

| 10 | 5 | 2.5 | 1.25 | m | m | m | m | m |

Syntaxin-11

His$_6$-Stx11 (ng) 5×10^6 Platelets

| 10 | 5 | 2.5 | 1.25 | h | h | h | h | h |

Syntaxin-11

His$_6$-SNAP-23 (ng) 1×10^7 Platelets

| 10 | 5 | 2.5 | 1.25 | m | m | m | m |

SNAP-23

His$_6$-SNAP-23 (ng) 5×10^6 Platelets

| 10 | 5 | 2.5 | 1.25 | h | h | h | h |

SNAP-23

Figure 3-15. Syntaxin-11 is the Most Abundant t-SNARE in Platelets. Recombinant his-tagged human syntaxin-2, -4, -7, -11, and SNAP-23 and GST-tagged murine syntaxin-11 were used to generate a standard curve for quantification (using Enhanced Chemi-Fluorescence western blotting) of each t-SANREs in the indicated number of murine platelets (m) and human platelets (h).
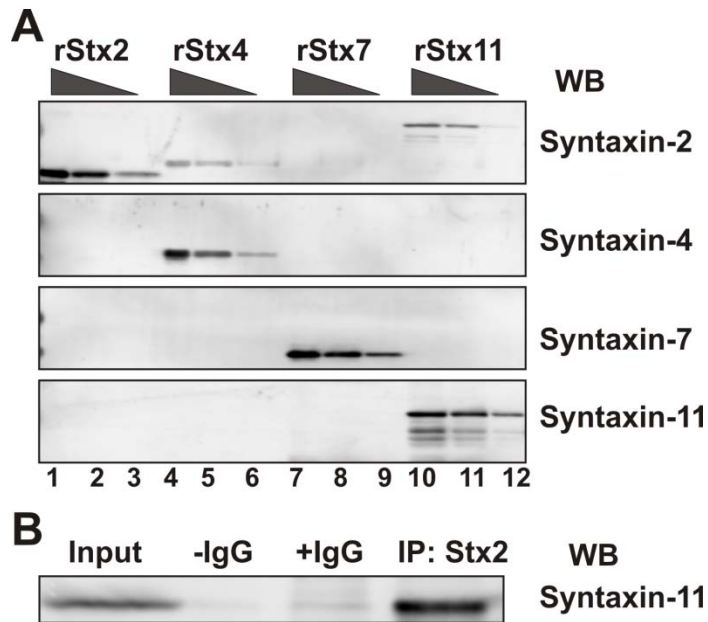
Figure 3-16. Endogenous Syntaxin-11 is Recognized by Syntaxin-2 Polyclonal Antibody. **A)** Recombinant his-tagged cytosolic domain of human syntaxin-2 (rStx2, lane 1-3), human syntaxin-4 (rStx4, lane 4-6), human syntaxin-7 (rStx7, lane 7-9), and GST-tagged human syntaxin-11 (rStx11, lane 10-12) were loaded with a series of amount: 20 ng, 10 ng, 2.5 ng per lane and probed by western blotting with the indicated antibodies. **B)** Human platelet extract was incubated with syntaxin-2 conjugated agarose beads. The specific bound proteins were eluted and subjected to western blotting by using indicated syntaxin-11 antibody. Controls include with or without IgG conjugated agarose beads.
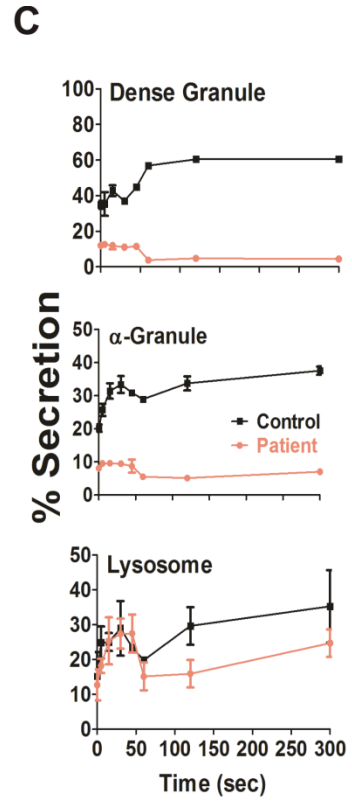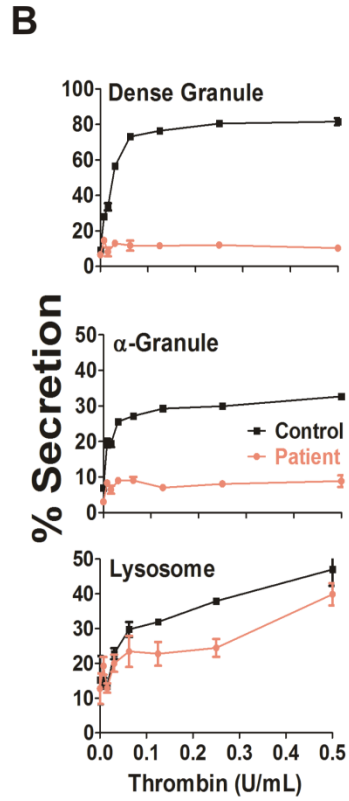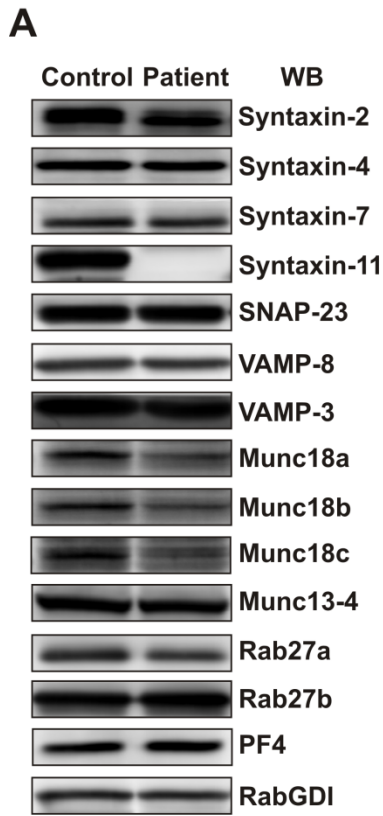
Figure 3-17. Syntaxin-11 Deficient Human Platelets Have Secretion Defects. Platelet extracts (5.0×10^7 platelets/lane) from control and FHL4 patients were probed by western blotting with the indicated antibodies (**A**). [^{3}H]-5HT labeled and washed platelets from FHL4 patient (red circle symbols) and normal control (black square symbols) were prepared as described in "Materials and methods". Aliquots of intact platelets (2.5×10^8 /mL) were incubated with 0.7 mM CaCl$_2$ at RT for 5 min and then incubated with the indicated thrombin concentration at RT for 1 min (**B**) or 0.05 U/mL thrombin for the indicated time points (**C**). Release of [^{3}H]-5HT from dense granules, PF4 from α-granules, and β-hexosaminidase from lysosomes was measured, and percent secretion was calculated. The points represent the average of triplicate measurements and the standard deviation is indicated. The statistical analysis was performed using ANOVA (GraphPad Prism 5), *p* values as indicated.
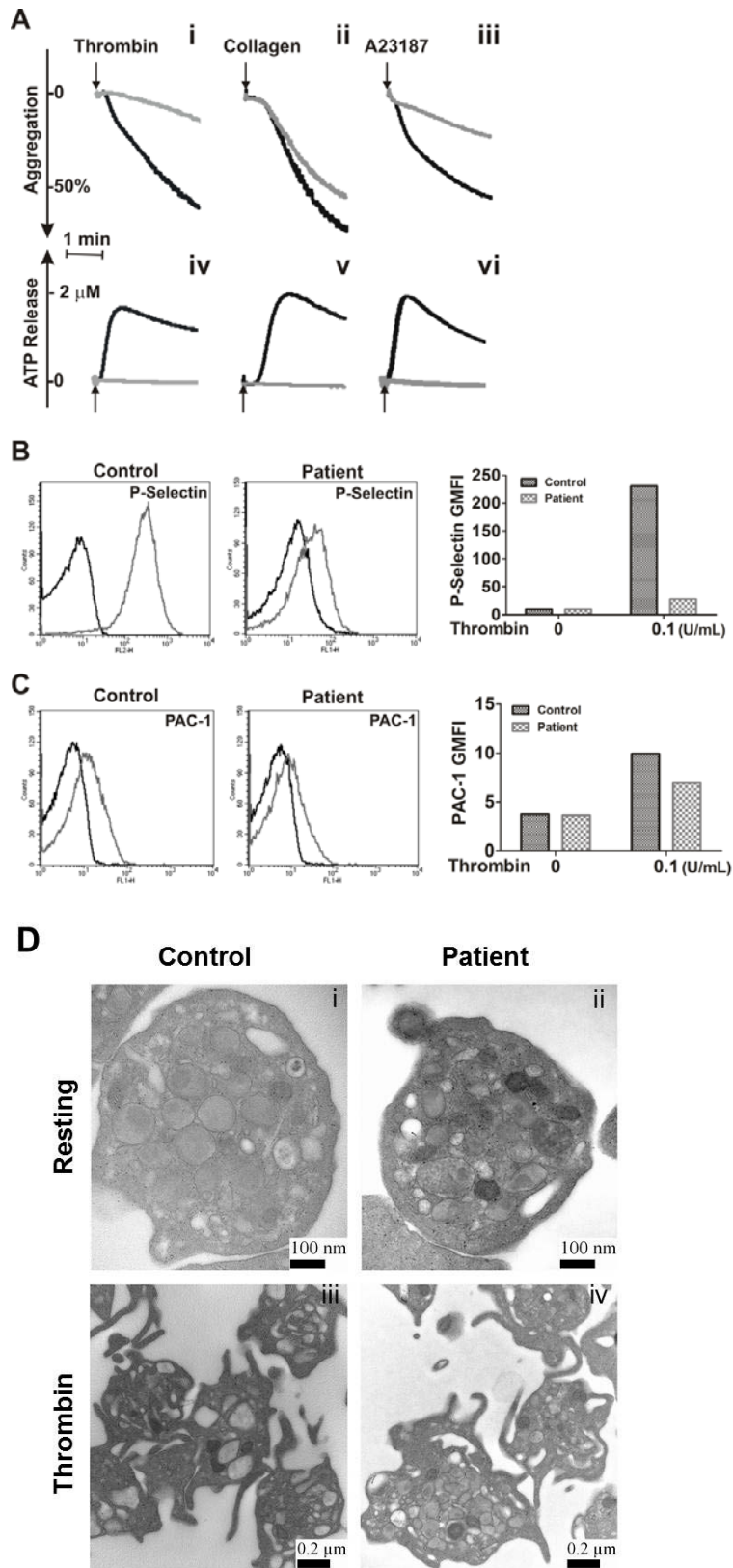
Figure 3-18. Loss of Syntaxin-11 in Platelets Affects Aggregation, ATP Release, and P-selectin Exposure. A) The washed platelets from control (black traces) and patient (gray traces) were stimulated with thrombin (0.1 U/mL, **i)**, collagen (10 µg/mL, **ii)**, and A23187 (100 nM, **iii**) for 2-3 min. The aggregation traces were monitored in the platelets (**i-iii**) concurrently the released ATP monitored by a luciferin/luciferase-induced luminescence (**iv-vi**). (**B**) and (**C**) Washed platelets (1×10^8/mL) from normal control (Control) and FHL4 patient (Patient) were stimulated with or without 0.1 U/mL thrombin for 1 min and then incubated with FITC-conjugated anti-P-selectin, or FITC-conjugated PAC-1 antibodies for 15 min at RT. The reactions were stopped by adding 10 volumes of 1% formaldehyde for 30 min and the fluorescent intensities were measured by flow cytometry. The data from each FACS run were plotted in histogram and graphed using Geo Mean Fluorescence Intensity (GMFI): P-selectin (**B**) and PAC-1 (**C**). **D)** Deficiency of syntaxin-11 does not affect resting platelet ultrastructure and cytoskeletonal rearrangement. Washed platelets (4×10^8/mL) were kept resting by adding PGI_2 (i-ii) or stimulated with 0.1 U/mL thrombin (iii-iv) for 5 min. The platelets were fixed in 0.1% glutaraldehyde for 15 mins at 37°C and 3% glutaraldehyde for 1 hour at 4°C. After osmication, the fixed platelets were dehydrated then polymerized in Spurr's resin. The thin sections of platelets were counterstained with uranyl acetate and the samples were analyzed using transmission electron microscope. The images were obtained using Gatan software and only the brightness and contrast were adjusted in Photoshop. The magnification of images was shown as indicated scale bars.
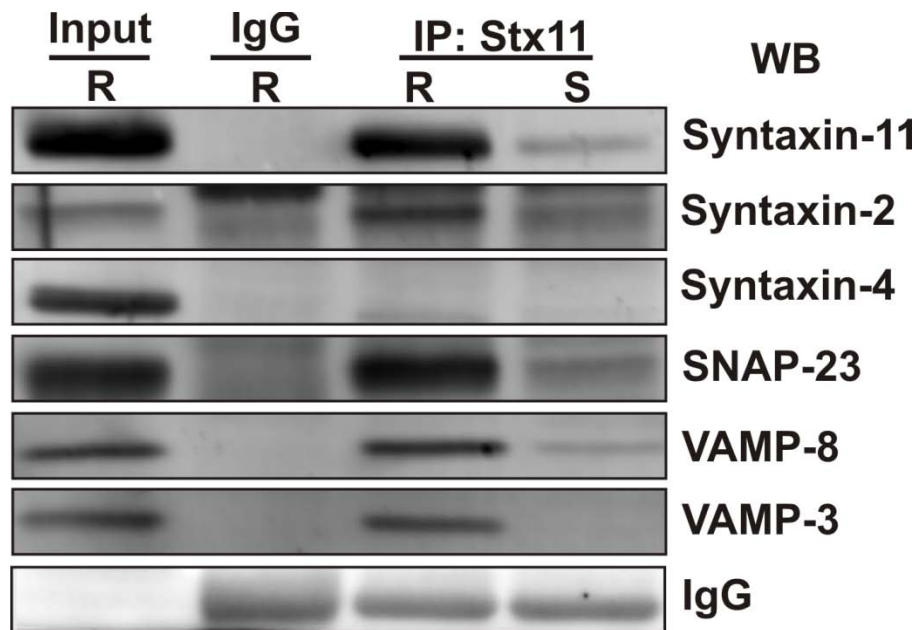
Figure 3-19. Syntaxin-11 is Associated with Platelet Core SNAREs. Platelet extracts from resting (R) or thrombin stimulated platelets (S) were prepared by solubilization with 1% Triton-100. After clarification, platelet extracts were incubated with syntaxin-11 polyclonal antibody or IgG control for 3 h at 4°C, then extension of 1h by adding Protein-A-Agarose. The bound proteins were eluted and separated by SDS-PAGE, followed by western blotting with the indicated antibodies.

Chapter FOUR

The Role of Tomosyn in Platelet Secretion

Introduction

Platelet secretion is critical for hemostasis and thrombosis. Hypoactive platelets cause bleeding problems while hyperactive platelets cause thrombosis, which eventually leads to heart attack or stroke. Control and regulation of platelet secretion is very important for normal platelet function. It has been generally believed that most regulated exocytosis processes are mediated by highly conserved SNARE proteins. VAMPs (v-SNARE), interact with two types of target membrane SNAREs: syntaxin-type and SNAP-25-type (t-SNAREs), serving as the minimal components to drive bilayer fusion [33]. An interaction between the v-SNARE and t-SNAREs is been thought to provide the specificity for individual fusion events, with additional accessory proteins regulating spatial and temporal aspects of the process [136, 137]. In platelets, the core SNARE machinery, which includes VAMP-8, SNAP-23, and syntaxin-11, are defined using genetic and biochemical analysis [40, 41, 46] and [Chapter 3]. Further efforts to look for t-SNARE interacting proteins are thought to be a method to identify regulatory components that control SNARE complex formation. These data will build a detailed picture of SNARE machinery necessary for platelet secretion. By using t-SNARE containing complexes as "bait", we found that tomosyn-1, granuphilin, and Munc18b, from platelet extracts, could specifically associate with different set of SNARE complexes. Tomosyn-1 had not been previously identified in platelets. Tomosyn-1, originally identified as a neuronal, syntaxin1A-binding protein, is a 130 kDa, cytoplasmic protein. There are two genes for tomosyn (-1 and -2) in mammals. Tomosyn-1 contains two domains: an N-terminal WD40 repeats and an R-SNARE coiled-coil domain in C-terminus, which are linked by a hypervariable region. The WD40 repeat domain is predicted to fold into a two, β-propeller structure, which is important for protein-protein interactions. Tomosyn-1 has three splice variants and shares 98% sequence identity between human and rat (shown on Fig.1). Over-expression of tomosyn-1 in neuronal or endocrine cells inhibits secretion, this together with its C-terminal, R-SNARE motif

suggests that tomosyn-1 plays a negative role in secretion. Consistently, knockdown of tomosyn-1 in β-cells increases insulin secretion [112]. Also, the loss of tomosyn-1 in neurons enhances neurotransmitter release [107]. Depletion of tomosyn-1 in *C. elegans* causes an increase in neurotransmitter release by enhancing vesicle priming [138, 139]. Although data supports a negative role for tomosyn-1 in exocytosis, there are several conflicting results from the neuronal field. Tomosyn-1 knockdown in SCG neurons has been shown to inhibit synaptic transmission [111]. Tomosyn-2 shares the same structure and alterative splicing pattern with tomosyn-1 and has been thought to have the similar function as tomosyn-1 [102, 103, 140]. Although data supports a negative role for tomosyn-1 in exocytosis, there are several conflicting results from the neuronal field. Tomosyn-1 knockdown in SCG neurons has been shown to inhibit synaptic transmission [111].

The goal in this Chapter was to identify SNARE regulators and characterize their function. To this end, we have identified tomosyn-1 in platelets and determined its novel function in platelets and hemostasis. Our findings suggest tomosyn-1 is a positive regulator for platelet secretion, thus important for hemostasis and thrombosis.

Results:

Tomosyn-1 is Present and Associated with SNAREs in Platelets

Although the core SNARE machinery has been studied in platelets, SNARE complex regulation and its coupling to signaling cascades in the platelet has not been as well studied. Among the core SNARE complex components (VAMP, syntaxin, and SNAP-23), only syntaxin contains a potential regulatory domain (Habc domain), suggesting syntaxin might be important for regulation of t-SNARE activation and/or SNARE complex formation. Thus, it seems logical that components which specifically interact with syntaxins may be potential regulatory proteins. To identify potential regulatory proteins from platelet extracts, we performed pulldown assays using syntaxin-containing complexes as "bait" and then analyzed the bound proteins by mass spectrometry. Four specific bands were resolved by SDS-PAGE and SYPRO Ruby staining (Figure 4-1A). Mass spectrometry identified all four (labeled as T1, T2, T3, T4)

(Table 4-1). Tomosyn-1 (T1 and T2) was found to associate with both syntaxin-2/SNAP-23 and syntaxin-4/SNAP-23 complexes, which is in agreement with the previous data that tomosyn-1 can form high affinity ternary complexes with syntaxin4/SNAP-23 [113]. To verify expression of tomosyn-1 in platelets, we probed the platelet lysate (Input) and pulldown samples with antibodies directed against a peptide sequence in tomosyn-1 (a region shared by all three isoforms). A specific, immunoreactive band was only seen when t-SNARE heterodimers were used as "bait" (Figure 4-1B), but not Munc18a and Munc18c containing complexes were used. This indicates that tomosyn-1 might not co-exist with Munc18a or Munc18c on SNARE complexes (Figure 4-1B), but tomosyn-1 could be present in the same complex with Munc18b (Figure 4-1A, -1B). To further determine which tomosyn-1 isoform is present in platelets. RT-PCR was performed using b-, m-, and s-tomosyn-1 specific primers. Only m-tomosyn-1 was detected (Figure 4-1C), suggesting m-tomosyn-1 is most abundant isoform in platelets.

Granuphilin, specifically interacted with Munc18a/syntaxin-2 complex, Munc13-4 and Munc13-1 also specifically bound to this complex but not to the Munc18c/syntaxin-2 complex suggesting that Munc18a, but not Munc18c, can interact with Munc13-4 and granuphilin in platelets (Figure 4-8). VAMP-8 and SNAP-23 were not shown to be present in Munc18a/syntaxin-2 complex. This work was begun before syntaxin-11's role was appreciated. Future studies on interacting proteins with syntaxin-11 containing complexes will be a fruitful direction to pursue to identify other regulatory proteins.

M-tomosyn-1 and Munc18a binding to syntaxin-1 has been shown mutually exclusive using recombinant proteins [104]. We found that tomosyn-1 and Munc18b were associated with SNAP-23/syntaxin-2 complex (Figure 4-1A, -1B). Our finding is consistent with studies from adipocytes, showing Munc18c and b-tomosyn-1 binding to syntaxin-4 [113]. Further studies will need to clarify whether other components are required so that Munc18 can co-exist with tomosyn-1 in the same complexes. It is generally thought that tomosyn-1 functions by interacting with t-SNAREs and blocking v-SNARE binding, thus prevent the formation of fusogenic SNARE complexes [98]. The syntaxin-2/SNAP-23 complex, but not syntaxin-4/SNAP-23, was associated with tomosyn-1, Munc18b, and VAMP-8 (R-SNARE). This data suggests that tomosyn-1

could exist in the same complex with VAMP. Similar observations have been reported by Widberg *et al.* [113]. In adipocytes, Munc18c, but not Munc18b, associates with tomosyn-1 and the SNARE complex (syntaxin-4/SNAP-23/VAMP-2), suggesting specific tomosyn-1/Munc18s pairings in different tissue are important for fusogenic SNARE complex formation.

VAMP-8, SNAP-23, and syntaxin-11 are the primary v- and t-SNAREs required for platelet secretion [40, 41, 46]. *In vitro* complex pulldown assay showed that tomosyn-1 was associated with syntaxin-2/SNAP-23 and syntaxin-4/SNAP-23 heterodimers (Figure 4-1A, -1B). Experiments were designed to determine if endogenous tomosyn-1 was capable of interacting with endogenous core SNARE machinery components, which include syntaxin-11, SNAP-23, and VAMP-8 in platelets. Both resting and thrombin-activated human platelets were lysed in Triton X-100 and immunoprecipitations were performed using control IgG and anti-tomosyn-1 monoclonal antibodies. Three t-SNAREs, syntaxin-2, syntaxin-4, and SNAP-23, were found in the tomosyn-1 immunoprecipitates, suggesting endogenous tomosyn-1 is associated with endogenous syntaxin-2 or -4 / SNAP-23 complexes. This is consistent with our *in vitro* complex pulldown data (Figure 4-2). Endogenous tomosyn-1 co-immunoprecipitated with syntaxin-11 from both resting and thrombin-activated platelet extracts, indicating tomosyn-1 might be involved in platelet secretion through interaction with functional t-SNAREs. Association with VAMP-3 and VAMP-8 was not detectable, which is consistent with the notion that R-SNARE motif of tomosyn-1 blocks v-SNARE binding to t-SNAREs. It is likely that VAMP-8 and tomosyn-1 are present in two different complexes since extra t-SNARE complexes were used in the pulldown assay. Taken together, these data demonstrate that tomosyn-1 is associated with core SNARE complex components, SNAP-23 and syntaxin-11 in activated platelets, suggesting tomosyn-1 may regulate functional SNARE complex formation *in vivo*.

Tomosyn-1 is Phosphorylated and Partially Associated with Membrane

Tomosyn-1 can form stable complexes with t-SNARE heterodimers through its C-terminal R-SNARE domain [141]. It also has been shown that neuronal tomosyn-1 is phosphorylated at Ser-724 by PKA and the PKA-catalyzed phosphorylation of tomosyn-1

enhances neurotransmitter release through increased formation of the SNARE complex [111]. Thus, tomosyn-1 could be regulated by phosphorylation. In platelets, PKA is activated in resting cells while PKC is activated upon. To address the potential role of tomosyn-1 phosphorylation in platelets, we investigated whether tomosyn-1 was phosphorylated *in vivo* by using ^{32}P metabolically-labeled platelets. Figure 4-3A shows that phosphorylated tomosyn-1 was immunoprecipitated from both resting platelets treated with or without PGI_2 and platelets activated by thrombin. The phosphorylation level is similar in resting and activated platelets. This result differs from that in neurons, suggesting that tomosyn-1 phosphorylation may not be important in platelets. It is also possible that PKA is responsible for tomosyn-1 phosphorylation in resting platelets while PKC is associated with tomosyn-1 phosphorylation upon platelet activation, thus the level of phosphoryation observed did not change. Further experiments will be required to address this point

     Tomosyn-1 is a cytosolic protein while SNAREs are membrane proteins. If tomosyn-1 regulates SNARE complex formation through direct interaction with t-SNAREs, a portion of tomosyn-1 should be detected associated with membranes. If tomosyn-1 is a negative regulator in platelet through competing binding site with VAMP, tomosyn-1 would expect to translocate from membrane upon platelet activation, which allows SNARE complex formation and membrane fusion. To test this scenario and determine whether tomosyn-1 is dissociated from the membranes upon platelet activation, we performed membrane fractionation experiments. Tomosyn-1 was found to be present in both membrane and cytosol fraction (Figure 4-3B). Membrane association did not change upon thrombin stimulation, suggesting that tomosyn-1 does not dissociate from membranes upon activation and secretion (Figure 4-3C). The membrane association increased upon NEM-treatment, suggesting tomosyn-1 may anchor to membrane through association with SNAREs. This observation is consistent with a previous report that tomosyn-1/syntaxin/SNAP-23 complexes can be dissembled by NSF (which is NEM sensitive) [104]. As a control, SNAP-23 and syntaxin-4 membrane association did not change since both proteins are membrane proteins. In summary, unlike neuronal tomosyn-1, which behaves as a negative regulator through PKA

phosphoryation, phosphorylation and membrane association of platelet tomosyn-1 do not change upon stimulation, suggesting tomosyn-1 may have a different role in platelets.

Tomosyn-1 is Important for Platelet Secretion

To address whether tomosyn-1 plays a role in platelet secretion, we examined release from intact, tomosyn-1 KO mouse platelets. To exclude non-specific effects to the secretory machinery due to a loss of tomosyn-1, washed platelet extracts from tomosyn-1 KO (Tomo KO) and wild type littermates (Wt) were prepared and probed by western blotting analysis. The protein levels of fifteen other secretory components were not significantly altered by the deletion of tomosyn-1 (Figure 4-4A). A thrombin titration experiment was performed using a 1 min incubation time (Figure 4-4B). The secretion from α-granules and lysosomes was significantly decreased by the loss of tomosyn-1 compared with wild type, suggesting that tomosyn-1 is required for release from these two granules. The release from dense granules was slightly inhibited, suggesting that other components are compensating for the loss of tomosyn-1 or that it is not required.

To confirm whether tomosyn-1 is required for platelet exocytosis, we further analyzed dense granule secretion from tomosyn-1 null platelets by using lumi-aggregometry. Figure 4-5, A-D and F-H shows the aggregation traces for washed platelets (control, black traces; patient, gray traces) stimulated with 10 µg/mL collagen, 100 µM PAR4, 100 nM A23187, convulxin, and thrombin (0.025, 0.05, 0.1 U/mL). As can be seen there is a significant defect in aggregation for the tomosyn-1 deficient platelets. Concomitantly, ATP release from dense granules was significantly inhibited as measured with luciferin/luciferase-induced luminescence (Figure 4-5, a-d and f-h). Figure 4-5, E and I show in bar graph format, the release of ATP from dense granule stimulated with different agonists. Secretion induced by the calcium ionphore, A23187, is impaired, suggesting that the secretion defect seen on tomosyn-1 deficient platelets is caused by the final SNARE-mediated fusion step and not at some upstream signaling step.

To further confirm that the secretion defect, we also performed flow cytometry analysis. P-selectin and LAMP-1 are integral membrane proteins in α-granules and lysosome. Their exposure at the platelet surface is widely used as a metric for α-granule

and lysosome exocytosis. Compared to normal control and resting platelets, the geometric mean fluorescence intensity (GMFI) of staining for P-selectin and LAMP-1 on tomosyn-1 deficient platelets only slightly increased upon stimulation with 0.1 U/mL thrombin (Figure 4-6, A-B). When quantified, the P-selectin and LAMP-1 staining on tomosyn-1 null platelets was 2 fold less than those of wild type controls, suggesting that release from α-granule and lysosome is significantly inhibited.

To explore whether loss of tomosyn-1 affects granule biogenesis, we compared the levels of several granule markers. The level of the dense granule marker, serotonin and the lysosome marker, β-hexosaminidase was not significantly different (Figure 4-4B). There were 2538 ± 66 CPM/2.5×10^8 from control platelets (n=12) and 3083 ± 91 CPM/2.5×10^8 from tomosyn-1 deficient platelets (n=24) for serotonin. The level of β-hexosamidase was 64 ± 6 U/2.5×10^8 (Wt, n=12) *vs.* 60 ± 4 U/2.5×10^8 (Tomo KO, n=12). The level of α-granule marker, PF4, was reduced 3 fold in tomosyn-1 deficient platelets: 1771 ± 184 pg/2.5×10^8 (Wt, n=12) *vs.* 573 ± 82 pg/2.5×10^8 (Tomo KO, n=12), suggesting loss of tomosyn-1 causes reduction in intra-platelet PF4 storage. Further studies are required to clarify if the observed reduction is caused by biosynthesis or trafficking. Because the secretion assay is determined as percentage secretion, the PF4 storage level does not affect the result that there is an α-granule release defect in the tomosyn-1 KO platelets.

To exclude that the observed secretion defect is caused by signaling, we examined two important platelet activation markers: platelet cytoskeletal reorganization and integrin $\alpha_{IIb}\beta_3$ activation. Activated $\alpha_{IIb}\beta_3$ was measured by flow cytometry analysis using the JonA antibody. In Figure 4-4C, the histogram displays both JonA binding curves for thrombin activated platelets (grey trace) from control and tomosyn-1 KO platelets. There was no significant difference between wild type and the tomosyn-1 KO platelets, which was confirmed by quantification (right panel of Figure 4-4C), suggesting that tomosyn-1 depletion does not impair platelet $\alpha_{IIb}\beta_3$ inside-out signaling. In sum, tomosyn-1 deficiency inhibited platelet secretion without affecting integrin activation, granule centralization, platelet aggregation, and granule genesis, indicating tomosyn-1 is important for platelet exocytosis.

Tomosyn-1 is Important for Hemostasis and Thrombosis

The primary function of platelets is in hemostasis. To study whether deletion of tomosyn-1 affects thrombosis and hemostasis, we used two *in vivo* assays. First, tail-bleeding time tests were performed on 5-6 weeks old littermates generated from tomosyn-1 heterozygous siblings. In Figure 4-7A, among 24 tomosyn-1 KO mice we tested, only three mice had short or normal bleeding times, the remaining 21 mice bled for more than ten minutes. The wild type with the same background showed normal bleeding time with a median bleeding time of 226.4 ± 37.3 seconds (n=19). This prolonged tail-bleeding time in tomosyn-1 KO mice demonstrates that tomosyn-1 is required for hemostasis and suggests that tomosyn-1 plays a positive role in platelet secretion and hemostasis.

To further confirm that this robust *in vivo,* phenotype was due to loss of tomosyn-1, we utilized the carotid artery, $FeCl_3$-injury thrombosis model. The model looks at thrombosis in response to endothelial cell damage by $FeCl_3$ and measured to the time required for carotid artery occlusion post-damage induction. In agreement with the prolonged tail bleeding times, there was a robust thrombosis defect in tomosyn-1 deletion mice as compared to wild type littermates (Figure 4-7B). The time to occlusion in wild type littermates was around 2.7 minutes. Surprisingly, under the same condition, the occlusion time for tomosyn-1 deficient mice was not observed, except one mouse around 7 minutes. These *in vivo* data provide strong evidence that tomosyn-1 is required for platelet secretion, hemostasis, and thrombosis.

Discussion:

In summary, tomosyn-1 is present and phosphorylated in platelets. Tomosyn-1 was associated with the endogenous functional t-SNARE heterodimer, syntaxin-11/SNAP-23 in platelets. Deletion of tomosyn-1 in platelets reduced platelet secretion and thus impaired hemostasis and thrombosis. It is clear that tomosyn-1 is present in platelets and important for platelet secretion, hemostasis, and thrombosis. In agreement with previous studies showing tomosyn-1 interacts with t-SNARE heterodimers [104, 113, 141], tomosyn-1 was found associated with syntaxin/SNAP-23 heterodimers (Figure 4-1A, -1B) *in vitro*. In particular, tomosyn-1 was found associated with syntaxin-

11/SNAP-23 heterodimers in platelets (Figure 4-2). Studies have shown that incubating cells with NEM could inhibit NSF [142, 143]. This enzyme has been suggested to catalyze the disassembly of tomosyn-1/SNARE complexes. We observed an increase in tomosyn-1 membrane association upon NEM-treatment, supporting the notion that tomosyn-1 was associated with membranes through formation stable complexes with t-SNAREs (Figure 4-3B, -3C). Tomosyn-1 is believed to be a negative regulator of neurotransmitter release through its R-SNARE motif and through its phosphoryation by PKA [98]. If this is the case in platelets, we would expect to see tomosyn-1 translocate to the cytosol and phosphorylated upon platelet activation. Surprisingly, we did not find that tomosyn-1 dissociated from membrane into the cytosol and tomosyn-1 phosphorylation was not changed upon stimulation. These data suggest that tomosyn's function in platelets may be different that in neurons. Our secretion data support this contention. Taken together, our i*n vitro* data does not support negative role for tomosyn-1 in platelets.

Tomosyn-1 is important for platelet secretion from α-granules and lysosomes. Further, tomosyn-1 is required for hemostasis and thrombosis. Overall, tomosyn-1 is important for physiological platelet function and hemostasis, suggesting that tomosyn-1 plays a positive role in platelet exocytosis, possible through interaction with syntaxin-11/SNAP-23.

Originally tomosyn-1 was shown to disrupt the neuronal Munc18a/syntaxin-1a complex by binding to syntaxin-1a and has thus been proposed to be a positive modulator of SNARE complex formation [104]. It is possible that tomosyn-1 is required for activating syntaxin by releasing syntaxin-11 from Munc18b into an open stage. Our results, in agreement of Widberg *et al* [113], showed that all the components can still be in a fusion complex which probably requires some conformational change for activation of fusion. Another possibility is that tomosyn-2 might be important for neurotransmitter release while tomosyn-1 is important for platelets and adipocyte exocytosis. The inhibitory phenotype shown in tomosyn-1 KO mice may reflect tomosyn-1 competition with tomosyn-2 [107]. Moreover, there are conflicting results from neuronal field. Tomosyn-1 knockdown in SCG neurons inhibits synaptic transmission [111]. It has also

been shown that down regulation of tomosyn-1 reduced insulin secretion from an insulin-secreting INS-1E cell line [144].

Table 4-1. MS of Syntaxin Complex Interacting Proteins

Sample	MS Score	Protein
T1	144	Tomosyn-1
T2	66	Tomosyn-1
T3	325	Munc18b
T4	259	Granuphilin

The specific interacting proteins from Figure 4-1, which were indicated on the top as T1-T4 were excised from the gel and subjected to trypsin digestion. The samples were spotted onto a matrix-assisted laser desorption ionization (MALDI) target plate. Both mass spectrometry (MS) and tandem time-of-flight (TOF-TOF) spectra were acquired and processed. The data were searched against the database using MASCOT software. The MASCOT search results were shown.

A

Munc18a	-	-	-	-	-	+	+
SNAP-23	-	+	+	+	+	-	-
Syntaxin	-	2	2	4	4	2	2
Plt. Ext.	+	+	-	+	-	+	-

116 kDa—

66 kDa —

T1, T2, T3, T4

B

Munc18	-	-	-	-	a	c	c	
SNAP-23	-	+	+	-	-	-	-	
Syntaxin	-	2	4	-	2	2	4	
Plt. Ext.	+	+	+	-	+	+	+	Input

Tomosyn

VAMP-8

C

Random Hexamers   Oligo d(T)₁₆

s   m   b   s   m   b
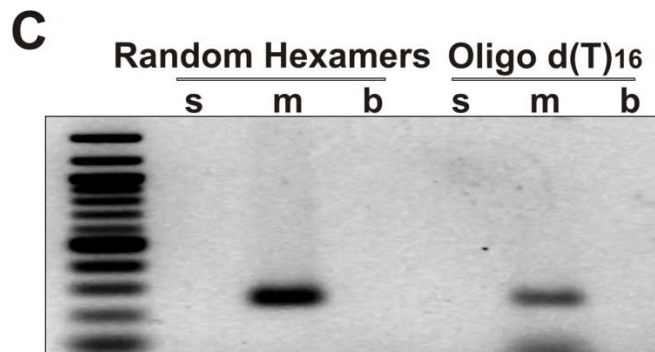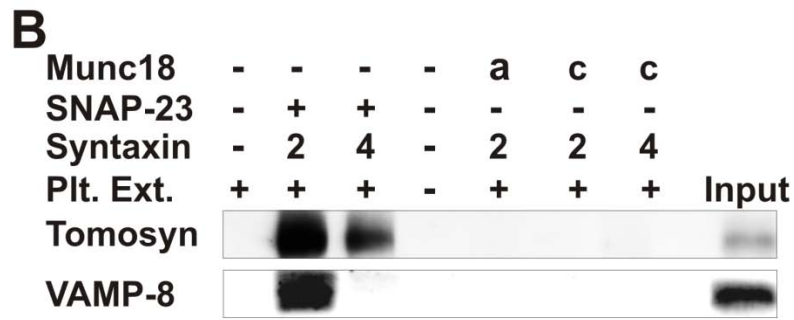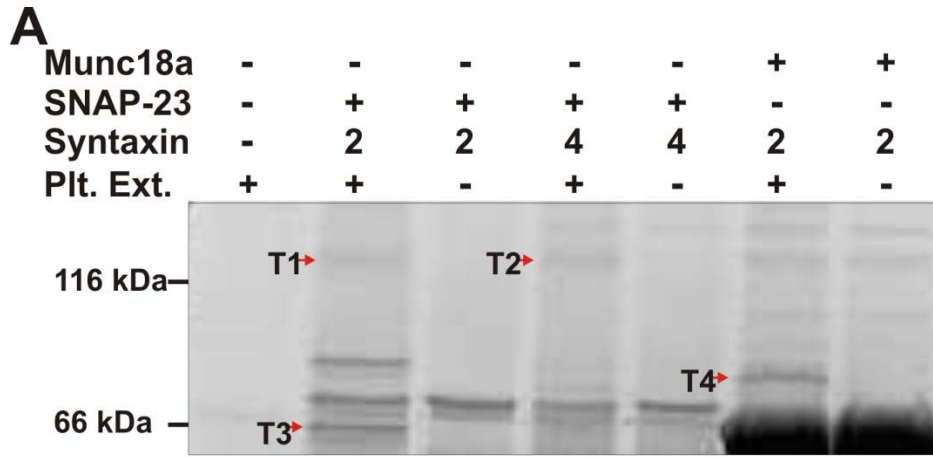
Figure 4-1. Tomosyn-1 is Present in Platelets. **A)** The indicated complexes were produced in *E.coli* using the pRSFDuet plasmid with a S-tag peptide fused to the C-terminus of the syntaxin. Complexes are bound to S-Protein-Agarose beads through the S-tag. After washing, the complex-bound beads were incubated with solubilized platelet extracts. The beads were washed again, and then the proteins were eluted with SDS-PAGE sample buffer. The eluted proteins were separated by SDS-PAGE and stained with Sypro Ruby. The specific interacting proteins which were indicated on the top as T1-T4 were excised from the gel and subjected to trypsin digestion. The samples were spotted onto a matrix-assisted laser desorption ionization (MALDI) target plate. Both mass spectrometry (MS) and tandem time-of-flight (TOF-TOF) spectra were acquired and processed. The data were searched against the database using MASCOT software. The MASCOT search results are shown on the Table 4-1. **B)** The indicated complexes were produced in *E.coli* using the pRSFDuet plasmid with a S-tag peptide fused to the C-terminus of the syntaxin. Solubilized platelet extracts were incubated with the different complexes and the resulting material was recovered on S-Protein-Agarose. After washing, the proteins were eluted and probed by western blotting with the antibodies to the indicated proteins. **C)** RT-PCR using platelet-derived RNA with tomosyn-1 isoform-specific primers shows only expression of PCR product corresponding to m-tomosyn-1 mRNAs while not to b- and s-tomosyn-1 mRNAs in human platelets. Marker = 1-kb DNA ladder.
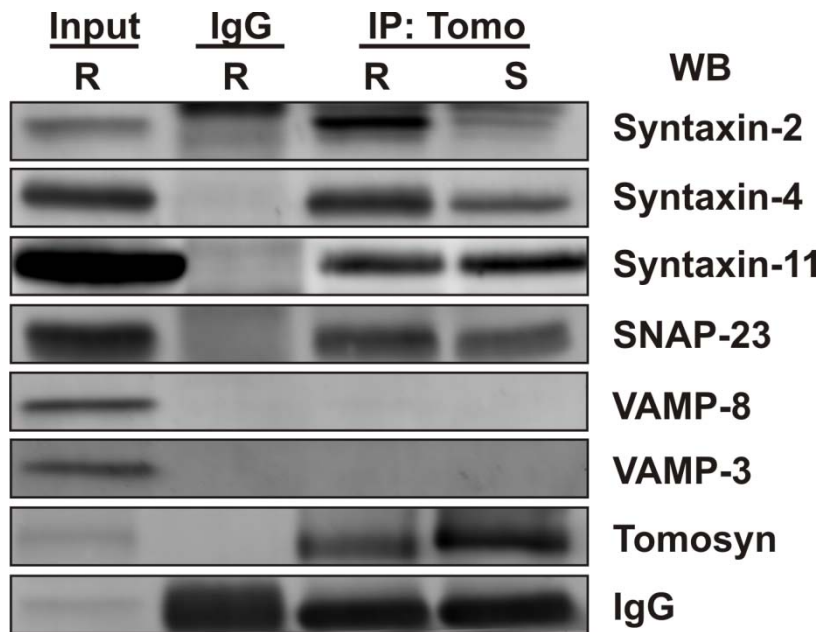
Figure 4-2. Tomosyn-1 is Associated with Platelet Core SNARE Fusion Complexes. Platelet extracts (Plt Ext.) from resting (R) or thrombin stimulated platelets (S) were prepared by solubilization with 1% Triton-100. After clarification, platelet extracts were incubated with anti-tomosyn-1 murine monoclonal antibody or an IgG (1:1 ratio mixture of murine and rabbit IgG) control for 4 h at 4°C. The immune complexes were recovered with Protein-G-Aagarose. The bound proteins were eluted and separated by SDS-PAGE, followed by western blotting with the indicated antibodies.

A

Tomo Ab	-	-	+	+	+
IgG	-	+	-	-	-
Thrombin	-	-	-	+	-
PGI$_2$	-	-	-	-	+

Phosphor image

-220 kDa

-97 kDa

WB: Tomosyn

-119 kDa

B

Thrombin	-	-	+	+	+	+
NEM	-	-	-	-	+	+
Cytosol	+	-	+	-	+	-
Membrane	-	+	-	+	-	+

Tomosyn

SNAP-23

RabGDI

Syntaxin-4

C

Legend: Tomo, SNAP-23, Stx4

Y-axis: Membrane Association %

X-axis: Resting, Thr, Thr/NEM
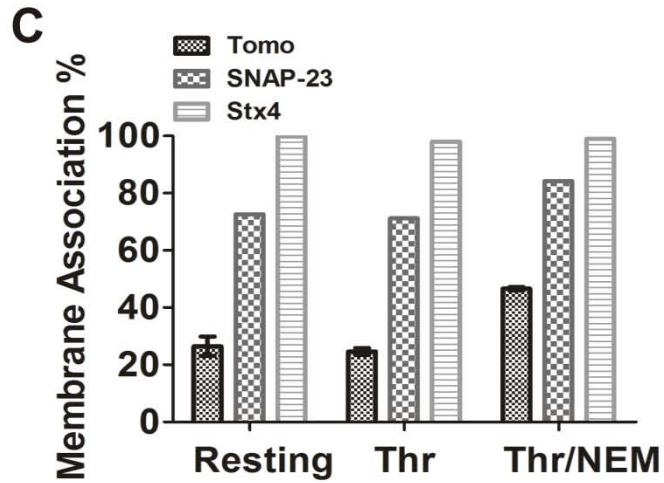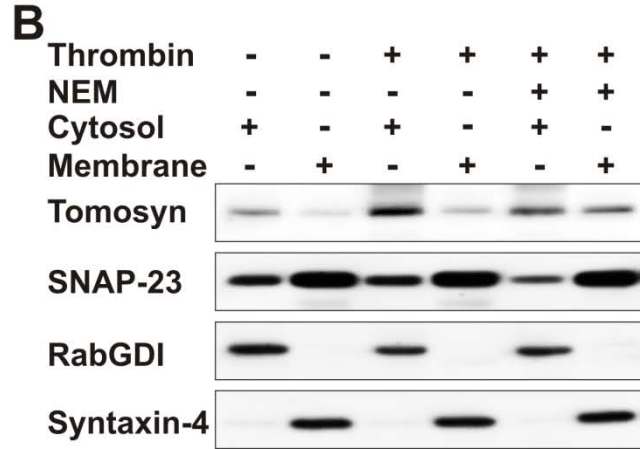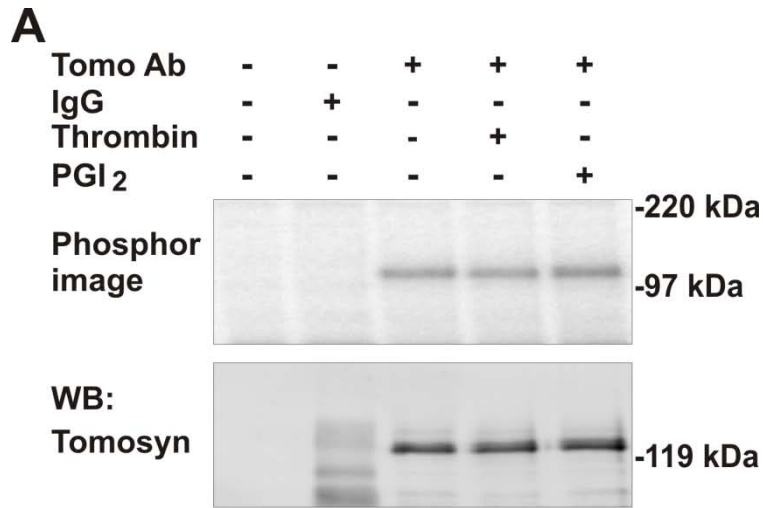
Figure 4-3. Tomosyn-1 is Phosphorylated in Both Resting and Activated Platelets and Membrane Association Increases upon NEM Treatment. A) Metabolically [^{32}P]-labeled platelets were stimulated with or without thrombin for 2 min. Platelets were solublized with 1% Triton-100 to make platelet extract. Protein A Sepharose, conjugated with anti-tomosyn-1 monoclonal antibodies were incubated with the labeled platelet extract. After washing, the bound proteins were analyzed with SDS-PAGE, followed by Phosphor Imaging and western blot for tomosyn-1. **B)** Resting and thrombin activated platelets treated with or without NEM were sonicated, followed by centrifugation to separate membrane and cytosol fractions. The equivalent amount of two fractions were loaded and analyzed with SDS-PAGE, followed by western blotting for the indicated proteins. ImageQuant 5.2 software was used to analysis of immunolabeled bands. The data are presented as means ± SD.

A

Wt	Tomo KO	WB
		Tomosyn
		Syntaxin-2
		Syntaxin-4
		Syntaxin-7
		Syntaxin-11
		SNAP-23
		VAMP-8
		VAMP-3
		VAMP-2
		Munc18a
		Munc18b
		Munc18c
		Munc13-4
		Rab27a
		Rab27b
		RabGDI

B

Dense Granule

α-Granule

% Secretion

- Wt
- Tomo KO

Lysosome

Thrombin (U/mL)
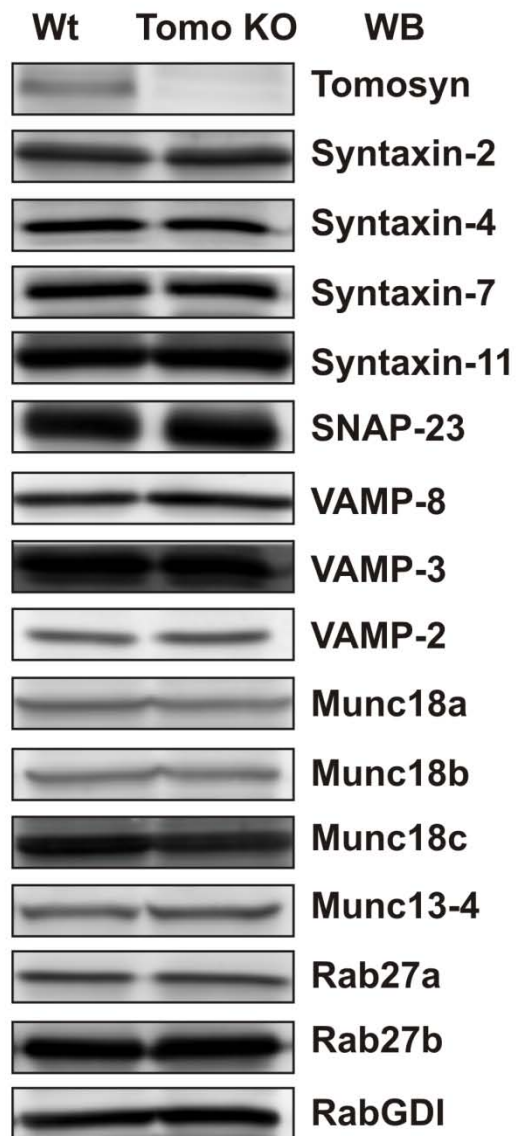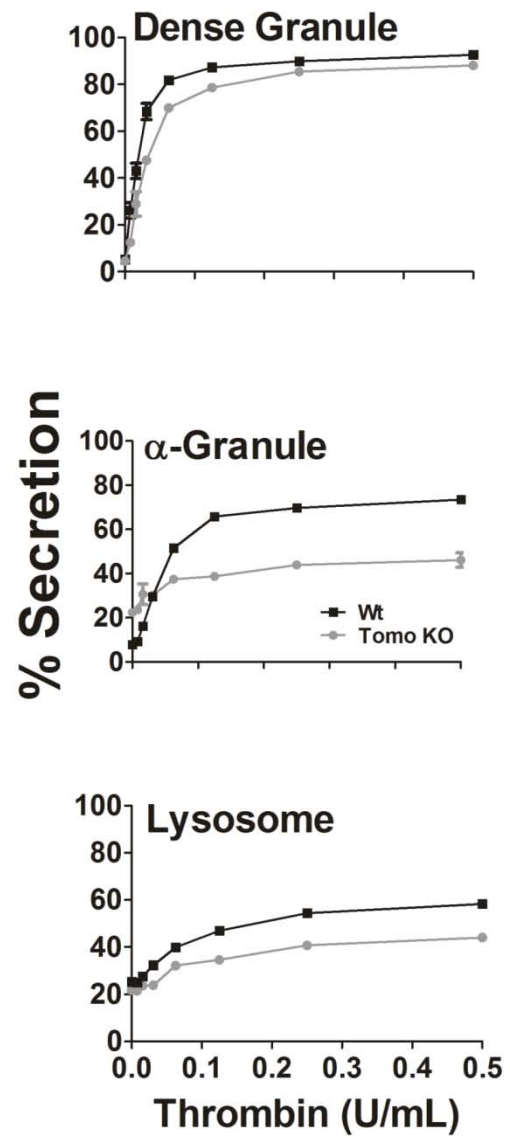
Figure 4-4. Tomosyn-1 Deficient Murine Platelets Have Secretion Defects. A) Platelet extracts (5.0×10^7 platelets/lane) from wild type (Wt) and tomosyn-1 KO (Tomo KO) mice were probed by western blotting with the indicated antibodies. [^{3}H]-5HT labeled and washed platelets from tomosyn-1 null (red circle symbols) and Wt (black square symbols) were prepared and incubated with 0.7 mM $CaCl_2$ at RT for 5 min, prior to activation with the indicated thrombin concentration for 1 min. Release of [^{3}H]-5HT from dense core granules, PF4 from α-granules, and β-hexosaminidase from lysosomes was measured, and percent secretion was calculated. The points represent the average of triplicate measurements and the standard deviation is indicated. The statistical analysis was performed by ANOVA (GraphPad Prism 5), $p<0.001$.
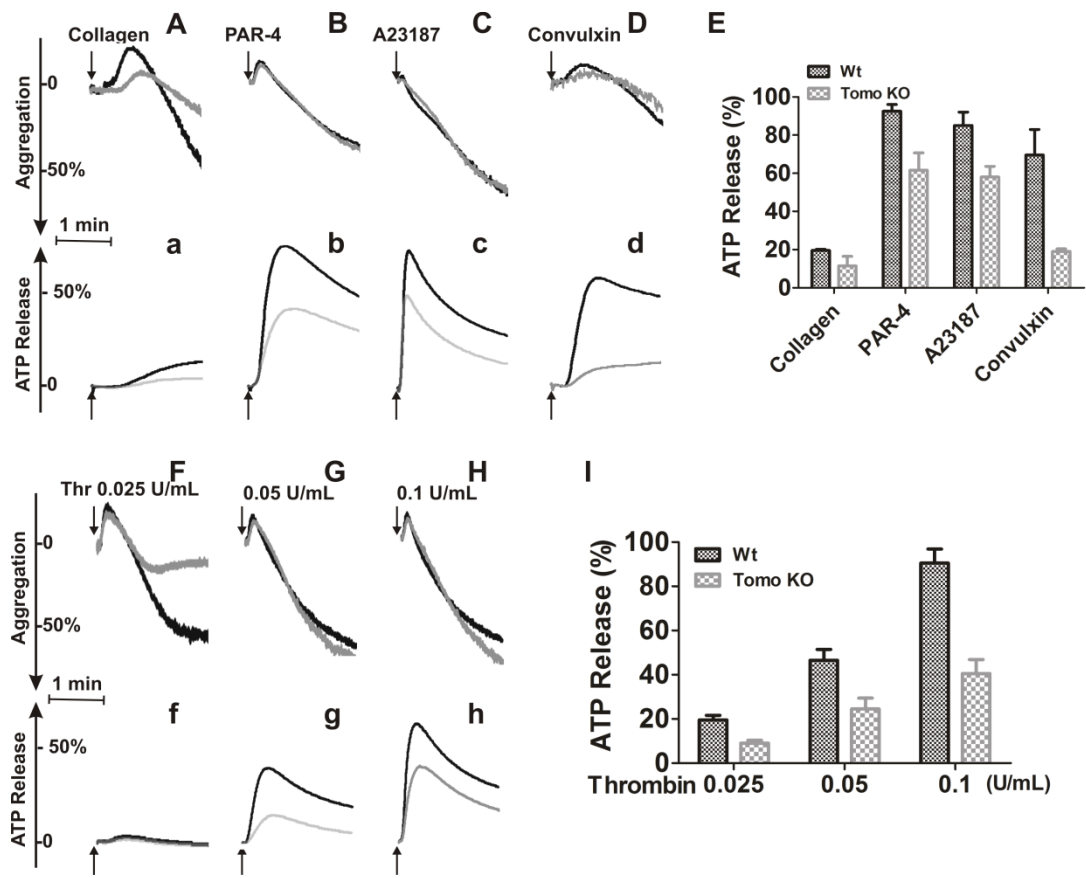
Figure 4-5. Deletion of Tomosyn-1 in Platelets Affects ATP Release but has Limited Effects on Aggregation. Washed platelets from Tomosyn-1 null (Tomo KO) and wild type (Wt) mice were stimulated with collagen (5 µg/mL, A), PAR4 peptide (100 µM, B), A23187 (10 nM, C), convulxin (10 µg/mL, D), or thrombin (0.025 U/mL, F; 0.05 U/mL, G; 0.1 U/mL, H) for 2-3 minutes. The aggregation traces were monitored (A-D and F-H) and concurrently the released ATP monitored by a luciferin/luciferase-induced luminescence (a-d and f-h). The absolute percentage ATP release comparing to background PPP was graphed (E and I).
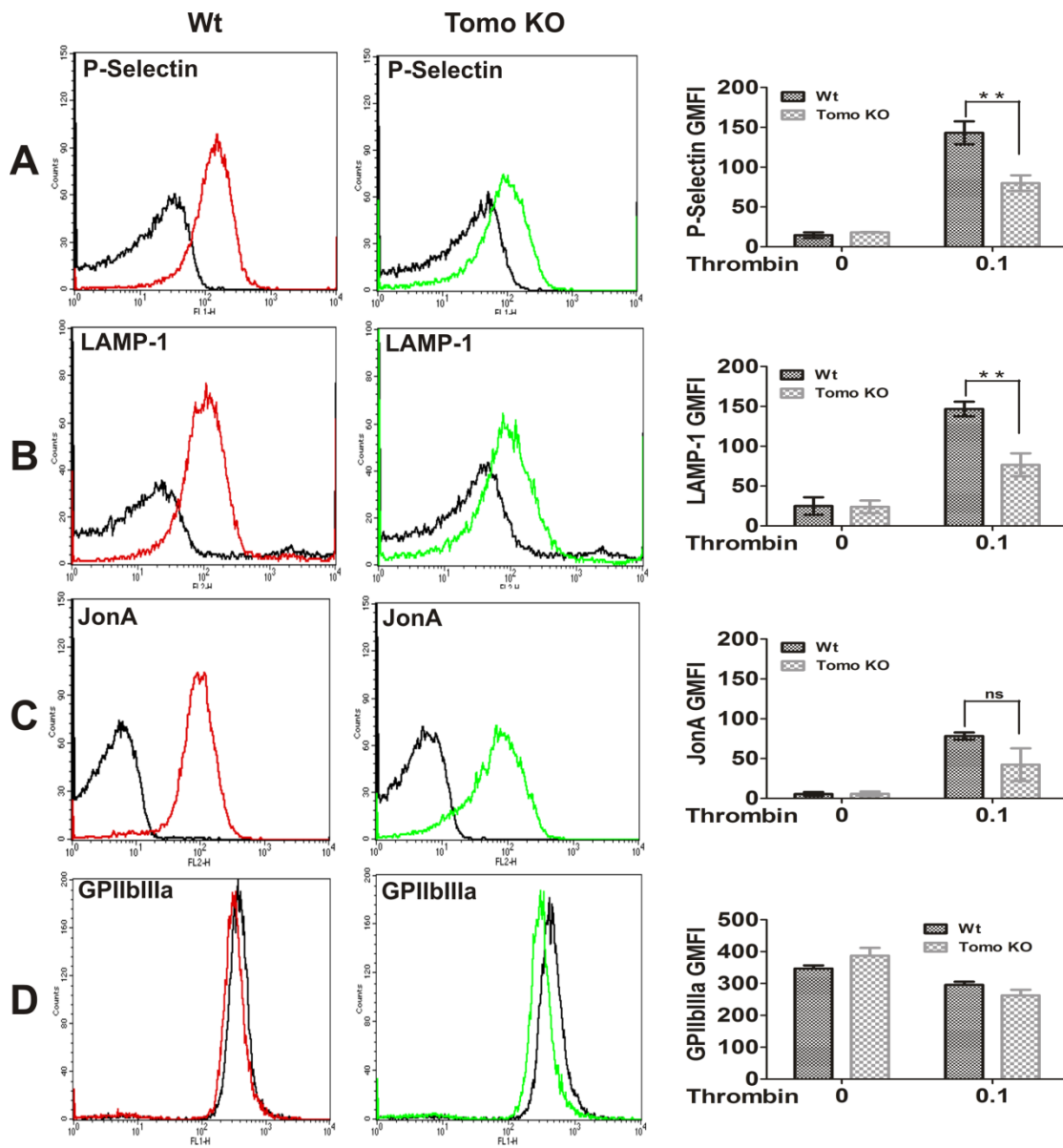
Figure 4-6. Depletion of Tomosyn-1 in Platelets Affects P-selectin and LAMP-1 Exposure but not Intergrin Activation. Washed platelets (1×10^8/mL) from wild type (Wt) and tomosyn-1 null (Tomo KO) mice were stimulated with or without 0.1 U/mL thrombin for 1 min and then incubated with FITC-conjugated anti-P-selectin, or FITC-conjugated LAMP-1, or FITC-conjugated anti-integrin αIIbβ3, or PE-conjugated JonA antibodies for 15 min at RT. The reactions were stopped by adding 10 volumes of 1% formaldehyde for 30 min and the fluorescent intensities were measured by flow cytometry. The data from a representative FACS run are shown and cumulative data was graphed using Geo Mean Fluorescence Intensity (GMFI): P-selectin (A), LAMP-1 (B), JonA (C), and GPIIbIIIa (D).
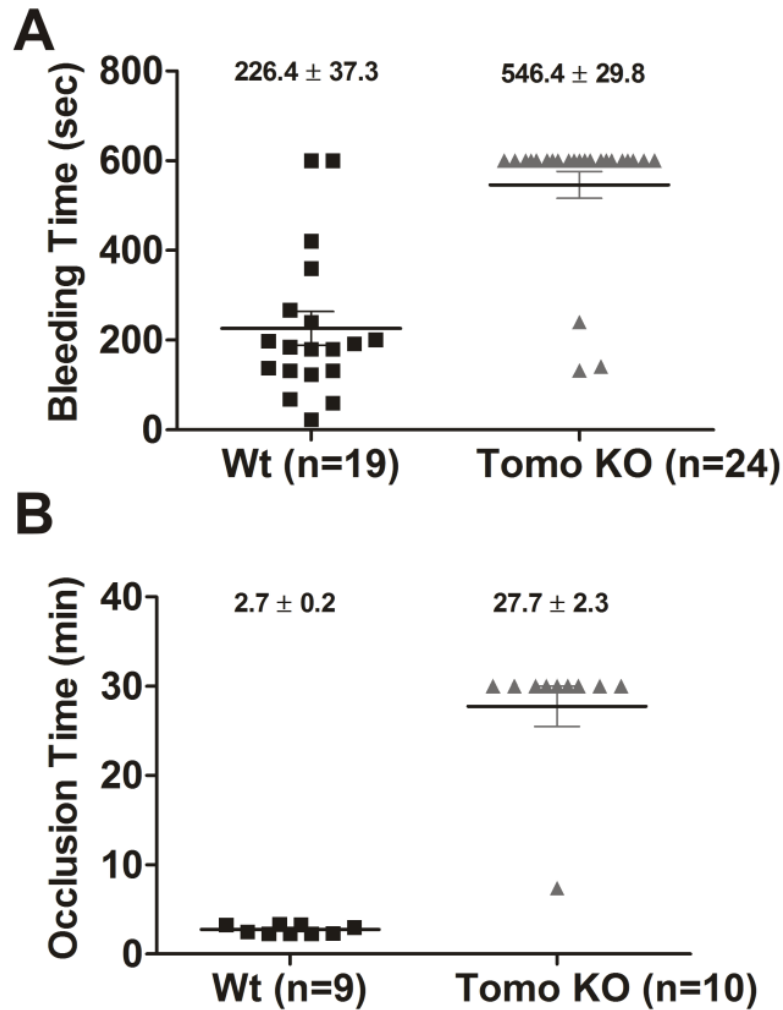
Figure 4-7. Tomosyn-1 Deficient Mice Have Bleeding Phenotype. Mice from Tomosyn-1 null (Tomo KO) and wild type (Wt) littermates were used for tail-bleeding time assay and $FeCl_3$-induced arterial thrombosis. The animal number, mean and standard deviation are indicated ($p < 0.0001$, ANOVA). **A)** Tail bleeding times were measured after tail transection. The tail tips were used for genotyping. **B)** Occlusion time in $FeCl_3$-induced arterial thrombosis test.

	Munc18/Syntaxin				SNAP-23/Syntaxin			
Munc18	c	c	a	a	-	-	-	-
Syntaxin	2	2	2	2	4	4	2	2
SNAP-23	-	-	-	-	+	+	+	+
Plt. Extract	+	+	-	+	-	+	-	+	-

Munc13-1

Munc13-4

Tomosyn

Vamp-8

SNAP-23

Munc18c
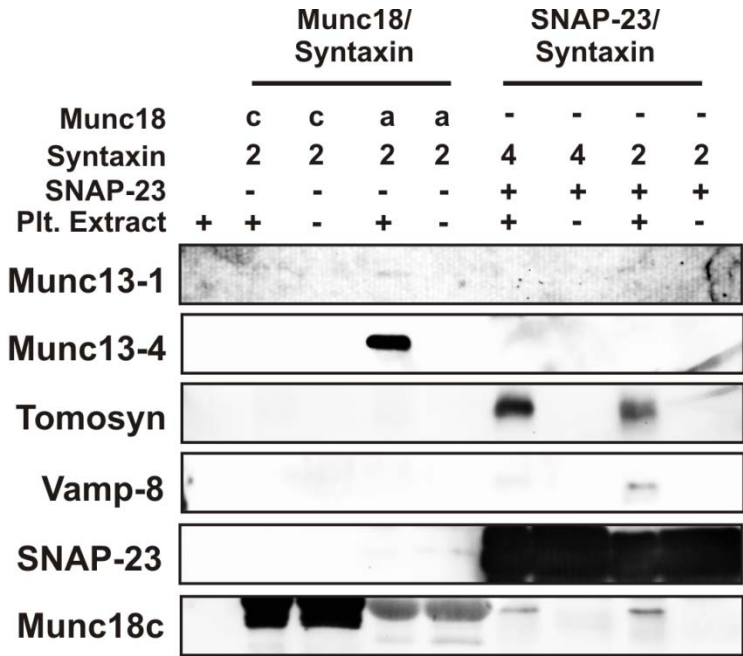
Figure 4-8. Western Blot Analysis of Interacting Proteins of Syntaxin Containing Complexes. The indicated complexes were produced in E.coli using the pDuet plasmid with a S-tag peptide fused to the C-terminus of the syntaxin. Solubilized platelet extracts were incubated with the different complexes and the resulting material was recovered on S-Protein-Agarose. After washing, the proteins were eluted and probed by western blotting with the antibodies to the proteins indicated on the left.

Chapter Five

Conclusions and Future Directions

The research presented in this dissertation demonstrates that syntaxin-11, rather than syntaxin-2 or syntaxin-4, is the primary t-SNARE required for platelet secretion. The enhanced α-granule secretion from syntaxin-2/4 double KO platelets suggests that syntaxin-2 and syntaxin-4 could serve as secondary syntaxins for platelet secretion. Given that endogenous syntaxin-11 was associated with SNAP-23 and VAMP-8 in platelets, it seems plausible that syntaxin-11 forms fusogenic SNARE complexes required for platelet secretion.

We have also added a novel component, tomosyn-1, to the list of proteins involved in platelet secretion. Tomosyn-1 is associated with syntaxin-11 and SNAP-23 both in activated platelets and resting platelets, suggesting tomosyn-1 may be interact with a fusogenic SNARE complex. The studies of tomosyn-1 KO mice show that tomosyn-1 is important for platelet secretion, at least for α-granule and lysosome release. Moreover, tomosyn-1 is required for hemostasis and thrombosis. Overall, tomosyn-1 is important for platelet function and hemostasis, suggesting that tomosyn-1 plays a positive role in platelet exocytosis. My data suggest that its role could be through interactions with syntaxin-11/SNAP-23 heterodimers. The mild defect of dense granule secretion contrasts with the robust impairment of hemostasis, suggesting that tomosyn-1 is required for normal platelet function and also suggests that there is more to uncover about the role of tomosyn-1 *in vivo*. Clearly the data presented here show that controlling platelet secretion without affecting signaling may be a therapeutic target for anti-platelet therapy to control serious cardiovascular diseases, such as heart attack and stroke.

The Core SNARE Machinery in Platelet Secretion

It is generally believed that most regulated exocytosis is a SNARE-mediated membrane fusion process. SNARE proteins are characterized by a conserved heptad repeat region of approximately 60-70 residues in length, referred to as SNARE motif. The energy from SNARE complex formation is believed to drive lipid bilayer fusion. A functional SNARE complex comprises four SNARE motifs from synaptic SNARE

proteins, forming a four-helix, coiled-coil structure [28, 145]. All physiological SNARE complexes cotain one R- and three Q-(a, b, c) SNAREs as described in Chapter 1. In platelets, both VAMP-8 (R) and SNAP-23 (Qbc) are required for platelet secretion, so the functional SNARE complex should be the R-Qa-Qbc configuration. This suggests that Vtib (Qb) is not likely to be a component of the functional SNARE complexes in platelets. The studies from Chapter 3 clearly demonstrated that syntaxin-11 is required for platelet exocytosis and thus is the most likely Qa-SNARE candidate. Studies also suggested that syntaxin-11, SNAP-23, and VAMP-8 are present in the same complex as demonstrate by immunoprecipation with syntaxin-11 antibodies (Chapter 3) and SNAP-23 antibodies [Karim *et al,* in preparation].

Syntaxin-11 is much more important for dense granule and α-granule release than for lysosome release, which suggests that syntaxin-11 mediates at least one common step that is important for both efficient and rapid dense granule release and less rapid but the more abundant α-granule release. Given the clinical bleeding diathesis observed with this patient (Filipovich, personal communication), this step must be vital for hemostasis. Syntaxin-11 also must play a role in lysosome release; but, unlike dense granule and α-granule release, secretion from lysosomes was not completely eliminated in syntaxin-11 deficient human platelets. Residual release from lysosomes indicates that a redundant syntaxin compensates for the lack of syntaxin-11, though less efficiently. The potential candidates are syntaxin-2 and -4, especially considering that the syntaxin-2/4 double depletion mouse platelets had mild secretion enhancement for α-granule and lysosome release. If syntaxin-2 or -4 serve as secondary syntaxins for lysosome release, it may not be obvious in the single and double knockouts. As a comparison, deletion of VAMP-2 and/or-3 had no overt effect yet they could be shown to play a role when analyzing the residual secretion from VAMP-8 KO platelets.

The data in Chapter 3, showing that syntaxin-2 and -4 are not critical for platelet secretion, contradict previous reports from our laboratory and others [40, 41, 44]. The discrepancy can be explained by a number of factors that stem from the use of antibodies as inhibitors in permeabilized platelets. The large antibody molecules could sterically block a functional SNARE or the antibody could cross react with other SNAREs. Based on the observation that β-hexosaminidase and PF4 levels from syntaxin-2 and -4 double

KO and syntaxin-11 deficient platelets are normal, syntaxin-2, -4, and -11 are almost certainly not required for granule biogenesis. Thus the requirement for syntaxin-11 in platelet secretion, shown in Chapter 3, could be attributed to a defect of vesicle priming and/or fusion. To define the exact mechanism for syntaxin-11 in platelet exocytosis, further studies are required, but clearly it is essential for secretion.

Platelet exocytosis represents a unique model to study vesicle trafficking because platelets have three different granules with the three different types of cargo, release rates, and delays post-stimulation. This might suggest that three different types of granule release events may use slightly different sets of SNAREs or regulatory proteins. Loss of the primary v-SNARE, VAMP-8, shows differential effects on the three granule secretion processes. Dense granule is the least affected in VAMP-8 deletion murine platelets. However deficiency of syntaxin-11 totally abolishes both α-and dense granule release but has a milder effect on lysosome release [Figure 3-17]. This suggests that three different granule release processes may use different SNARE regulators or that certain t-SNARE regulators are more critical for the rapid release. The supportive evidence for SNARE regulators playing a critical role in granule release comes from the studies of Munc13-4 and tomosyn-1. The loss of Munc13-4 in platelets has a greater effect on dense granule secretion than on α-granule and lysosome release [48]. The newly described regulatory protein tomosyn-1 (Chapter 4) appears to play a more important role for α-granule and lysosome release than dense granule release [Figure 4-4]. Further studies on regulatory proteins will identify whether different regulatory proteins interact with specific SNAREs, thus playing a differential role in specific granule release.

Based on the classic neuronal SNARE complex (syntaxin-1/SNAP-25/VAMP-2), it is expected that both Qa and R-SNARE need TMDs. However, syntaxin-11 does not contain a TMD. Since studies from Chapter 3 demonstrate that syntaxin-11 is required for platelet secretion, we hypothesize that the functional SNARE complex in platelets is syntaxin-11 (Qa), SNAP-23 (Qbc), and VAMP-8 (R). This is supported by the fact that syntaxin-11 associates with SNAP-23 and VAMP-8 in (Figure 3-19). Thus it would appear that syntaxin-11 forms a functional SNARE complex required for all three granule release. Moreover it has been demonstrated that syntaxin-11 is required for exocytosis

from lymphocytes [146]. Although the exact membrane anchoring is still unknown, studies [37] have suggested that the majority of syntaxin-11 is anchored to membranes and may be so *via* palmitoylation of C-terminal cysteine residues. This is significant because studies show that t-SNARE membrane anchor activity, and thus fusogenicity, is governed by the overall degree of hydrophobicity of the added modification [39]. A t-SNARE complex anchored only by phosphatidyl-ethanolamine (PE)-linked SNAP-25 (involving up to four PE molecules per SNAP-25) and syntaxin-1 cytoplasmic domain is enough to drive membrane fusion [39]. Studies [36, 39, 147] have suggested that long prenyl groups ($\sim C_{55}$) can replace the TMD of VAMP-2 in proteoliposome fusion. To test our hypothesis, two types of proteoliposomes: t-SNARE (SNAP-23 and PE-linked syntaxin-11 containing different number of C-terminal cysteine residues or a C-terminal domain replaced by the TMD of syntaxin-2) containing vesicles and v-SNARE (VAMP-8) containing vesicles can be tested for fusogenicity [33]. Fusion efficiency is expected to be correlated with the number of modified C-terminal cysteine residues of syntaxin-11 and concentration of cholesterol in liposome. Considering syntaxin-11, but not syntaxin-2 and -4, is required for platelet secretion and that platelet secretion is believed to happen at the OCS, which is cholesterol rich membrane region, one expectation should be that lipid-modified syntaxin-11 is more fusogenic than that with chimeric syntaxin-2 TMD.

Finally the latest discovery of Vti1b in platelets opens another possibility. The typical core fusion complexes are likely to have a similar parallel four-helical bundle structure, but in some cases the two α-helical SNARE domains provided by SNAP-25 may be replaced by the SNARE domains of other members of the syntaxin family. For example, VAMP-8 complexes with syntaxin-7 (Q_a), Vti1b (Q_b), and syntaxin-8 (Q_c) and VAMP-4 complexes with syntaxin-16, Vti1a, and syntaxin-6 [148, 149]. At this point, we cannot fully exclude that syntaxin-2 is in the fusion complex for the final step since endogenous syntaxin-2 (Q_a) might be present in the endogenous syntaxin-11 (Q_a) precipitates [Figure 3-19]. Another candidate is Vti1b (Q_b). Although Vti1b is negatively regulated by syntaxin-11 in macrophage [77], it might be in similar complexes in platelets and thereby compensate for the lack of a syntaxin-11 transmembrane domain. Further studies are required to address these possibilities.

Taken together, a model consistent with our data shows the functional core SNARE machinery includes VAMP-8 on granule membranes and SNAP-23 and syntaxin-11 on plasma membrane in platelets (Figure 5-1). Syntaxin-11 is the most abundant t-SNAREs of four known syntaxins in platelets (Table 5-1); suggesting syntaxin-11 could be the critical t-SNARE just like VAMP-8 is the primary v-SNAREs [46]. In sum, all the evidence support that syntaxin-11 is the primary t-SNARE required for platelet secretion.

The SNARE Complex Regulation for Platelet Secretion

Munc18s are cytosolic proteins that belong to the evolutionarily conserved SM family which is required for vesicle trafficking events [79, 136, 150]. SM proteins act as syntaxin-specific chaperones and may contribute to matching cognate v- and t-SNARE to form fusogenic *trans*-SNARE complexes [151]. Among the three SM isoforms present in platelets: Munc18 (a, b, c) [47, 63, 78], Munc18b is the most abundant [Al-Hawas *et al.* in preparation]. Moreover when using t-SNARE heterodimer [Figure 4-1] or SNARE complex [47], Munc18b is the only Munc18 identified in the pull-downs from human platelet extracts. More importantly, human platelets from Munc18b mutant patients (FHL5) show a robust secretion defect from all three granules [50]. Data from our laboratory show that platelets from Munc18b deficient patients have defects in the release of serotonin, PF4, and β-hexosaminidase [Al-Hawas *et al.* in preparation]. It will be interesting to know if the platelet secretion impairment in certain FHL5 patients is caused by dominant-negative Munc18b mutations (or loss of the protein due to protein instability). If this is true, inhibitory peptides of based on Munc18b could be a new drug candidate to modulate thrombosis. Consistently, peptides based on a conserved region of Munc18 did prove to be effective inhibitors of secretion when introduced into permeabilized human platelets [47].

Since Munc18b is required for all three granule secretion events, further exploring the mechanism of Munc18b in platelet secretion will help to identify other SNARE machinery components and understand how Munc18b specifically interacts with t-SNAREs or v-SNARE to promote specific SNARE complex formation. This might also shed light on how secretion is coupled to the platelet signaling pathways. Munc18b may

be a syntaxin-11-specific partner since our unpublished data showed that platelets from FHL5 patient (which had no Munc18b) had reduced levels of syntaxin-11. Syntaxin-11-deficient platelets from a FHL4 patient display a robust secretion defects while Munc18b protein level is only partially reduced [Figure 3-17A]. This prompts speculation that syntaxin-11 is an absolutely required component for secretion while Munc18b is required for syntaxin-11 stability (Figure 5-1). This may indicate that Munc18b functions as a specific chaperone for syntaxin-11.

Given that loss of Munc18b affects syntaxin-11, it is formally possible that loss of syntaxin-11 contributes to the observed phenotype in FHL5 patients, which lack Munc18b. This would be consistent with the phenotype from syntaxin-11 deficient platelets from a FHL4 patient in this thesis. One hypothesis is Munc18b serves solely a syntaxin-11 chaperone. To test this hypothesis, more characterization of FHL5 platelets is required. A correlation analysis should be performed to link the types of Munc18b mutations to the levels of secretion defect to determine whether the severe secretion defect is caused by certain Munc18b mutations. Another experiment could use mutagenesis to generate two kinds of Munc18b mutations that differentially affect syntaxin-11 protein levels and then introduction into megakaryocytes lacking endogenous Munc18b and syntaxin-11. If our hypothesis is right, we would expect to see the secretion defect corrects with the syntaxin-11 protein levels. However, it should be noted that Munc18b heterozygous patients show a partial secretion phenotype and no reduction in syntaxin-11, so it seems possible that Munc18b could play other roles as a limiting component of the secretion pathway (Al Hawas et al. in preparation). Further studies are required to discriminate whether Munc18b serves solely a chaperone or is also a core regulator for functional specific SNARE complex formation in platelet secretion.

Although syntaxin-11 stability is dependent on Munc18b, Munc18b is not solely a syntaxin-11-specific partner. Loss of syntaxin-11 in platelets did not significantly affect Munc18b protein levels as shown in Figure 3-17A, suggesting that syntaxin-11 is not required for Munc18b expression and stability. Moreover Munc18b can specifically interact with syntaxin-2/SNAP-23 complex but not the syntaxin-4/SNAP-23 complex [Figure 4-1], supporting the idea that Munc18b might also be a chaperone for syntaxin-2.

Tomosyn in Platelets

By using syntaxin-containing complexes, we purified tomosyn-1 from platelet extracts. Tomosyn-1 is a cytosolic protein, was originally identified as a syntaxin-binding protein. It has been shown to disrupt the neuronal Munc18a/syntaxin-1a complex and thus was proposed to be a positive modulator of SNARE complex formation [104]. The results from this work support the hypothesis that tomosyn-1 is a positive regulator for platelet granule secretion. Results in this work clearly show that tomosyn-1 KO mice have a bleeding diathesis and tomosyn-1 is required for platelet secretion from at least α-granules and lysosomes. Overall, tomosyn-1 is important for platelet function and for hemostasis though the mechanism is still elusive. In agreement with previous studies [104, 113, 141], tomosyn-1 is associated with the membrane through interactions with syntaxin/SNAP-23 heterodimers in platelets. Tomosyn-1 has also been thought to bridge the fusion machinery to a signaling event. It has been shown that phosphorylation of tomosyn-1 by PKA [98] is important for regulation of SNARE complex formation upon stimulation in neuron. Indeed, tomosyn-1 is phosphorylated in platelets but this phosphorylation does not appear to change during platelet activation. This suggests that tomosyn-1 phosphorylation may not be a viable secretion control pathway in platelets. Tomosyn-1 also can form complexes with syntaxin-11 and SNAP-23. Widberg *et al* [113] and our syntaxin-1\SNAP-23 pull down experiments (Figure 4-1A) show that the fusion components, which include VAMP-8, syntaxin-11, SNAP23, Munc18b, and tomosyn-1 can form a large complex. It is possible that tomosyn-1 is required for activating syntaxins by releasing syntaxin-11 from the Munc18b/closed syntaxin stage into an open conformation.

However, the mechanism that tomosyn-1 plays in different tissues is elusive. The studies of tomosyn-1 KO mice clearly show that tomosyn-1 is a negative regulator of neurotransmitter release but a positive regulator for platelet exocytosis. It is possible that this inconsistency is caused by tissue-specific factors that have yet to be identified. Consistently, phosphorylation of tomosyn-1 appears different in neuron than in platelets. Tomosyn-1 is similarly phosphorylated in both resting and activated platelets while neuronal tomosyn-1 is phosphorylated by PKA only upon stimulation. The difference could be caused by the specific kinases in different tissue. Upon stimulation, PKA is the

active form in neurons while PKC is activated in platelets. It is possible that tomosyn-1 is phosphorylated by PKA in resting platelets and by PKC in activated platelets, but the sites of phosphorylation are different. Another possibility is that tomosyn-2 might be important for neurotransmitter release while tomosyn-1 is important for platelets and adipocytes exocytosis. The inhibitory phenotype shown in tomosyn-1 KO mice may be a result of tomosyn-1 competition with tomosyn-2 [107]. Future studies should focus on the different mechanism for tomosyn-1 as negative role in neurotransmitter release and as positive role in platelet exocytosis. It is important to determine if tomosyn-1 serves as place holder for VAMPs and/or is keeping the t-SNAREs in an activated state. These experiments will help us understand the differential roles of tomosyn-1 in different cell types. The phenotype observed from tomosyn-1 KO mice is similar to that in the granuphilin KO mice [92]. Finally, it is also possible that tomosyn-1 sequesters t-SNAREs from the functional fusion complex or secretion active zones using unknown mechanisms.

The last possible mechanism for tomosyn-1 regulating platelet secretion can be through interaction with cytoskeletal components. Future studies are required to understand the molecular mechanism of tomosyn-1 on platelet secretion and cardiovascular diseases. Because endothelial cells are another key regulator for initiation of thrombus formation by providing vWF for platelet adhesion and activation, bone marrow transplantation will allow differentiation of the role of tomosyn-1 in platelets and endothelial cells. We expect that vWF release from Weibel-Palade body (WPB) will be defective considering tomosyn-1 is ubiquitously expressed in different tissue. The robust bleeding problem and impaired thrombosis formation could be due to a defect in endothelial cells.

Platelet Rabs

Rab GTPases and their effectors, which coordinate vesicle attachment and the subsequent assembly of cognate v- and t-SNARE complexes, play a key role in exocytosis [152]. The Rab27 family is involved in both dense granule biogenesis in megakaryocytes and platelet secretion [90]. In platelets, it has been shown that the majority of Rab27 is associated with dense granules while a smaller portion is associated

with the plasma membrane. Rab27a/b double KO murine platelets display a severe defect in serotonin secretion [86]. The exact role of Rab27a/b in the regulation of dense granules instead of α-granules remains unclear. Presumably the multiple-functions of Rab27 act through recruiting different effectors. One of Rab27's effectors, Munc13-4, is required for platelet secretion [48, 49], suggesting Rab27s and their effectors are required for dense granule exocytosis. It is also possible that Rab27s interact with unknown factors which are important for dense granule package and trafficking, given the fact that deficiency of Munc13-4 also affects α-granule release. The role of Munc13-4 and Rab27s in platelets is not fully understood since Munc13-4 is not required for granule biogenesis while Rab27s are[48]. Munc13-4 is also associated with α-granule release while Rab27s are not. Further exploration of the mechanisms for Rabs in platelet secretion will clarify whether different granules require specific sets of Rab proteins or effectors.

Rab27 Effectors in Platelets

Munc13s are widely believed to serve as key vesicle priming factors. The strongest evidence for this is from genetic ablation of both Munc13-1 and Munc13-2, which completely abolishes the synaptic vesicle priming and release [87, 88]. Mice lacking Munc13-4 have a bleeding diathesis and release from dense granules is totally abolished while the release from α-granules and lysosomes is severely impaired [48]. This indicates that Munc13-4 mediates at least one step that is uniquely required for rapid dense granule release, and that this step is also critical for hemostasis *in vivo*. The residual secretion from α-granules and lysosomes could be compensated for by other Munc13 family members or other Rab27 effectors. The syntaxin containing complex pull-down experiment in this work (Chapter 4) discovered that both of Munc13-1 and Munc13-4 could interact with Munc18a/syntaxin-2 complex specifically in platelets. However, only Munc13-4 is critical for platelet secretion [48]. Compared with other Munc13s, Munc13-4 does not contain a C1 domain, but does have two C2 domains and a MHD domain. The observations that mutations in the MHD domain or deletion of the C2B domain [89] and Munc13-4 peptides without C2B domain potently inhibit platelet secretion [48] suggest that both the MHD domain and the C2B domain are important for

its function. It is possible that the MHD domain is required for interaction with important secretory machinery components while the C2B domain is important for anchoring to the plasma membrane or for regulating membrane fusion in response to Ca^{2+} signaling. Identifying the key interacting partners of Munc13-4 will be an important step for us to understand the SNARE complex formation or membrane fusion upon stimulation. One possible candidate is Munc18b since patients with mutations in Munc18b have blocked secretion of all three granules. This suggests that Munc18b is downstream in the secretion pathway and is shared by all three granule release events.

Other Munc13 family members, DOC2α or granuphilin are candidates for possible contributors to the steps required for the residual release of α-granule and lysosome content upon loss of Munc13-4. Granuphilin is a known Rab27 effector; its presence in platelets was first identified by using Munc18a/syntaxin-2 complex (Chapter 4). The role of granuphilin in platelets has not been investigated. It will be interesting to know whether granuphilin is negatively regulating platelet secretion considering deficiency of granuphilin enhances β-cell exocytosis [92]. Future studies should determine the mechanism of Rab27 effectors on platelet exocytosis. One expectation is that dense granules contain a specific molecule, such as Rab27, which is important for the specific release pathway that it is required for. Identification of new interacting partners that are directly or indirectly associated with Rab27 effectors and the SNARE/Munc18 complex will help us to dissect the molecular mechanism of vesicle priming.

Endocytosis

Endocytosis is important for platelet function though it is uncharacterized at the molecular level. Several granule cargo proteins are taken up by platelets from plasma *via* an endocytic pathway. Several mechanisms of endocytosis have been suggested. It has been shown that plasma proteins can bind to a platelet surface receptor and subsequently be internalized *via* a clathrin-dependent process. The best example is uptake of fibrinogen *via* integrin $\alpha_{IIb}\beta_3$ [153-157]. The endocytosis of factor V by megakaryocytes is through a two-receptor system mediating specific endocytosis by binding to a specific factor V receptor and then to the low-density lipoprotein receptor-related protein-1 (LRP-1) [158]. Some plasma proteins, such as immunoglobulins and albumin, can incorporated

into α-granules *via* pinocytosis [159]. Exploration of endocytosis in platelets will unveil a pathway, fusion machinery, and connections to exocytosis. Our observation that platelets from syntaxin-2/4 double KO mice have lower levels of fibrinogen (Figure 3-13) supports the notion that there is an endocytosis defect caused by the loss of these two syntaxins. This indicates that syntaxin-2 and -4 may be more important in platelet endocytosis than in exocytosis. Thus the syntaxin-2 and -4 double KO mouse models will be an ideal tool to study platelet endocytosis. It will also be interesting to determine whether both pathways share common SNARE proteins. If they have common SNAREs, what other factors coordinate the equilibrium between the two different pathways. Given syntaxin-11 was discovered as an endosomal trafficking t-SNARE [37], we expect that syntaxin-11 may be involved in the late stage of endocytosis pathway, for example the fusion between late endosome with lysosome. Alternatively, identification of the tissue specific factors that direct syntaxin usage in endocytosis or exocytosis will not only be an important biological concept, but may also lead to the discovery of a new therapeutic target for cardiovascular diseases.

Hemostasis and Thrombosis

Platelets play a vital role in hemostasis upon vascular injury. Damage to endothelial cells causes the exposure of agonists, such as collagen and vWF, which initiate platelet adhesion and activation. Activated platelets release granule contents from three different types of granules. Each granule type carries specific molecules that promote thrombus formation. Dense granules contain serotonin, ATP, ADP, and calcium, which are important for vascular contraction and thrombogenesis [1, 13]. α-granules contain many kinds of factors, such as PDGF, VEGF, chemokines, adhesive molecules (*eg*. vWF and fibrinogen), and coagulation factors (*eg*. Factor v). These factors are not only important for clot stabilization but also play a role in wound repair. The released cargo from lysosomes is thought to be involved in fibrinolysis [1, 5]. Although platelet secretion is essential for hemostasis and thrombosis, little is known about the regulatory elements that control it. Platelet secretory machinery contains three core SNAREs and more than four regulatory proteins that control when, where, and how SNARE complexes assemble upon stimulation [5, 13, 47, 48, 50, 64]. Deficiency of the

primary t-SNARE or v-SNARE results in bleeding problems. For example, VAMP-8 null mice form smaller and delayed thrombus [13, 46] while syntaxin-11 and Munc18b deficient patients have severe bleeding problems [160]. Loss of regulatory proteins, such as Munc18b [50], Munc13-4 [48], and tomosyn-1 [Chapter 4], causes prolonged bleeding time and impairs thrombosis formation. Future studies on how these regulatory proteins affect hemostasis and thrombosis beyond the regulation of SNARE complex formation might lead to new drug discovery to control thrombosis without damage to normal hemostasis.

The Platelet Secretion in FLH Diagnosis

Hemophagocytic lymphohistiocytosis (HLH) is a rare immune disorder characterized by proliferation of cells of the mononuclear phagocyte system (histiocytes) and hyperinflammation [161-163]. The typical clinical features include fever, hepatosplenomegaly, lymphadenopathy, jaundice, rash, and laboratory findings of lymphocytosis, histiocytosis and hemophagocytosis. This clinical syndrome comprises the primary (genetic) HLH and secondary (acquired) HLH. The genetic HLH can be classified as FHL and immune deficient associated HLH, such as Griscelli syndrome 2 (GS-2), X-linked proliferative syndrome (XLP), Chediak-Higashi syndrome (CHS) according to the newest classification [161]. Acquired HLH appears as a severe complication of certain virus infection, such as Epstein–Barr virus, of autoimmune diseases [161].

Five FHL (1-5) have been categorized. FHL1 is associated with a potential gene locus (9q21.3-q22) with unidentified specific gene [164]. The perforin gene (PFR1) mutations at 10q21-22 locus were described to be the first causative gene for FHL2 [165]. Perforin is essential for T-cell function and is released from secretory granules and is required for delivering granzyme to induce apoptotic death of target cells. This pore-former plays a critical role in maintaining immune homeostasis [166]. Recently genes mutated in *Munc13-4* (FHL3), *Syntaxin-11* (FHL4), and *Munc18b* (FHL5) have been shown to be involved in cytotoxic granule exocytosis from CTL and NK cells [89, 167, 168]. Concomitantly these gene are also critical for platelet exocytosis and deficiency of these proteins cause granule release defects [48, 50] and Chapter 4.

Detection of perforin, Munc13-4, syntaxin-11, and Munc18b expression in NK cells with FACS or western blotting analysis are reliable methods to screen for FHL2-5. However, these analyses are not available to some patients with extremely reduced NK cell numbers. Compared to NK and CTL, platelets are more abundant. There are clinically established tools to analyze platelet function, such as aggregometry and electron microscopy. Platelet function analysis is a practicable and reliable method to diagnose at least FHL3-5. Combination of platelet function analysis will also provide suggestions for the cause of other genetic HLH. However, it may not apply for acquired HLH.

Although a consensus set of criteria for diagnosis of HLH was established in 2004 by the Histiocyte Society and was revised in 2007 [163], diagnosis of HLH is still difficult since most criteria are not specific for HLH. In reality, HLH patients may be missed because some initial symptoms are rather nondescript [161], may be disguised as a normal infection, or may overlap with some immune deficient disorders. It is critical to find reliable methods to screen early HLH in order to initiate life-saving therapy.

Based on our study of FHL4 patients with mutations in the syntaxin-11 gene, the extent of the platelet secretion defect may be determined by the loss of syntaxin-11. It seems promising that the simple FACS analysis of the intra-platelet syntaxin-11 level could be used as a primary screen of patients with bleeding problems to exclude the possibility of FHL4 or severe FHL5 cases. A small amount of whole blood is required for the FACS analysis. Murata *et al*. [169] have shown that FACS analysis of intraplatelet Munc13-4 protein is a sensitive and reliable method to screen for FHL3. Here we propose that FACS analysis of platelets may be a good way to rapidly screen for three isotypes of FHL: 3, 4, and 5. This technique is easy to combine with the analysis of P-selectin exposure allowing determination of whether there is a secretion defect from α-granules upon thrombin stimulation. In addition, almost all medical centers have the facility to perform the platelet aggregation and ATP release from dense granules using lumi-aggregometry. Thus analysis of platelets from suspected FHL patients is a simple and reliable way to assist in the diagnosis of FHL subtypes and other associated HLH. If platelet function analysis is normal, at least FHL3-5 can be excluded. Analysis of CTL functions, genetic testing, and screening for primary immunodeficiencies such as XLP and CHS should be followed.

Finally, western blotting analysis of protein level of Munc13-4, Munc18b, syntaxin-11, and Rab27 in platelets can confirm the above FACS analysis for FHL diagnosis. Munc13-4 is a 120 kDa cytosolic protein and is expressed much more abundantly in platelets than in peripheral blood mononuclear cells (PBMCs) [169]. Mun18b, a 67 kDa cytosolic protein, is extensively expressed in platelets. Syntaxin-11 is a 35 kDa membrane associated protein. The maximal platelet number required for detection of syntaxin-11 is around 5×10^5, which means around 0.5 µL normal whole blood are needed. Since the different sizes of these proteins are enough to be separated from SDS-PAGE, western blotting would be a good method to determine which protein is missing. Although it takes around 6 hours, the results are accurate and reliable. The same membrane could also be probed for Rab27a, which would further provide information for screening other genetic causes of HLH. No more than 1 mL of whole blood is required to for western blotting analysis.

Summary

The work presented here, for the first time, gives a picture of how syntaxin-11 and tomosyn-1 are used to mediate platelet exocytosis. Syntaxin-11 is the primary t-SNARE required for platelet exocytosis while syntaxin-2/4 may play a secondary role in the absence of syntaxin-11 but not as efficiently as syntaxin-11. Although the precise mechanism of syntaxin-11 function in platelet exocytosis, we can clearly pinpoint that syntaxin-11 is an important, core element of the secretory machinery. The work presented elucidates a complete picture of the core secretory machinery in term of which isoforms of t- or v-SNARE are required for platelet secretion. Syntaxin-11, SNAP-23, and VAMP-8 are the functional SNARE proteins for platelet exocytosis. In addition to the core SNARE secretory machinery components, a novel syntaxin-binding protein in platelets, tomosyn-1 was shown to be required for platelet secretion. Tomosyn-1 is associated with the functional t-SNARE complex, syntaxin-11/SNAP-23 heterodimers. Future work will focus on how granule secretion is regulated and how the core machinery is coupled to the platelet signaling cascades that are activated by agonists. The answers to these questions will yield a better understanding of the control mechanism of platelet exocytosis and give directions for pharmaceutical design.

Table 5-1. SNAREs and Their Regulators in Human and Murine Platelets

t-SNAREs	Molecules/Platelet*	
	Human	Murine
VAMP-2†	<806	7,629 ± 89
VAMP-3†	4,588 ± 658	3,080 ± 10
VAMP-7†	3,766 ± 696	2,712 ± 230
VAMP-8†	6,590 ± 370	8,360 ± 110
SNAP-23	19,374 ± 5384	5,363 ± 798
Syntaxin-2	1,579 ± 429	1,766 ± 121
Syntaxin-4	9,633 ± 75	1,625 ± 184
Syntaxin-7	3,861 ± 564	3,707 ± 372
Syntaxin-11	51,717 ± 5459	46,966 ± 5516
Munc13-4#	ND	318 ± 47
Tomosyn-1	1029 ± 80	1152 ± 46

ND indicates not determined.

*Number of molecules per platelet was calculated using the molecular weight for VAMP-2 (13 kDa), VAMP-3 (11 kDa), VAMP-7 (25 kDa), VAMP-8 (12 kDa), SNAP-23 (23 kDa), Syntaxin-2 (33 kDa), Syntaxin-4 (34 kDa), Syntaxin-7 (30 kDa), Syntaxin-11 (33 kDa), Munc13-4 (125 kDa), Tomosyn-1 (128 kDa).

#From Ren *at al.*[48]

†From Graham *at al.* [13]
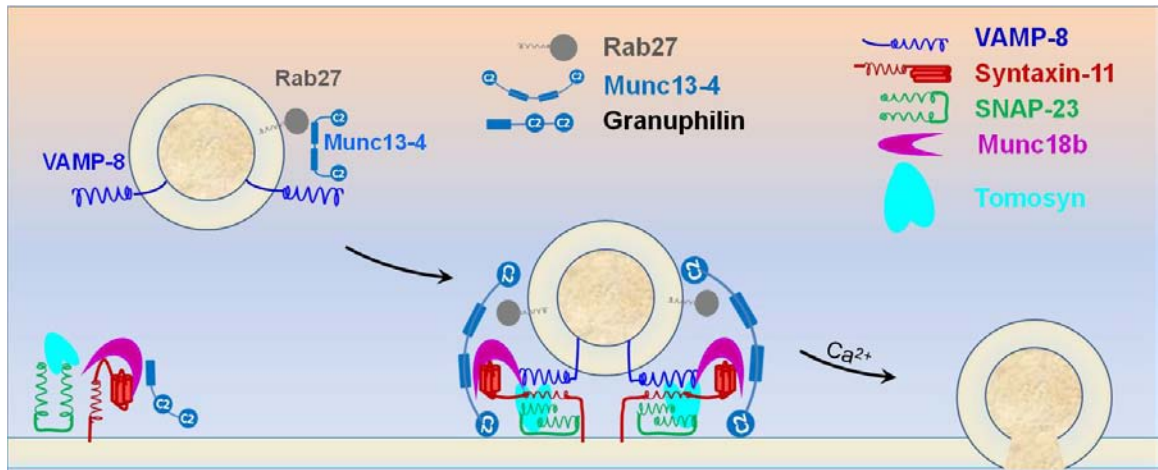
Figure 5-1. A Model of Platelet Secretory Machinery. Platelet exocytosis is mediated by SNAREs and their regulatory proteins upon physiological stimulation. The core SNARE machinery includes v-SNARE (VAMP) on vesicle membrane and t-SNAREs (syntaxin-type and SNAP-25-type) on target membrane. The primary SNAREs required for platelet secretion are VAMP-8 (in blue), syntaxin-11 (in red), and SNAP-23 (in green) in platelets. Munc13-4 (in blue), Munc18b (in purple), tomosyn-1 (in cyan) are required for regulation of vesicle priming, probably facilitating fusogenic SNARE complex formation, thus leading to membrane fusion and releasing granule contents upon stimulation.

Abbrieviations

5-HT	serotonin (5-hydroxytryptamine)
ADP	adenosine biphosphate
ATP	adenosine triphosphate
BCA	bicinchoninic acid
β-TG	β-thromboglobulin
DAG	diacylglycerol
DMP	dimethyl pimelimidate
ECF	enhanced chemi-fluorescence
EGF	epidermal growth factor
ELISA	enzyme-linked immunosorbent assay
FACS	fluorescent-activated cell sorting
FBS	fetal bovine serum
GST	Glutathione S-transferase
FHL3	familial hemophagocytic lymphohistiocytosis type 3
FHL4	familial hemophagocytic lymphohistiocytosis type 4
FHL5	familial hemophagocytic lymphohistiocytosis type 5
FITC	fluorescein isothiocyanate
HPS	Hermansky-Pudlak syndrome
HRP	horseradish peroxidase
HUVEC	human umbilical vein endothelial cell
IP	immunoprecipitation
IP_3	inositol tri-phosphate
IPTG	isopropyl-beta-D-thiogalactopyranoside
LAMP	lysosomal associated membrane protein
LDL	low density lipoprotein
lgl	legthal ginat lavea
LTP	long term potential
MBP	maltose binding protein
MHD	munc homolog domain

MI	myocardial infarction
MVB	multivesicular body
NK	Natural Killer
NSF	N-ethylmaleimide sensitive factor
OCS	Open Canalicular System
PAGE	polyacrylamide gel electrophoresis
PAR	Protease-activated receptor
PE	platelet extract
PF4	platelet factor IV
PGI_2	prostaglandin I_2
PI_3P	phosphatidyl inositol tri-phosphate
PKA	protein kinase A
PKC	protein kinase C
PMA	phorbol 12-myristate 13-acetate
PMSF	phenylmethanesulphonylfluoride
PRP	platelet rich plasma
PVDF	polyvinylidene fluoride
RRP	ready releasable pool
RT	room temperature
SLO	streptolysin-*O*
Slp	synaptotagmin like protein
SNAP-23	synaptosome-associated protein of 23 kDa
SNAP-25	synaptosome-associated protein of 25 kDa
SNARE	soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptor
SNP	single nucleotide polymorphism
SPD	Storage pool disease
TCA	tricholoracetic acid
TF	tissue factor
TGN	*trans*-Golgi network
TIRF	total internal reflection fluorescence

VEGF	vascular endothelial growth factor
VAMP	vesicle associated membrane proteins
vWF	von Willebrand factor
WPB	Weibel-Palade body
Wt	wild-type

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Peered Review Publications:

1, Zhang G, Xiang B, <u>**Ye S**</u>, Chrzanowska-Wodnicka M, Morris AJ, Gartner TK, Whiteheart SW, White GC 2nd, Smyth SS, Li Z. Distinct Roles for Rap1b Protein in Platelet Secretion and Integrin αIIbβ3 Outside-in Signaling. J Biol Chem. 2011;286(45):39466-77. Epub 2011 Sep 22.

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