THE ROLE OF TMEM16F IN CALCIUM FLUX, PS EXPOSURE AND MICROPARTICLE FORMATION IN PLATELETS

by

Debora Kamin Mukaz

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

Summer 2014

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 - 1346

THE ROLE OF TMEM16F IN CALCIUM FLUX, PS EXPOSURE AND MICROPARTICLE FORMATION IN PLATELETS

by

Debora Kamin Mukaz

Approved: _____
Donna S.Woulfe, Ph.D.
Professor in charge of thesis on behalf of the Advisory Committee

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

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

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

ACKNOWLEDGMENTS

First and foremost, I would like to thank my advisor Dr Woulfe. She has tremendously supported me throughout these past two years and helped me gain more confidence in my research skills. I also can't thank her enough for being there whenever I needed advice and when my experiments did not work out. I am truly thankful to have had her as a mentor.

I would also like to thank my committee members Dr Randy Duncan and Dr Terry Paoutsakis. I extremely appreciated their insights and thank them for challenging me in my scientific process.

I am also grateful to Pani Apostolidis for helping me with the FACS Aria, Ben Rohe for training me on the Accuri C6, Dr Deni Galileo for also helping me with the flow cytometer and Dr Gary Laverty for teaching me how to use the PTI fluometer.

I also owe a huge thanks to my labmates, Aasma, Ann, Shreya, Dongjun and Linda. Aasma, thanks for the big help and I would like to especially acknowledge her help with the western blot picture of HEK293 cells transfected with TMEM16F and the analysis of the calcium data; Ann, thank you for drawing the blood and being a friend and Shreya, thank you for listening to me when I was sometimes frustrated. Dongjun, thanks for your help with the PS exposure and microparticle experiments.

I'd also like to thank the Black Graduate Student Association (BGSA) for being there and giving me a clear perspective on life of a graduate student.

I'd like to thank my family. My parents, Joseph Mukaz Sandjamb and Charlotte Kaj Kamay, for their undying spiritual, emotional and financial support, my

sister, Sara, for listening to me even though she did not understand half of what I was talking about, and my cousin, Da Arlette, for being there whenever I needed it. Je vous aime beaucoup et merci de votre aide.

Finally, I would like to thank my God without whom I would not be here.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

PS: Phosphatidylserine

GPVI: Glycoprotein VI

PAR: Protease-Activated Receptor

ER: Endoplasmic Reticulum

SOCE: Store-Operated Calcium Entry

TMEM16F: Transmembrane 16F

TMEM16F+/-: TMEM16F heterozygote

TMEM16F KO: TMEM16F knockout

THR+CVXN: Thrombin and Convulxin

THR+COL: Thrombin and Collagen

UNTX: Untreated

PRP: Platelet-Rich Plasma

ABSTRACT

In platelets, TMEM16F, an eight-transmembrane protein, is a calcium activated-cation channel highly permeable to calcium. The TMEM16F gene has been shown to be mutated in Scott's syndrome, a rare bleeding disorder characterized by a defect in the scrambling activity of platelets and microparticle generation. In this thesis, I investigated the effect of TMEM16F on calcium signaling, microparticle formation and phosphatidylserine exposure in platelets under physiological conditions. First, I investigated whether TMEM16F was required for optimum calcium signaling in platelets. TMEM16F+/- mouse platelets treated with a combination of thrombin and collagen displayed a significant decrease in calcium entry measured as percent above basal calcium entry compared to the wild-type platelets ($p=0.02$). Further analysis of calcium entry revealed that there was a delay in the SOCE for the TMEM16F+/- platelets compared to the WT platelets ($p=0.05$). To determine whether TMEM16F is required for maximal PS exposure, binding of Annexin V to activated platelets from WT vs. TMEM16F knockout mice (TMEM16F KO) was compared. To measure PS exposure, Annexin V binding to platelet surfaces was quantified by flow cytometry: when treated with thrombin and convulxin, there was a significant decrease in maximal Annexin V binding achieved by 10μM A23187 of TMEM16F knock-out (KO) platelets compared to the WT ($p=0.0005$). Microparticle generation from TMEM16F KO platelets compared to WT platelets was also analyzed by flow cytometry. On average, after thrombin and convulxin treatment, there was a significant decrease of the percentage of microparticles over total particles

(microparticles and platelets) generated by the TMEM16F KO mice compared to the WT mice ($p=0.05$). These data demonstrate that TMEM16F regulates calcium influx, PS exposure and microparticle formation in platelets. Interestingly, when the channel function of TMEM16F was blocked with CaCCinhA01, there was a significant decrease in the calcium influx compared to the untreated platelets ($p=0.01$) while no significant change was seen in the PS exposure, suggesting that TMEM16F might have two independent functions in platelets: one as a channel and the other as a regulator of the scrambling activity of platelets. Furthermore, preliminary results of calcium entry analyzed in HEK293T cells and CHOK cells stimulated with ionophore showed an increase in calcium entry when cells transfected with TMEM16F were compared to untransfected cells. However, analysis of the scrambling activity revealed that heterologous expression of TMEM16F was not sufficient to induce PS exposure after treatment with A23187. These results suggest that there are additional mechanisms to promote TMEM16F scramblase activity in platelets.

Chapter 1

INTRODUCTION

1.1 Platelets

Human platelets are disc-shaped, anuclear cells that have a size of 2-4μM (Harrison, 2005) and have a lifespan of 7 to 10 days (Nayak et al., 2013). Platelets have multiple functions, including the maintenance and regulation of vascular tone, inflammation, and host defense and tumor biology (Harrison, 2005). However, platelets mainly regulate hemostasis, which is the stoppage of blood loss (Rivera, 2009). Platelets also play a role in thrombosis, which is a pathological dysregulation of the normal processes of hemostasis (Furie and Furie., 2009). Hence, understanding how platelets mediate hemostasis and thrombosis can potentially lead to new treatments for patients with hemostatic disorders and those at risk for pathological thrombosis, the underlying events that lead to heart attack and stroke.

1.2 Hemostasis

Hemostasis is divided into two different stages: primary hemostasis and secondary hemostasis. (Gale, 2011) Primary hemostasis occurs when platelets adhere to the damaged vascular wall and aggregate to form a temporary platelet plug, while secondary hemostasis is the stabilization of this plug throught the additional formation of a fibrin meshwork, termed a fibrin clot (McMichael, 2005).

1.2.1 Formation of the Platelet Plug

Under normal conditions, circulating platelets are inactivated and do not bind to the endothelium (Rasche, 2001) because Prostaglandin I_2 and Nitric Oxide released from the vessel wall inhibit that interaction (Broeders et al., 2005). However, upon injury and subsequent exposure of the subendothelial matrix, platelets arrest at the site of injury and get activated by binding to proteins such as vWF (von Willebrand Factor) via GPIbα and integrin αIIbβ3 receptors and collagen via GPVI and integrin α2β1, thus forming a platelet monolayer. During the initial phase of platelet plug formation, platelets also bind and get activated by thrombin through their protease-activated receptors (PARs) (Brass et al., 2007). After the initial activation steps, more platelets are recruited to the sites of injury through the activation of different G-protein-coupled receptors (GPCRS) by thrombin via PARs, thromboxane A2 (TXA2) via TP and ADP via P2Y12 (Brass, 2003). Finally, the platelet plug is stabilized by the activation of the integrin αIIbβ3 binding to fibrinogen, thereby allowing stabilization of the plug through actin-myosin contractile networks (Brass et al., 2007).

1.2.2 Blood Coagulation Cascade

In order for the hemostatic clot to be formed, the temporary platelet plug formed during primary hemostasis has to be stabilized by a fibrin meshwork (Brass et al., 2007). Fibrin is generated from fibrinogen cleaved by thrombin, which is an end-product of the blood coagulation cascade (Wolberg, 2007). There are two pathways in the blood coagulation cascade; the intrinsic pathway and the extrinsic pathway. While the extrinsic pathway requires Tissue Factor(TF) for initiation (Mackman et al., 2007), the intrinsic pathway was characterized as such because it does not require non-blood

factors, but is initiated upon contact with the glass of the assay tube(Davie et al., 1964). Upon vascular injury, the blood coagulation cascade is initiated in vivo via the extrinsic pathway (Versteeg et al., 2013). The exposed TF present on a variety of cells such as monocytes, neutrophils, endothelial cells and platelets (Mackman et al, 2007) binds and cleaves Factor VII (FVII) to form FVIIa. FVIIa will lead to the sequential generation of FIXa and FXa (Chu, 2001; Versteeg et al., 2013). FXa will then assemble with FVa in the presence of calcium to form the prothrombinase complex: this complex will produce a small amount of thrombin that will amplify the blood coagulation cascade through the intrinsic pathway (Savage and Ruggeri, 2007)

During the intrinsic pathway (or contact pathway), FXII is cleaved to generate FXIIa by two factors of the contact system, kininogen and prekallikrein (Gailani and Renné, 2007). The contact system gets activated by binding to negatively charged compounds such as dextran sulfate (Nakazawa et al., 2002) or extracellular RNA (Kannemeier et al, 2007). FXIIa in turn leads to the activation of FXI, which becomes FXIa. FXIa, then, activates FIX into FIXa (Gailani and Renné. 2007). FIXa binds to the extrinsic thrombin-activated FVIII(FVIIIa) (Savage and Ruggeri, 2007) to form the tenase complex that activate FX into FXa (Louvain-Quintard et al.2005). FXa will then assemble with extrinsic thrombin-activated FV (FVa) to generate more prothrombinase that will catalyze the formation of thrombin from prothrombin. Thrombin is important for fibrin generation from fibrinogen (Savage and Ruggeri, 2007).

1.3 Calcium Signaling in Platelets

Calcium has been known to be important for clot formation since it is needed for the formation of the Xase and prothrombinase complexes that allow for thrombin

formation (Figure 1). An increase in cytosolic calcium in platelets happens through two different pathways: the release of calcium from the ER stores and the extracellular entry of calcium. In platelets, calcium release from the stores and its ensuing intracellular increase in concentration happen upon stimulation of receptors by agonists such as thrombin, collagen and ADP (Varga-Szabo et al., 2009). Collagen triggers an increase in intracellular calcium through its immunoglobulin platelet receptor, GPVI that binds to the γ-chain FC Receptor [FCR] (Watson et.al, 2005). The crosslinking of GPVI and FCRγ leads to the autophosphorylation and phosphorylation of the FCRγ ITAM (Immunoreceptor Tyrosine-based Motif) by the Scr kinases, Lyn and Fyn (Ezumi et al., 1998). Once the ITAM is activated, binds and activates the tyrosine kinase Syk (Watson et al., 2005), which will initiate a downstream signaling that lead to the activation of PLCγ2 (Jarvis et al., 2002). PLC γ2 catalyzes the formation of inositol-1-4, 5-triphosphate (IP3) and 1, 2-diacyl-glycerol (DAG) from phosphoinositide-4, 5-biphosphate (PIP2). The formed IP3 will bind to the IP3 receptors (IP3R) in the ER to release calcium. (Varga-Szabo et al., 2009)

Thrombin, on the other hand, activates the PARs (Brass et al, 2007). Only 3 out of the 4 known members (PAR1, PAR3 and PAR4) have been shown to be mediate thrombin responses in platelets (Kahn et al., 1998). While human platelets express PAR1 and PAR4 with PAR1 seemingly playing a more prominent role (Andersen et al., 1999); rodent platelets express PAR4 and PAR3 (Brass, 2003). The PARs activation by thrombin will stimulate another PLC isoform, PLCβ that will also allow for the formation of IP3 and subsequent release of calcium from the ER stores (Varga-Szabo et al., 2009)

ADP released from the injured vessel wall is necessary for the processes of hemostasis and thrombosis (Hechler et al., 1998), although its effects are weaker and reversible compared to thrombin and collagen. Nevertheless, because it is stored at high concentration in the platelet dense granules, it strongly potentiates hemostatic and thrombotic responses in platelets (P2 receptors and platelet function, Hechler and Gachet, 2011). In platelets, ADP induces calcium signaling in platelets by activating its receptor P2Y1, which is a GPCR found in most human and mouse cells (Abbracchio et al. 2006.). Like Thrombin, ADP mediates calcium signaling in platelets through PLCβ (Varga-Szabo et al., 2009).

The depletion of the calcium stores triggers a process known as the Store-Operated Calcium Entry (SOCE), which accounts for the majority of calcium entry in non-excitable cells, including platelets (Varga-Szabo et al., 2009; Targos et al., 2005). As calcium gets reduced, STIM1, a calcium sensor on the ER, senses it through its calcium-sensing motif, EF hand (Zhang et al., 2005). The EF hand is a helix-loop-helix motif with a configuration that is favorable to calcium and magnesium binding (Lewit-Bentley and Rety, 2010). Upon depletion of calcium stores, STIM1 activates extracellular calcium entry (Zhang et al., 2005) through Orai1 (Bergmeier et al., 2009), the main SOCE channel in platelets (Varga-Szabo et al., 2011).

1.4 Procoagulant Activity of Platelets

The assembly of the Xase and the prothrombinase complexes requires the exposure of phosphatidylserine (PS) and calcium (Figure 1).

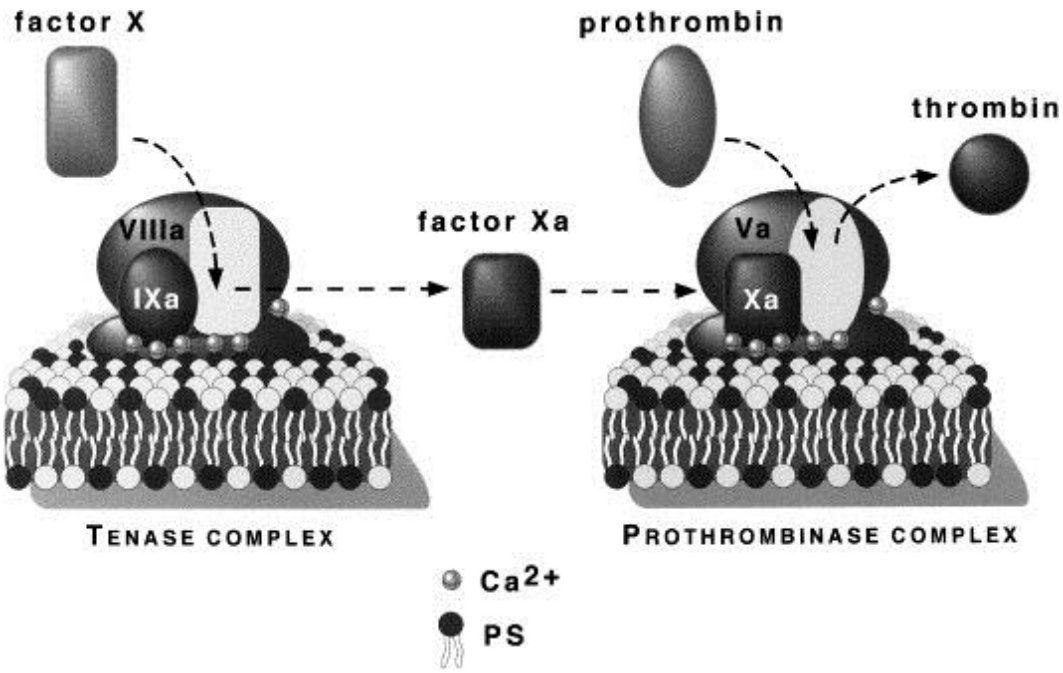


Figure 1. The exposure of phosphatidylserine (PS) provides a surface for the assembly of the tenase and prothrombinase complexes of the blood coagulation cascade; both of which are needed for the generation of thrombin(Zwaal et al., 2004)

The negatively-charged PS exposed on the outer leaflet of the plasma membrane during platelet activation (Lhermusier et al., 2011) provides a surface that will enhance the generation of thrombin (Solum,1999) allowing for the formation of the fibrin clot and the activation of more platelets. Hence, the exposure of PS on the surface of the plasma membrane links the blood coagulation cascade to platelet activation, thus, facilitating the procoagulant activity of platelets. Calcium is important for the procoagulant activity of platelets since it is required for the formation of the Xase and prothrombinase complexes (Figure 1). Moreover, an increase in intracellular calcium activates the scramblase needed for PS exposure and the calpain needed for microparticle formation. The role of calcium entry in platelet

microparticle formation and scramblase activity will be further expanded in the next sections (Sections 1.4.1-1.5).

1.4.1.1 Microparticle Formation

One of the consequences of cells exposed to agonists that elicit a very large increase in intracellular calcium is the formation of microparticles (Mause et al., 2010). Microparticles are budding microvesicles or blebs with sizes ranging from 0.1μm to 1μm that derive from the plasma membrane of various cell types (Owens et al., 2011). Though the exact mechanism of microparticle shedding in platelets remains obscure, it is known that upon increase in intracellular calcium (Mause et al., 2010), they arise from the disruption of the plasma membrane cytoskeleton by calpain, a calcium-dependent protease (Fox et al. 1990). More than 80% of circulating microparticles have a platelet origin (Morel et al., 2011) and they have been associated with a number of diseases, including the bleeding disorder Scott's syndrome where patients have a decreased number of microparticles (Sims et al., 1989). The fact that Scott's syndrome patients have a defect in both the PS expressed on the surface of their platelets and the number of microparticles suggests that the mechanisms of PS exposure and microparticle formation are linked (Sims et al., 1989). However, the exact cellular mechanism that connects PS exposure and microparticle generation remains unclear. Nevertheless, the increase in intracellular calcium activates both the calpain and the scramblase needed for PS exposure (Lhermusier et al., 2011), indicating that calcium signaling is common to both processes.

As expressed earlier, platelets are the mediators of hemostasis and thrombosis. Platelet microparticles facilitate the hemostatic and thrombotic processes by providing a large surface of PS+ membrane on which to assemble the coagulation proteases at

the site of injury or thrombotic initiation. In fact, microparticles were originally shown to have procoagulant properties because an increase in their number led to more prothrombinase(Sims et al., 1989) and tenase binding sites(Chang et al., 1993), which are important for thrombin formation. Later on, it was confirmed that virtually all platelet microparticles expose PS on their surface (Owens et al., 2011). Because microparticles have procoagulant properties and allow for the formation of thrombin needed for clot formation, an increase in their number have been associated with a number of prothrombotic diseases such as acute coronary syndrome, venous thromboembolism or heparin-induced thrombotic thrombocytopenia(Ardoin et al.,2007); denoting the importance of a regulated microparticle formation. Conversely, the fact that Scott syndrome patients have a decrease in circulating (platelet) microparticles may also contribute to their bleeding disorder, further demonstrating the role of platelet microparticles in hemostasis. Because microparticles have procoagulant properties and allow for the formation of thrombin needed for clot formation, an increase in their number have been associated with a number of prothrombotic diseases such as acute coronary syndrome, venous thromboembolism or heparin-induced thrombotic thrombocytopenia(Ardoin et al.,2007); denoting the importance of a regulated microparticle formation.

1.4.2 Plasma Lipid Bilayer Asymmetry

The plasma membrane of platelets and other eukaryotic cells is an asymmetrical lipid bilayer (Fadeel et al., 2010) whose main property is amphiphilic meaning the lipids that make the bilayer have a polar head group and non-polar tails (Alberts et al., 1989). The main types of phospholipids in the plasma membrane are

phosphatidylserine (PS), phosphatidylcholine (PC), phosphatidylethanolamine (PE) and sphingomyelin. The phospholipids on the plasma lipid bilayer are asymmetrically distributed; the outer leaflet of the membrane is mostly made up of cholinephospholipids(PC and sphingomylin), while the inner side of the membrane is in majority composed of aminophospholipds (PS and PE) (Cooper,2000). A tightly controlled regulation of lipid bilayer asymmetry of the cell is important, since a disruption of said asymmetry facilitates cellular mechanisms such as apoptosis (Savill, 2002) or procoagulant activity of platelets (Fadeel et al., 2010).

1.4.3 Maintenance and Disruption of the Plasma Lipid Bilayer

In resting cells, the lipid asymmetry of cells is regulated by two ATP-requiring enzymes or translocases; the Flippase and the Floppase(Clark et al., 2011; Figure 2). The Flippase transports PS and PE from the outer to the inner membrane against their concentration gradient (Seigneuret and Devaux, 1984; Clark et al., 2011; Devaux et al., 2008; Daleke et al., 2007) while the Floppase transport both PC and sphingomyelin (Clark et al., 2011). Though it has been shown that the Flippase is a P4 ATPase (Lhermursier et al., 2011; Coleman et al., 2013), the nature of the Floppase in platelets is still controversial. In erythrocytes, the Floppase has been shown to be an ABCC1 transporter (Coleman et al., 2013), but the expression of ABCC1 in platelets has yet to be confirmed (Jedlitschky et al, 2012).

Agonist-mediated platelet activation leads to an increase in intracellular calcium that allows for the disruption of the plasma lipid asymmetry and the exposure of PS (van Kruchten et al., 2013) In platelets, it is known that PS exposure is calcium-dependent, but not ATP-dependent, and thus is mediated by a Scramblase, rather than a Floppase or Flippase (Lhermusier et al., 2011). In 2010, a cDNA was cloned and

found to be present in platelets, required for platelet scramblase activity, and mutated in patients with Scott Syndrome, which is characterized by a deficiency in scramblase activity. This cDNA encodes TMEM16F, an 8-transmembrane domain calcium channel (Suzuki et al., 2010).

Scott's syndrome is a rare bleeding disorder characterized by the impaired procoagulant activity of platelets due to defective scrambling activity. Interestingly, the defect in scrambling activity in Scott's syndrome platelets is accompanied by not only the expected decrease in PS exposure on the surface of platelets, but also diminished microparticle shedding (Weiss and Lages, 1997). While laboratory tests usually show that Scott's syndrome patients do not have an abnormal platelet count, structure, secretion, aggregation or coagulation factors (Zwaal et al., 2004); it has been shown that these patients have a reduced capacity to generate thrombin and fibrin (Wielders et al., 2009) due to a decrease in the binding surface for the prothrombinase (Miletich et al., 1979) and the tenase complexes (Ahmad et al., 1989).

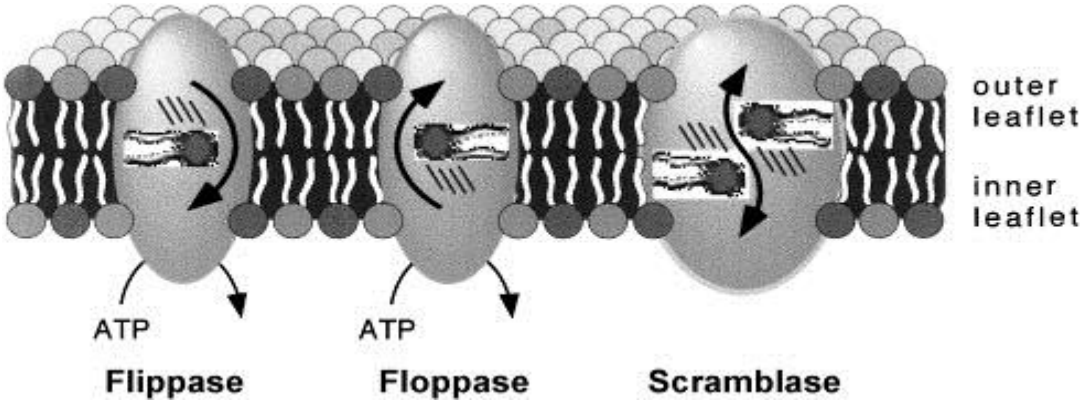


Figure 2. The phospholipid asymmetry across the plasma lipid bilayer is maintained by the ATP-dependent proteins Flippase that moves phospholipids to the cytosolic side of the plasma membrane and the Floppase that transports them from the inner leaflet to the outer leaflet of the membrane. The Scramblase disrupts the asymmetry by transporting the phospholipids bidirectionally across the membrane.(Zwaal et al., 2004)

1.5 TMEM16F

Although the nature of the scramblase responsible for the transport of PS from the inner leaflet to the outer leaflet has been a subject of controversy, a 2010 study (Suzuki et al. 2010) showed that a patient with Scott syndrome had a mutation at the splice acceptor site of the gene encoding for TMEM16F that caused a frameshift and a premature termination of the protein. A different study (Castoldi et al., 2011) further supported the role of TMEM16F in the scrambling activity of cells. The study demonstrated that a different Scott's patient had 2 mutations, at intron 6(IV6+1G→A) and exon 11(IV12-1G→T), that lead to a frameshift and premature termination of the protein. The TMEM16F gene is found on chromosome 12 and has 20 exons that encodes for a 910-aa protein (Castoldi et al., 2011). In dogs, genomic analysis of

animals affected with a canine form of Scott's Syndrome (CSS) revealed that the deficiency in canine procoagulant activity was linked to markers on chromosome 27. Although the gene responsible for CSS has yet to be definitively identified, canine TMEM16f falls within close proximity to these markers on chromosome 27 (Brooks et al., 2010). TMEM16F is an eight-transmembrane protein with its N and C-termini inside the cytoplasm (Galietta et al., 2009). TMEM16F is expressed in a variety of cells (Suzuki et. al, 2010; Ehlen et. al, 2013) including platelets, where it has been shown to be a calcium-activated cation channel that is highly permeable and selective for calcium (Yang et al., 2012). Since TMEM16F has been shown to be defective in Scott's syndrome that leads to defective procoagulant activity of platelets and because TMEM16F has been shown to be a calcium-activated calcium channel, we hypothesized that, in platelets, the loss of TMEM16F will affect PS exposure on the surface of cells, microparticle formation and the increase in intracellular calcium after agonist stimulation.

Chapter 2

MATERIALS AND METHODS

2.1 Animals

C57BL/6 TMEM16F knockout mice were generously provided and generated in the laboratory of Dr Andrea Vortkamp. Wild type (WT) mice were produced from the breeding of TMEM16F heterozygous (TMEM16F+/-) mice. Animal care and sacrifice followed protocols approved by the Institutional Animal Care and Use Committee at the University of Delaware. TMEM16 KO mice had a thrombotic and bone defect.

2.2 Platelet Rich Plasma (PRP) Isolation

Blood was drawn from the inferior vena cava of mice with 100µl of 150U/ml heparin per mouse. 0.02 U/ml of Apyrase in 1ml HEPES-Tyrode's buffer (137 mM NaCl, 20 mM HEPES, 5.6 mM glucose, 1g/L BSA, 1mM $MgCl_2$, 2.7 mM KCl, 3.3. mM NaH_2PO4) were added to the blood, which was spun at 800 rpm for 3 minutes at room temperature. Platelet Rich Plasma (PRP) was obtained and the concentration was measured using a Beckam Coulter particle counter.

2.3 Fluo-4-AM Loading in Platelets

Blood was drawn from the inferior vena cava of mice. Platelet Rich Plasma (PRP) was obtained and the concentration was measured. 5µM of Fluo-4-AM

(Invitrogen) were added to 10^6 platelets in 200 µl of Tyrode's buffer then incubated for 10 minutes at 37 degrees Celsius.

2.4 PS Exposure in Platelets

The PRP was obtained from WT, TMEM16F +/-, TMEM16KO mice then the concentration was measured and adjusted to $0.5x10^8$ platelets/ml. Samples were aliquoted in 100µl volume, stained with FITC-conjugated Annexin V (BD Biosciences). Samples were incubated for 15 minutes with 0.5 U/ml thrombin (Chrono-Log), 0.5µg/ml convulxin (Chrono-Log), 0.5 U/ml thrombin and 0.5µg/ml convulxin combined, and calcium ionophore A23187(1uM or 10µM)(Sigma). The samples were then analyzed by flow cytometry (FACS Calibur)

2.5 Microparticle Generation

The PRP was obtained from WT, TMEM16F +/-, TMEM16KO mice then the concentration was measured and adjusted to $0.5x10^8$ platelets/ml. Samples were aliquoted in 100µl volume, stained with Alexa-Fluor-conjugated Annexin 647 (Invitrogen) and FITC-conjugated CD41 488(Biolegend). Samples were incubated for 30 minutes with thrombin, convulxin and calcium ionophore, A23187 and analyzed by flow cytometry (FACS Aria and Accuri C6).

2.6 Total Calcium Influx in Platelets

1 ml of HEPES-Tyrode's buffer was added to Fluo-4-AM loaded platelets from WT and TMEM16+/- mice from which 200µl were aliquoted into 200µl of Tyrode's buffer. $CaCl_2$ was then added to the platelets to a final concentration of 1mM. The basal reading of Ca^{2+} was measured for 1 minute by the Accuri C6 cytometer then 0.5 units/ml of thrombin and 10µg/ml of collagen or 1µM of calcium

ionophore, A23187, were added to the mixture and Ca^{2+} entry was measured for 9 minutes. Using the FACS Express Software, the percent of total Ca^{2+} entry above basal was measured and plotted for the WT vs TMEM16F+/- mouse platelets.

2.7 Stored-Operated Calcium Entry in Platelets

1 ml of HEPES-Tyrode's buffer was added to Fluo-4-AM loaded platelets from WT and TMEM16F+/- from which 200µl were aliquoted into 200µl of Tyrode's buffer. The basal reading of Ca^{2+} was measured for 1 minute by the Accuri C6 cytometer then EGTA was added to a final concentration of 200 µM and Ca^{2+} was measured for 1 minute. Next, Thapsigargin was added to a final concentration of 1µM and a reading of 1 minute was done. Finally, Ca^{2+} was added to the platelets to a final concentration of 0.5mM and Ca^{2+} entry was measured for 7 minutes. Using the FACS Express Software, the percent of total Ca^{2+} entry above basal was measured and plotted for WT vs. TMEM16F+/- mouse platelets.

2.8 Inhibition of Channel Activity of TMEM16F

10^6 platelets in 200 µl of Tyrode's buffer were loaded with Fluo-4-AM from WT mice. 5 minutes during incubation time, 20µM of CaCCinh-A01 (Sigma) was added. 1 ml of Tyrode's buffer was added to freshly incubated platelets from which 200µl were aliquoted into 200µl of HEPES-Tyrode's buffer. Ca^{2+} was then added to the platelets to a final concentration of 1mM. The basal reading of Ca^{2+} was measured for 1 minute by the Accuri C6 cytometer then 0.5 units/ml of thrombin and 10µg/ml of collagen or 1µM of calcium ionophore, A23187, were added to the mixture and Ca^{2+} entry was measured for 9 minutes. Using the FACS Express Software, the percent of

total Ca^{2+} entry above basal was measured and plotted for the untreated vs.CaCCinh-A01 treated mouse platelets.

2.9 Western Blot

PCDNA-TMEM16F-Flag was generated through TA cloning in a PCRII vector, and then sequenced by Genewiz Molecular Biology (Figure 3). HEK293T cells were transfected with pcDNA-TMEM16F-Flag, PAR4-GFP and pCDNA-TMEM16F-Flag and PAR4-GFP together using TransIT-293 transfection reagent (Mirius). 48hours after transfection, cells were lysed and immunoblotted with an antibody to TMEM16F (1:1000) (Santa Cruz), with an antibody to GFP (1:500) (MLB).

```
CLUSTAL 2.1 multiple sequence alignment

Known           CTCTCTGGCTAACTAGAGAACCCACTGCTTACTGGCTTATCGAAATTAATACGACTCACT 60
Cloned          CTCTCTGGCTAACTAGAGAACCCACTGCTTACTGGCTTATCGAAATTAATACGACTCACT 60
                ************************************************************

Known           ATAGGGAGACCCAAGCTTACCATGGACTACAAAGACGATGACGACAAAGGATCCCCGGGC 120
Cloned          ATAGGGAGACCCAAGCTTACCATGGACTACAAAGACGATGACGACAAAGGATCCCCGGGC 120
                ************************************************************
```

Figure 3. Comparison of sequenced pCDNA-TMEM16F-Flag clone with known Homo sapiens anoctamin 6(TMEM16F) mRNA(Genbank: BC098410)

2.10 Total Intracellular Calcium in Transfected HEK 293T Cells

Transfected HEK293T cells were lifted in HBSS (Sigma) with 1mM EDTA then resuspended in complete DMEM (Corning CellGro) with 10% FBS. $2x10^5$ cells were loaded in Tyrode's buffer with 2uM of pluronic acid (Invitrogen) in a 1:1 ratio with Fluo-4-AM (Invitrogen) for 20 minutes at $37^{O}C$. A final concentration of 0.5 mM of calcium and the agonist was added (2μM A23187, 2mM AYP, 1U/ml thrombin) were added to the cells. Calcium entry was then analyzed with the Accuri C6 flow cytometer and quantified using the FCS Express Software.

2.11 Total Intracellular Calcium in Transfected CHOK Cells

$1.8x10^7$ transfected CHOK cells together were lifted in HBSS (Sigma) with 0.5 M EDTA then resuspended in 10 ml complete media HAM's F-12 with 10% FBS. Cells were then incubated with 4uM Fura-2-AM (Invitrogen) at 37^oC for 30-45 minutes. A final concentration of 0.5 mM of calcium and the agonist was added (2μM A23187, 2mM AYP, 1U/ml thrombin) were added to the cells. Calculations of Ca^{2+} concentration were determined using the PTI fluometer and FelixGX software.

2.12 Statistics

Statistical analyses were done as t-tests using GraphPad Prism software and QuickCalcs of GraphPad website. Statistical significance was defined by $p \geq 0.05$. Data were measured as means±SE.

Chapter 3

RESULTS

3.1 Significant PS Exposure Defect in TMEM16F KO Platelets

To analyze the role of TMEM16F in PS exposure of platelets, we tested for the binding of AnnexinV on the surface of platelets from WT mice versus those with a knock-out in the gene for TMEM16F (TMEM16F KO). Using flow cytometry, PS exposure was measured as a percentage of maximal Annexin V binding yielded by WT platelets treated with 10μM A23187 (Figure 4). When compared to the WT platelets after dual agonist (Thr+Cvxn) treatment, TMEM16F KO showed a significant decrease in PS exposure platelets ($p=0.0005$). The percentage of maximal Annexin V binding decreased by almost 4 fold in the TMEM16 KO platelets compared to the WT platelets. The defect was milder in the TMEM16F+/- platelets since there was only a 2-fold decrease in the percentage of maximal Annexin V binding when compared to the WT platelets. In calcium ionophore (A23187) - treated platelets, the same general trend was observed, with the PS exposure defect being more severe in the TMEM16F KO than the TMEM16F+/-. These data suggested to us that the presence of TMEM16F likely affects the procoagulant activity of platelets since the gene knockout creates a defect in PS exposure: studies are continuing to be pursued in our laboratory.

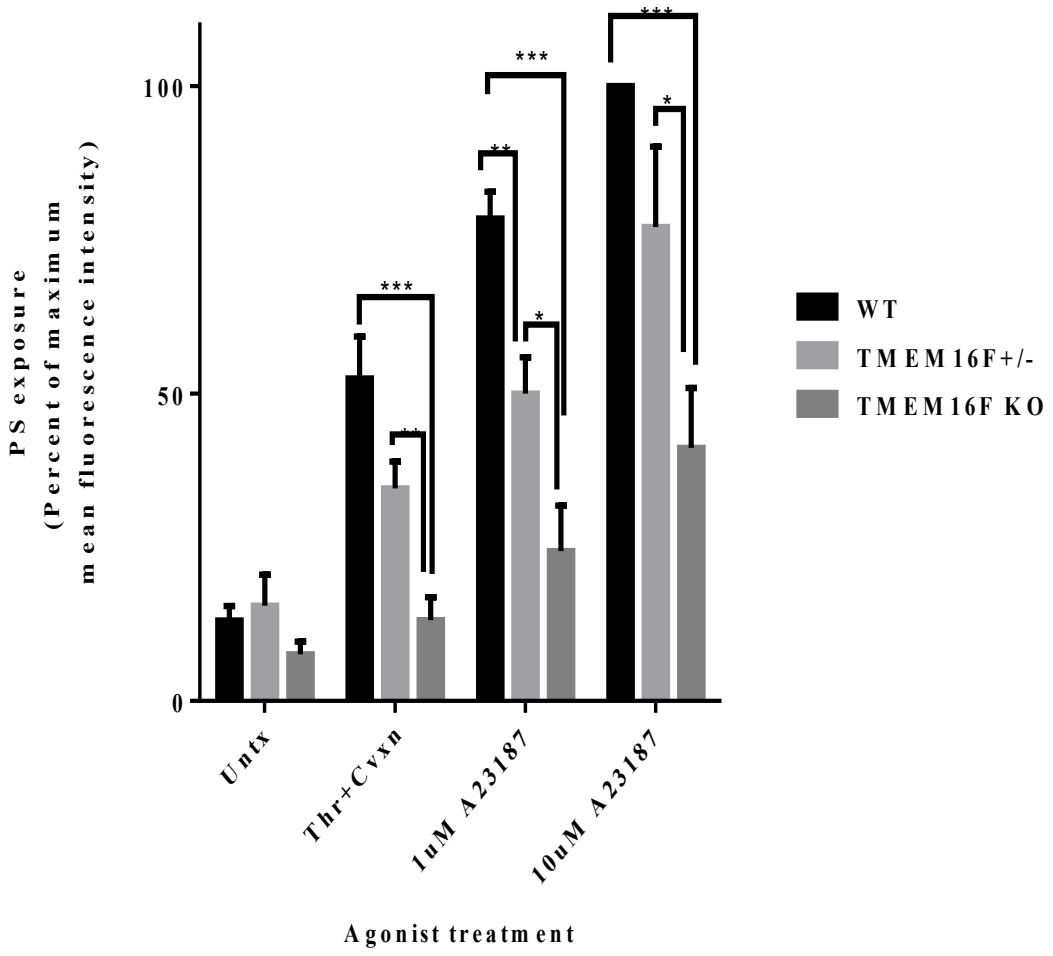
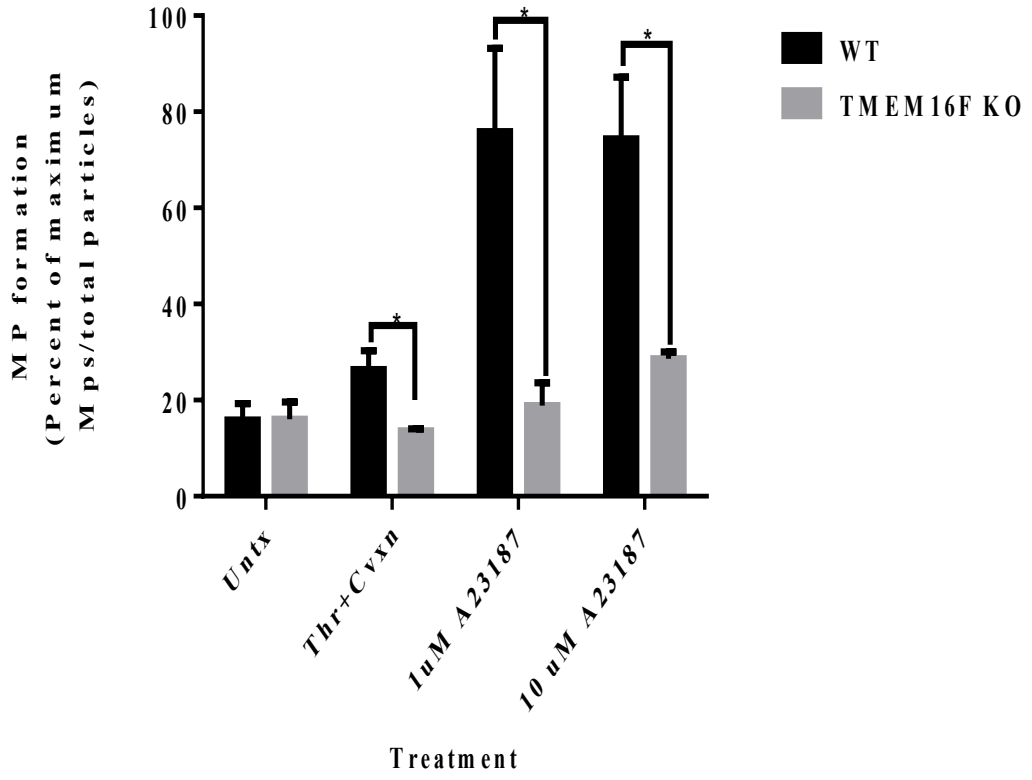


Figure 4. Comparison of Annexin-binding in the WT, TMEM16F+/- and TMEM16F KO mouse platelets as a percentage of maximum fluorescence intensity. Data was normalized to the mean fluorescence intensity of WT platelets treated with 10μM A23187. Significance level: *: $p \leq 0.05$, **: $p \leq 0.01$;***: $p \leq 0.001$

3.2 A Defect in MP Formation Detected in TMEM16F KO Platelets

Since microparticles have been shown to have procoagulant properties, we set out to determine whether the presence of TMEM16F affected microparticle formation. Using flow cytometry, microparticle formation was quantified as the ratio of the number of microparticles over the number of total number of particles measured as the sum of microparticles and platelets present in the sample (Figure 5A). When platelet-rich plasma (PRP) was treated with a combination of thrombin and convulxin (Figure 5), there was a significant decrease in the percent of microparticles formed by platelets from the TMEM16F KO mice compared to WT after thrombin and convulxin stimulation ($p=0.05$) and there was a significant decrease after treatment with 1μM of A23187 ($p=0.04$) and 10μM A23817 ($p=0.03$). The number of microparticles per platelets in the TMEM16F KO was compared to the WT. There was no significant decrease from the WT to the TMEM16F when the PRP was treated with thrombin and convulxin. We plan on doing an additional experiment to get an accurate picture of the significance since only 2 experiments were conducted under identical conditions with the same platelet input.

A


MP formation
(Percent of maximum
Mps/total particles)
100
80
60
40
20
0
*
*
*
Untx
Thr+Cvxn
1uM A23187
10 uM A23187
Treatment
WT
TMEM16F KO

B

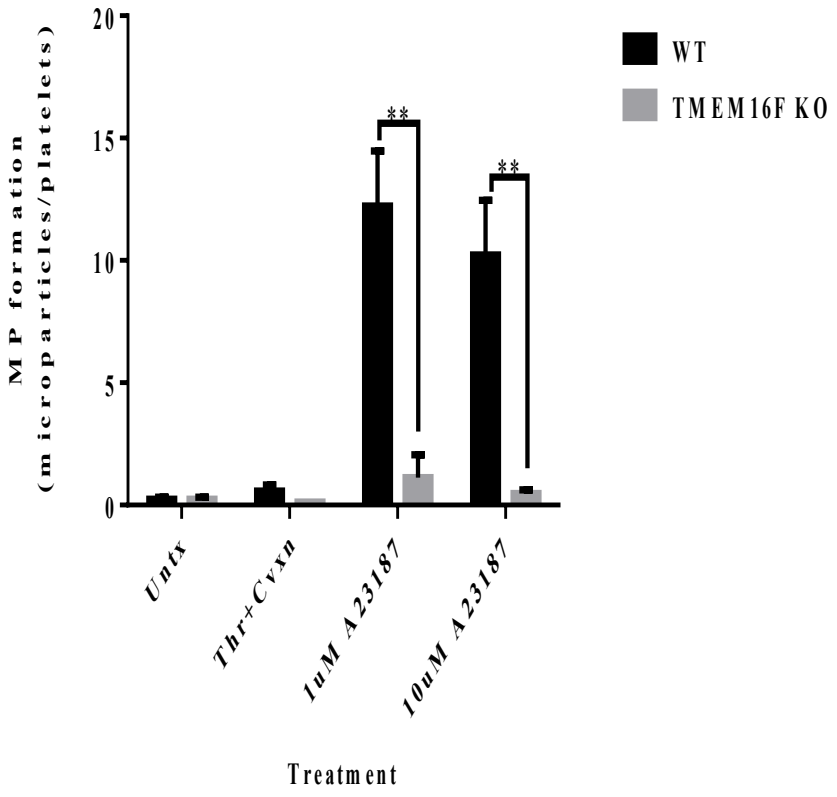


Figure 5. (A) Microparticle formation in the WT and TMEM16F KO mice as a percentage of microparticles formed over total particles defined as the platelets and microparticles. (B) Number of microparticles/platelet. Data was measured as a mean±SE. Significance was defined by $p \leq 0.05$. Significance level: *: $p \leq 0.05$, **: $p \leq 0.01$

3.3 Defect in Annexin + Microparticles Generated from TMEM16F KO Platelets

To determine if TMEM16F was associated with the exposure of PS on the surface of microparticles, flow cytometry was used to measure Annexin V binding to microparticles, quantified as mean fluorescence intensity (Figure 6). After treatment with thrombin and convulxin, TMEM16FKO microparticles bound less Annexin V (as a function of mean fluorescence intensity) compared to the WT. Additionally, there was a significant decrease in Annexin V binding in the TMEM16F KO microparticles compared to the WT after calcium ionophore stimulation (1μM A23187: $p=0.02$;10μM A23187: $p=0.008$).

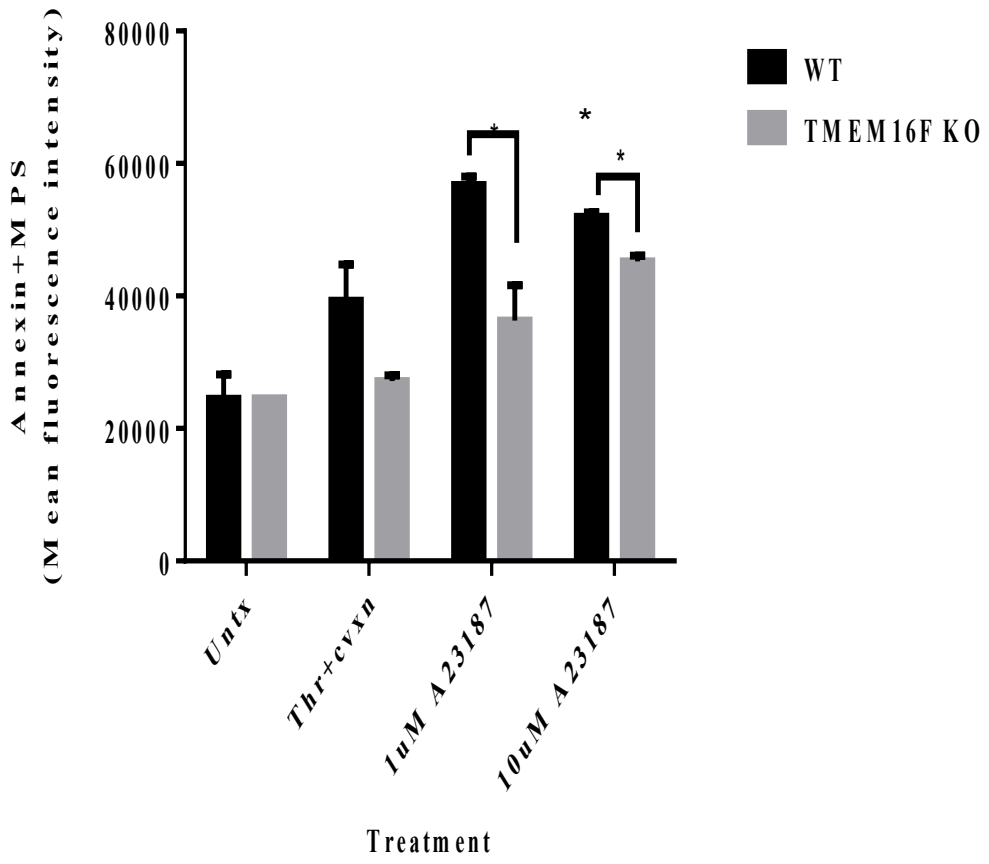
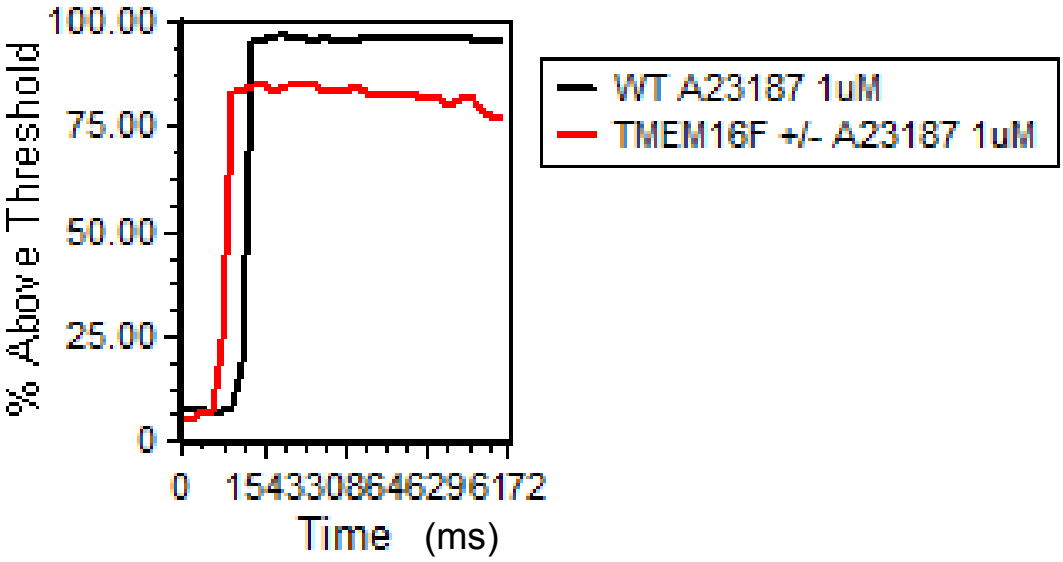


Figure 6. Annexin-binding on microparticles from WT, and TMEM16F KO mice measured as mean fluorescence intensity. Data was measured as a mean±SE. Significance was defined by $p \leq 0.05$. Significance level: *: $p \leq 0.05$, **: $p \leq 0.01$

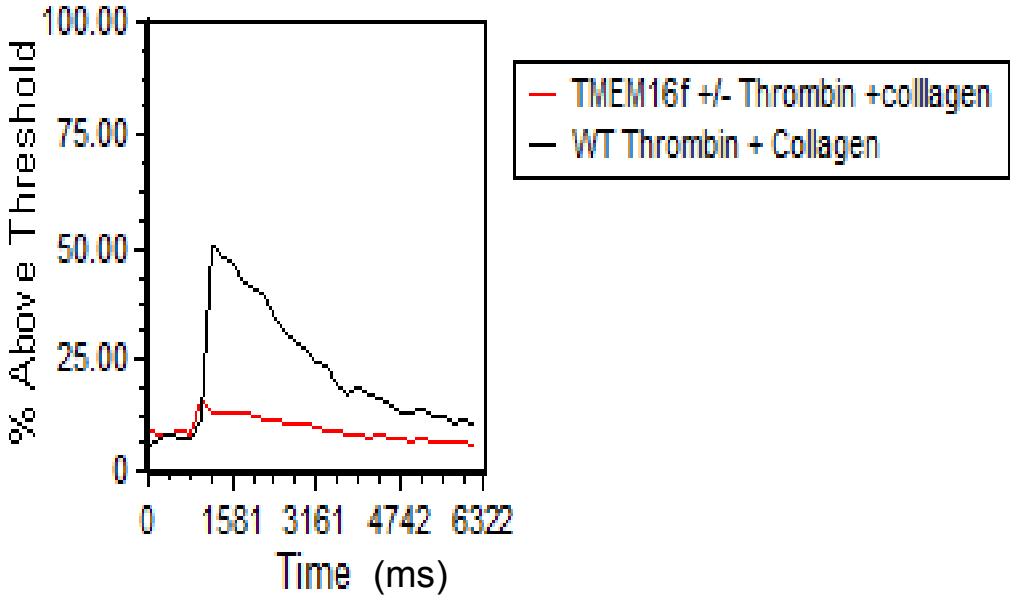
3.4 Significant Calcium Entry Defect of the TMEM16F+/- Platelets

An increase in intracellular calcium has been shown to be needed for PS exposure (Lhermusier et al., 2011) and MP formation (Mause et al., 2010). Therefore, we examined the requirement for maximal expression of TMEM16F in platelets for maximal increase in intracellular platelet calcium. Total intracellular calcium mobilization above basal was analyzed by assessing calcium-dependent fluo4 fluorescence following Fluo4AM loading of mouse platelets. After dual agonist stimulation, the WT platelets had a higher increase in calcium entry compared to the TMEM16F+/- platelets, whose calcium increase was not much higher than the basal (Figure 7.A). The average calcium signal (Figure 7.C) in the WT platelets immediately following stimulation was significant ($p=0.02$) and almost~ 2 -fold higher than that of the TMEM16F+/- platelets. As for the A23187 agonist treatment, there was not a significant difference between the WT and the TMEM16F+/- (Figures 7.B-C). The loss of calcium entry in the TMEM16F+/- platelets following dual agonist treatment suggests that this pathway is important in vivo, since thrombin and collagen are natural agonists generated (or exposed) at sites of injury or pathological thrombosis. Thus, TMEM16F potentially regulates thrombin and collagen-mediated calcium signals to regulate hemostasis and thrombosis.

A


100.00
75.00
50.00
25.00
0
% Above Threshold
0
1543308646296172
Time (ms)
WT A23187 1uM
TMEM16F +/- A23187 1uM

B


100.00
75.00
50.00
25.00
0
% Above Threshold
0
1581
3161
4742
6322
Time (ms)
TMEM16f +/- Thrombin +colllagen
WT Thrombin + Collagen

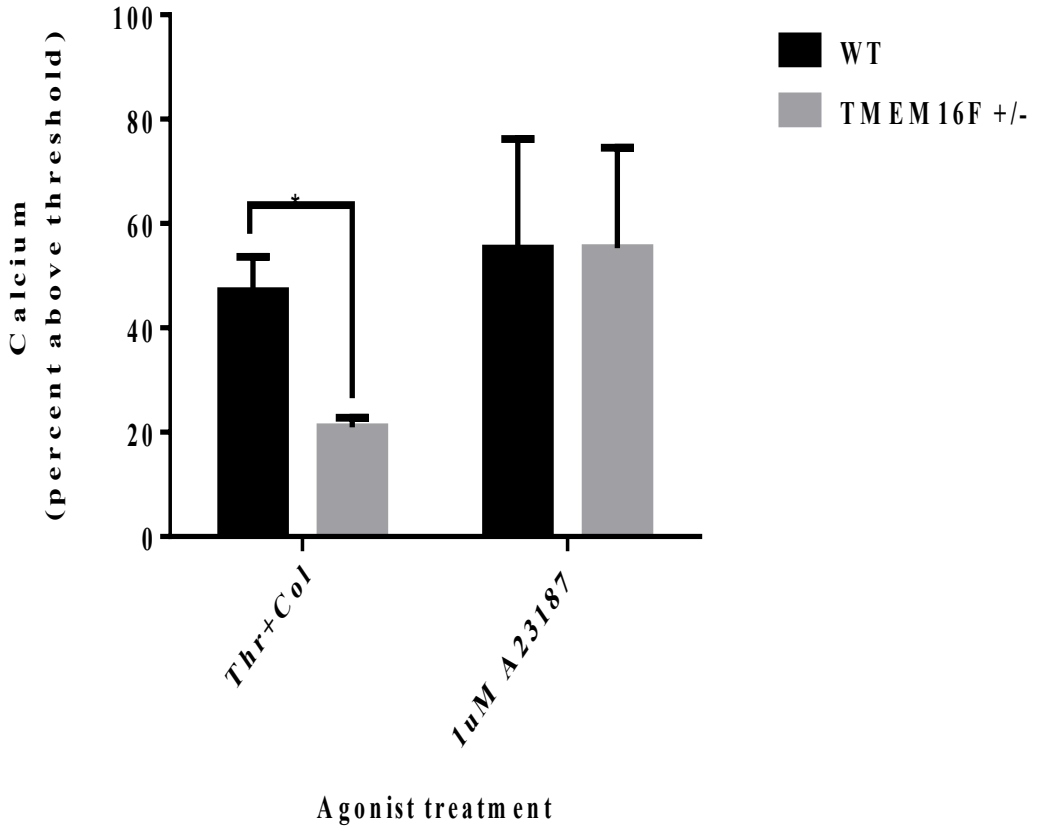
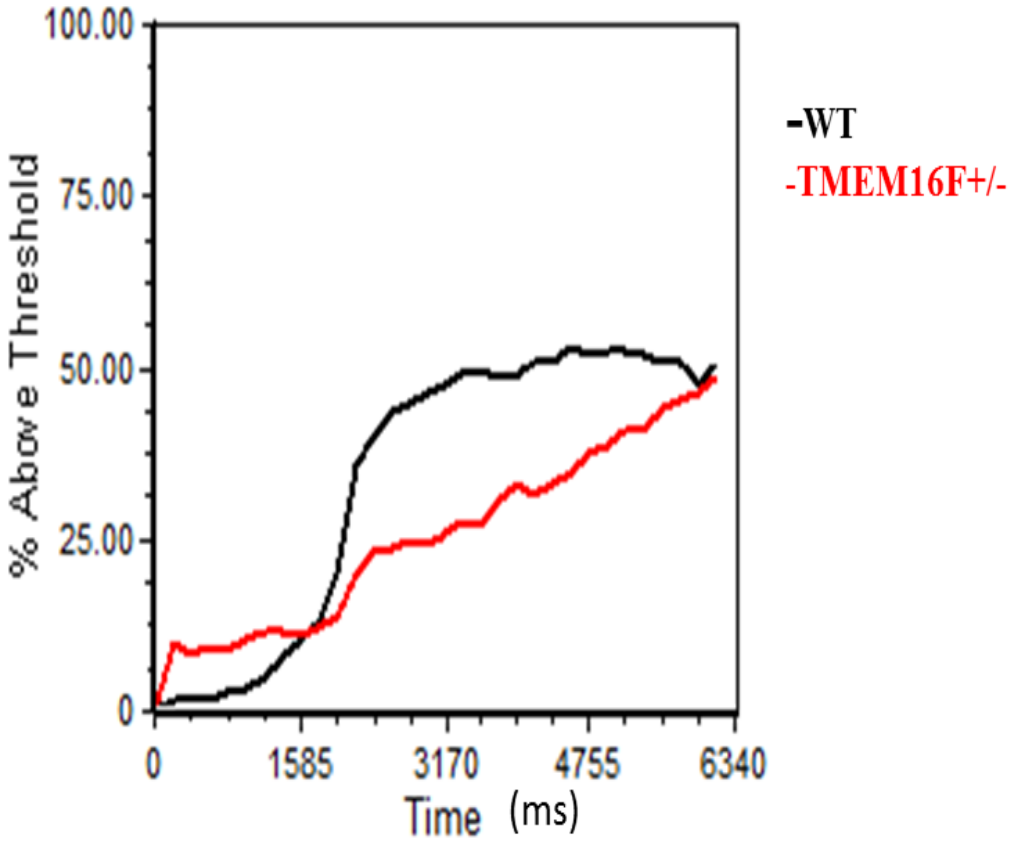


Figure 7. (A-B) FCS Express analysis of the flowcytometer data for the WT mouse platelets compared to the TMEM16F+/- mouse platelets treated with 1μMA23187 or 0.5U/ml thrombin and 10μg/ml collagen together. (C) Maximal percentage above basal for the different treatments of the WT mouse platelets compared to the TMEM16F+/- platelets. Significance level: *: $p \leq 0.05$

3.5 Impairment of the Store-Operated Calcium Entry (SOCE) in the TMEM16F+/- Platelets

Upon stimulation with thrombin and collagen, calcium gets released from the ER stores after stimulation of IP_3R receptors. The depletion of calcium from the ER stores triggers store-operated calcium entry through the plasma membrane, or SOCE. To test whether TMEM16F specifically regulated SOCE, platelets were treated with thapsigargin, an inhibitor that blocks the reuptake of calcium in the ER stores through the membrane protein transporter SERCA, thus increasing cytoplasmic calcium levels. Flow cytometry analysis of thapsigargin-treated platelets showed that calcium entry was defective in TMEM16F+/- platelets compared to the WT platelets (Figure 8.A). Further quantification revealed that, upon extracellular Ca^{2+} addition following ER store depletion, there was a significant delay in calcium entry in TMEM16F+/- platelets compared to the WT platelets($p=0.05$) (Figure 8.B.). These results demonstrate that a decrease in platelet TMEM16F expression affects extracellular calcium entry of the SOCE.

A


100.00
75.00
50.00
25.00
0
% Above Threshold
0
1585
3170
4755
6340
Time (ms)
-WT
-TMEM16F+/-

B

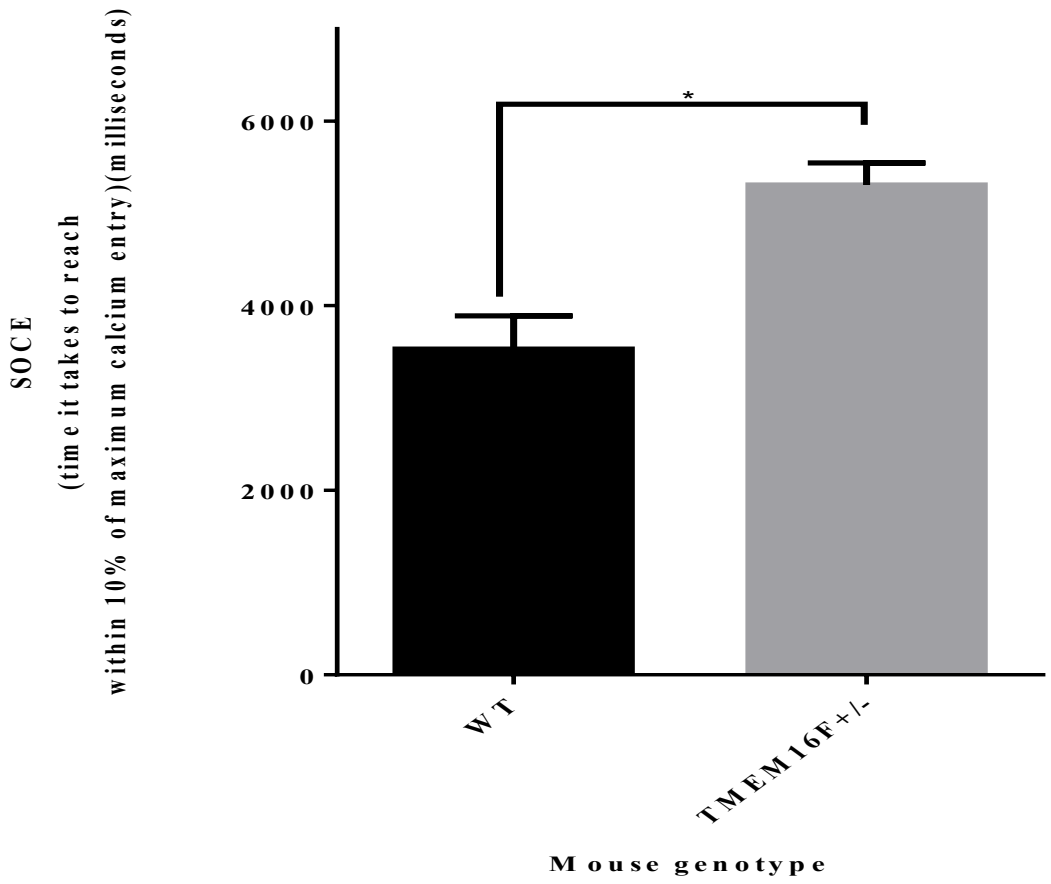
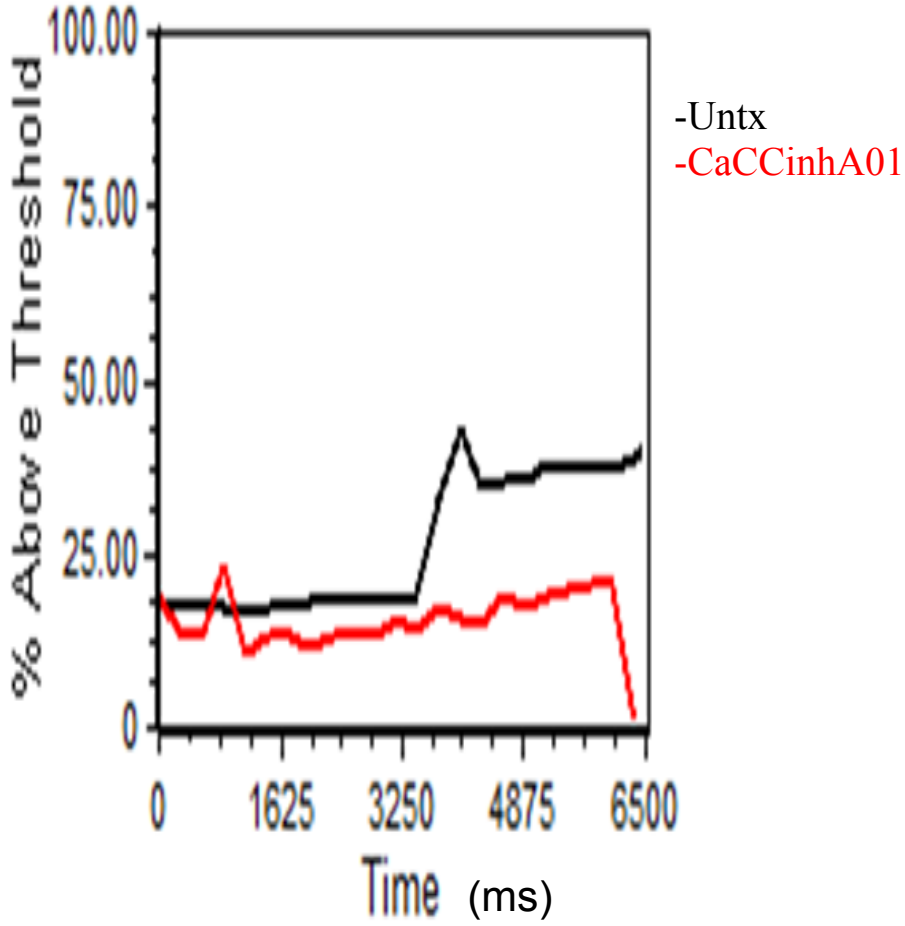


Figure 8. (A) FCS Express analysis of the SOCE Accuri data for the WT mouse platelets compared to the TMEM16F+/- mouse platelets. Platelets were treated with consecutively with 200µM EGTA, 1µM TG and 0.5 mM CaCl2 (Ca2+). (B) Time elapsed before 10% within Ca^{2+} maximal fluorescence peak is reached. Data measured as mean±SE. Significance level: *: $p \leq 0.05$

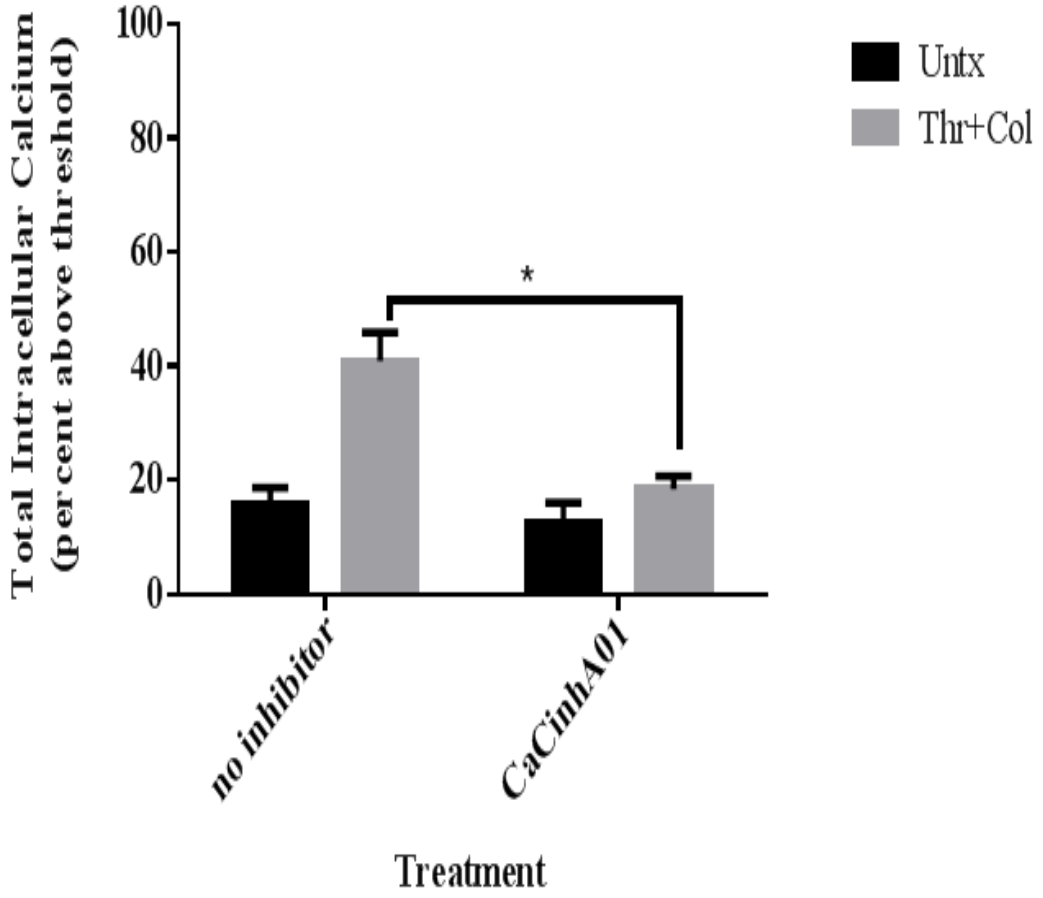
3.6 The CaCCinh A01 Inhibitor Blocked the Calcium Channel Activity of TMEM16F, but not PS Exposure

In this experiment, we set out to determine whether blocking the channel of TMEM16F will also block scramblase activity, and thus, PS exposure. Since PS exposure is defective in both the TMEM16F KO and TMEM16F+/-, and thus a decrease due to blocking scramblase activity would not be detectable, we studied the linkage between channel and scramblase activity by applying a channel blocker to platelets of WT mice. To determine whether calcium entry is required for TMEM16F scramblase activity, we blocked calcium entry with CaCCinhA01 (Figure 9.A-C). When CaCCinhA01 was added to platelets, calcium entry stimulated by thrombin and collagen was completely inhibited. ($p=0.01$) (Figures 9.A-B) To determine whether PS exposure is coupled to the role of TMEM16F as a calcium channel, PS exposure of WT platelets was tested in the presence vs. absence of CaCCinhA01 after stimulation with thrombin and collagen. The results show that there is no change in the PS exposure of platelets treated with thrombin and collagen after inhibition of the channel activity of TMEM16F. This suggests that the role of TMEM16f in PS exposure following thrombin and collagen stimulation is not linked to its role as a calcium channel under these conditions.

A


100.00
75.00
50.00
25.00
0
% Above Threshold
0
1625
3250
4875
6500
Time (ms)
-Untx
-CaCCinhA01

B


Total Intracellular Calcium (percent above threshold)
100
80
60
40
20
0
*
Untx
Thr+Col
no inhibitor
CaCinhA01
Treatment

C

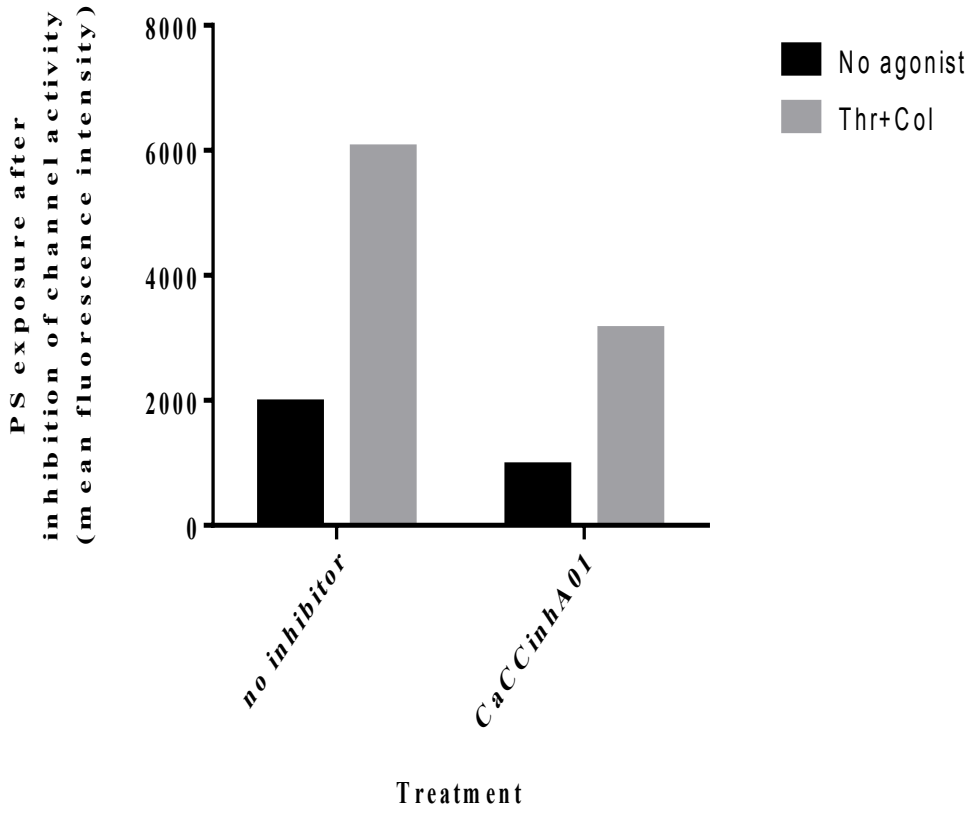
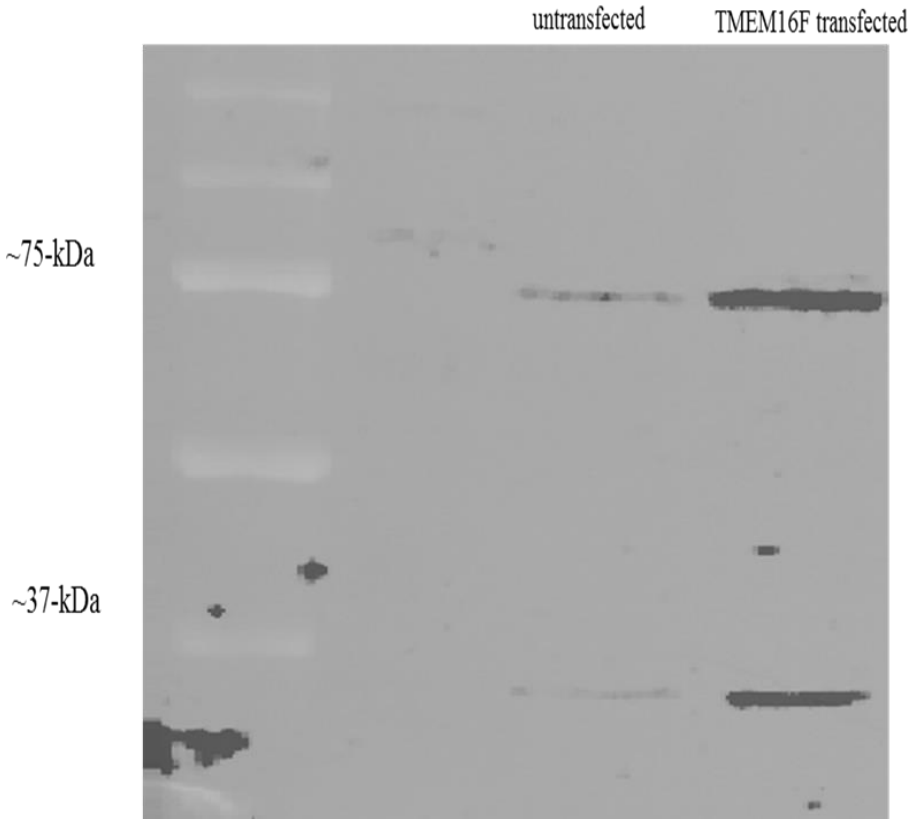


Figure 9. (A) FCS Express analysis of the Accuri data for the mouse platelets compared to the platelets preincubated with 20μM CaCCinhA01 for 5 minutes. (B) Percent of calcium fluorescence above threshold after thrombin (0.5u/ml) and collagen (10μg/ml) stimulation. Data measured as mean±SE. Significance level: *: $p \leq 0.05$. (C) PS exposure after thrombin (0.5u/ml) and collagen (10ug/ml) treatment measured as the percentage of mean fluorescence intensity above basal (untreated).

3.7 Expression of TMEM16F Delays Decay of Calcium Entry and Potentiates Calcium Entry, but not Sufficient to Mediate PS Exposure

HEK293T cells were transfected with TMEM16F and its expression was compared to untransfected HEK293T cells (Figure 10 A). A transient increase in TMEM16F expression in HEK293T cells was achieved, allowing comparison of the channel and scramblase activities of TMEM16F expression with non-expressing untransfected cells. When untransfected HEK293T cells were treated with A23187 (Figure 10B), they displayed a faster rate of calcium decay compared to the cells transfected with TMEM16F. Furthermore, expression of TMEM16F in CHOK.1 cells resulted in a greater rise in A23187-initiated peak intracellular calcium concentration when assessed by Fura-2-dependent fluorimetry than untransfected CHOK cells (Figure 11B, C). However, when the PS exposure was measured, there was no significant increase of PS compared to the basal after stimulation with A23187 in either the untransfected or the TMEM16F-transfected CHOK cells (Figure 11D). Overall, these results indicate that while TMEM16F regulates calcium entry in the heterologous system, its expression is not sufficient to mediate PS exposure.

A



B

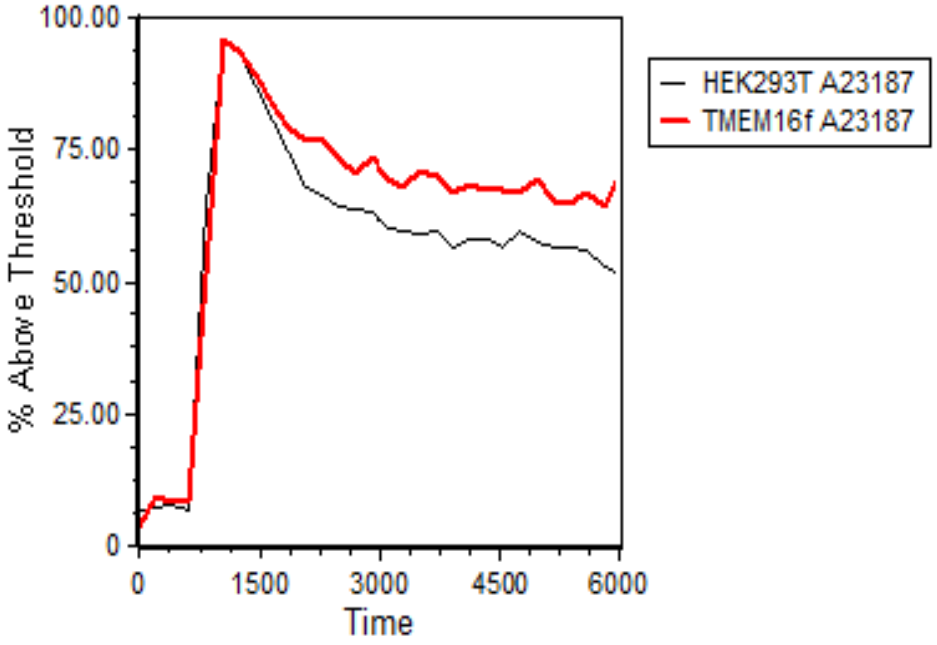
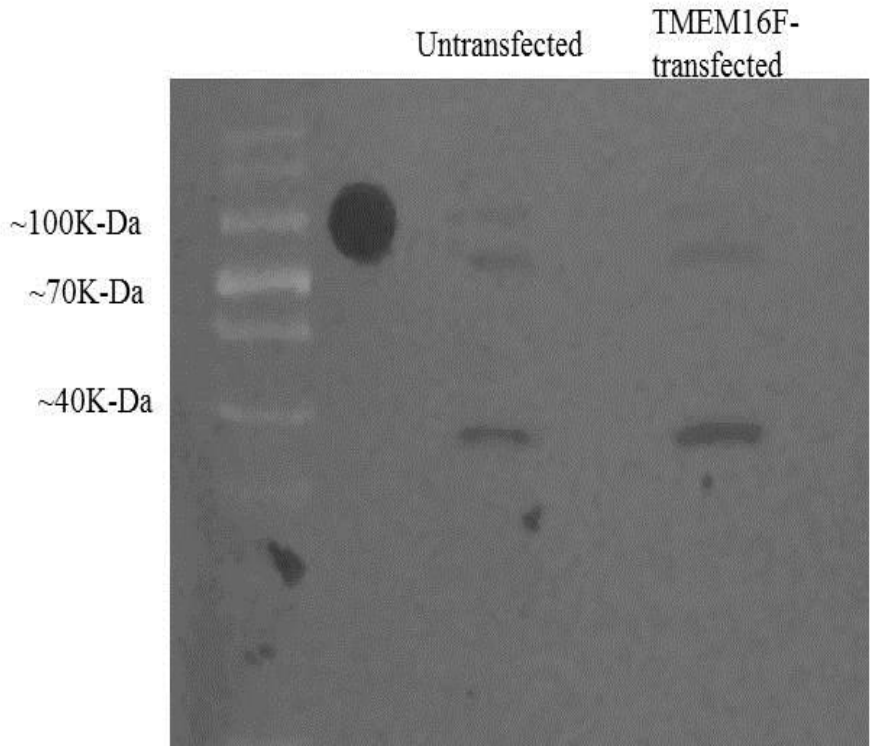
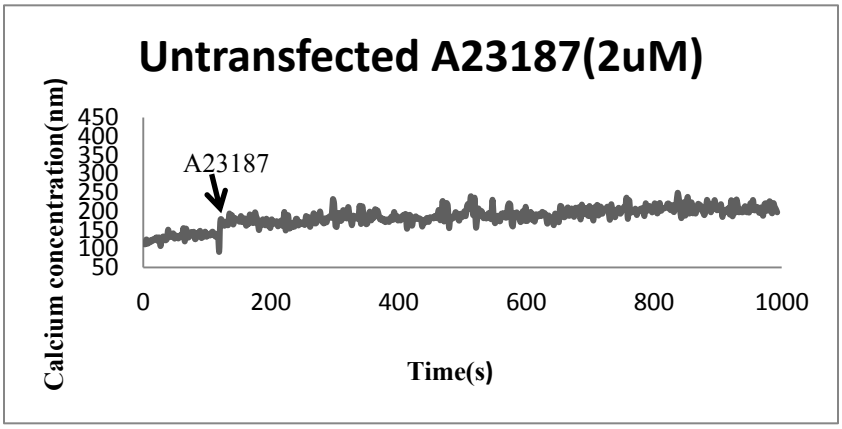


Figure 10. (A) TMEM16F- transfected HEK293T cells (lane 2: untransfected and lane 3: transfected). Immunoblotting with anti-TMEM16F. 2 bands are seen at size around size 75-kDa and 37-kDa. Expected molecular mass for TMEM16F size is 106-kDa. (B) FCS analysis of HEK293T cells treated with 2µM A23187.

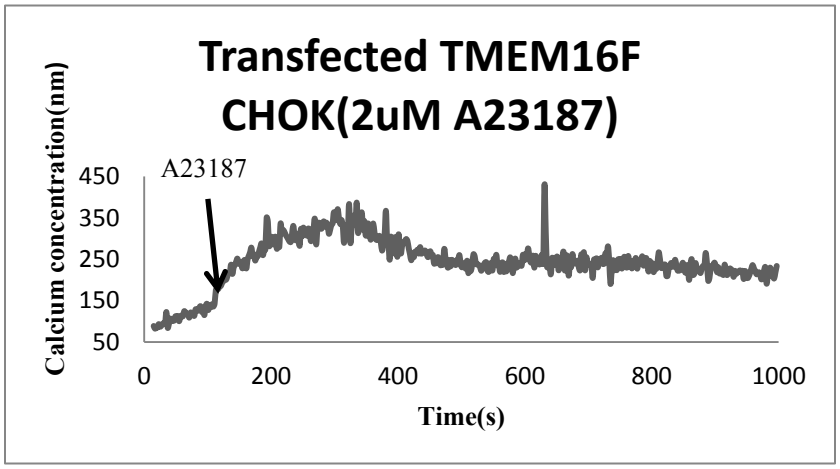
A



B


Untransfected A23187(2uM)
Calcium concentration(nm)
450
400
350
300
250
200
150
100
50
A23187
0
200
400
600
800
1000
Time(s)

C


Transfected TMEM16F
CHOK(2uM A23187)
Calcium concentration(nm)
450
350
250
150
50
A23187
0
200
400
600
800
1000
Time(s)

D

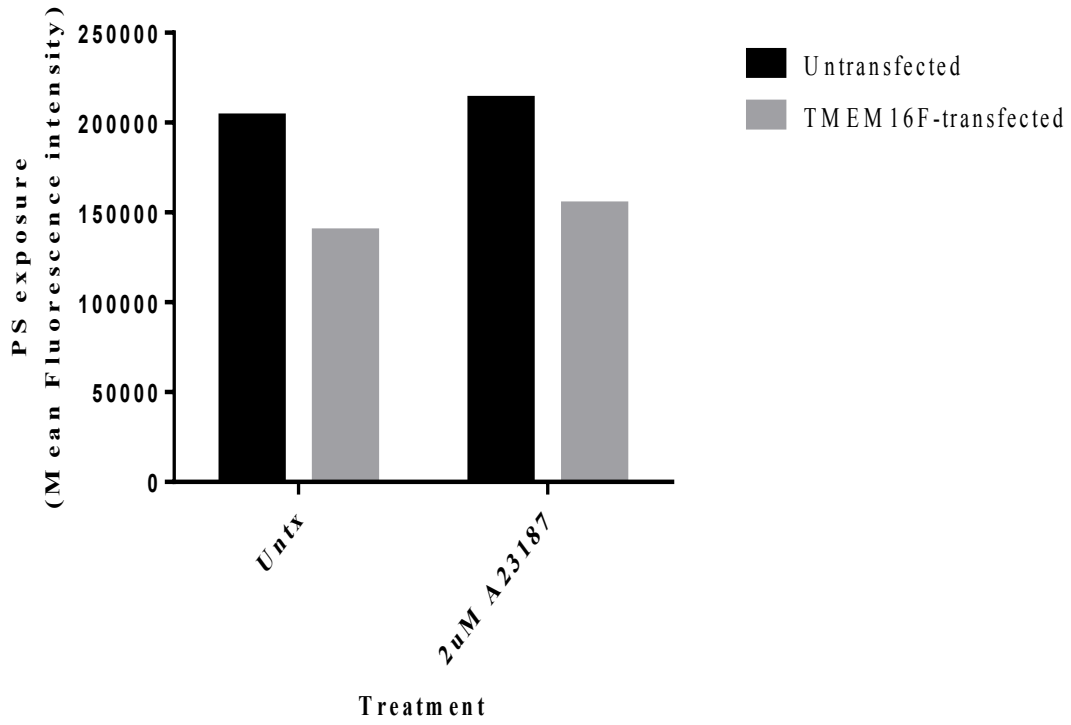


Figure 11. (A) TMEM16F- transfected CHOK cells (lane 3: untransfected and lane 4: transfected). Immunoblotting with anti-TMEM16F. (B-C) Calcium concentration was measured in untransfected and TMEM16F-transfected CHOK cells stimulated with 2μM A23187. PTI fluometry and Felix GX software were used. (D) PS exposure of untransfected and TMEM16F-transfected CHOK cells after treatment with 2μM A23187 measured as the percentage of mean fluorescence intensity above basal (untreated).

Chapter 4

DISCUSSION

The role of TMEM16F in the scrambling activity of platelets has been highly debated. In order for the hemostatic plug to be stabilized, thrombin must be generated to cleave fibrinogen and thereby generate a stable fibrin clot (Greenberg et al., 1985). This process is localized and accelerated by the exposure of phosphatidylserine (PS) on the surface of platelets, since PS provides the negatively charged surface needed for the formation of the tenase and prothrombinase complexes required for the production of thrombin (Weiss et al., 1997). TMEM16F was cloned as a candidate for this platelet scramblase by screening a BAF/3 cell line for high constitutive PS exposure (Suzuki et al. 2010). Further confirmation that TMEM16F was involved in platelet lipid scrambling was derived from the identification of 2 novel TMEM16F mutations in a patient with Scott syndrome (Castoldi et al., 2011), a disease in which patients display a defect in procoagulant activity of platelets due to impairment in their scrambling activity and subsequent PS exposure (Zwaal et al., 2004). Due to these observations, it was argued that TMEM16F was the scramblase responsible for the redistribution of PS on the outer leaflet of the plasma membrane. At this time, our laboratory began investigating the role of TMEM16F in platelet procoagulant activity and thrombin generation. We set out to determine whether TMEM16F was both necessary and sufficient for PS scrambling by both evaluating PS exposure in TMEM16F knock-out platelets and by reconstituting expression of TMEM16F in a heterologous system. During this time, a study done by Jan's group and published in Cell (Yang et al., 2012) showed that TMEM16F KO mice that had the same

characteristics as patients with Scott's syndrome still displayed some PS exposure upon stimulation of their platelets by the calcium ionophore, A23187. The authors of this study argued that TMEM16F was either part of a complex, or that there are compensatory Scramblases that can compensate for loss of TMEM16F under extremely high calcium conditions. Subsequent to publication of this study, we broadened our own studies to include understanding of the role of TMEM16F in microparticle formation. Furthermore, we also looked at platelet activation under physiological conditions because A23187 is not a natural agonist of platelet receptors, but this was the only activator of PS exposure evaluated in the study by Jan's group. Our results demonstrated that loss or reduction of TMEM16F expression impaired PS exposure in platelets, and thus is required for optimal scramblase activity in platelets. TMEM16F may be the scramblase or may be part of a larger scrambling complex. Other components of this complex or an independent scramblase may compensate for TMEM16F loss at very high calcium concentrations, such as that displayed by 10 μM A23187 stimulation in TMEM16F KO platelets.

Patients affected by Scott's syndrome also display a decrease in the number of microparticles (Dachary-Prigent et. al.1997). Microparticles are microvesicules resulting from the budding of the plasma membrane (Hugel et al., 2005). An increase in intracellular calcium triggers microparticle generation through the activation of calpain (VanWijk et al. 2003). Calcium-activated calpain mediates microvesiculation by cleaving cytoskeletal proteins and remodeling the submembrane cytoskeleton. (Pasquet et al., 1996). Calcium-dependent activation of kinases by the C5b-9 complement complex has also been shown to participate in microparticle generation (Wiedmer et al., 1991). Moreover, calcium-dependent phosphatase inhibition also

increased the number of microparticle generated (Wiedmer et al. 1991), suggesting the important role of phosphorylation in microparticle formation. We demonstrated that a loss or decrease in expression of TMEM16F on the surface of platelets negatively affected microparticle formation from platelets. This may simply be due to the activity of TMEM16F as a calcium channel, such that when TMEM16F is deleted, less calcium entry is permitted, so that total intracellular calcium does not reach the threshold required to activate calpain. However, our studies showing that the calcium inhibitor CaCCInhA01 blocked calcium entry, but did not block PS exposure, suggest that TMEM16F modulates PS Scramblase activity by a mechanism not coupled to the flux of Calcium through the channel. In other words, the dual activities of TMEM16F as a calcium channel and as a Scramblase may be independent of one another. The role of TMEM16F in PS+ microparticle shedding from platelets likely also regulates procoagulant activity, since TMEM16f-/- mice have a documented decrease in thrombin generation (Ann Fowajuh, unpublished results and Jan, 2012).

The channel activity of TMEM16F has been extensively studied and remains a topic of great contention. TMEM16F has been labeled a volume-sensitive outwardly rectifying Cl- channel (Almaça et al., 2009), a calcium-activated chloride channel (Shimizu et al., 2013), outwardly rectifying chloride channel (Martins et al., 2011) and a calcium-activated cation channel (Yang et al., 2012). However, because intracellular calcium coincides with microparticle formation and PS exposure in activated platelets (Chang et al., 1993) and considering the fact that TMEM16F has been identified as a calcium-activated calcium channel in platelets(Yang et al., 2012), we wanted to establish whether TMEM16F mediated the calcium flux needed for both of these processes to occur. Our results indicated that not only did TMEM16F regulate total

intracellular calcium, but it was specifically required for thrombin and collagen-stimulated calcium entry via store-operated calcium entry (SOCE). As noted above, although platelets treated with the calcium-activated channel blocker displayed a decrease in calcium entry, their PS exposure was similar to that of untreated platelets. As noted, a possible explanation for these results may be that TMEM16F has two independent functions in platelets: one as a regulator of the scrambling activity of platelets and the other as a calcium channel. This explanation is further supported by the fact that preliminary data of calcium entry analyzed in HEK293T cells (Figure 10.B) stimulated with ionophore displayed an increase in calcium entry if transfected with TMEM16F compared to untransfected controls. However, expression of TMEM16F was not sufficient to induce PS exposure in these cells, even in the presence of calcium ionophore. One possible explanation for these results may be that platelet-specific mechanisms that are not found in the heterologous system are needed for TMEM16F to regulate PS exposure in those cells. This may suggest that TMEM16F is part of a scrambling complex, a component of which is not expressed in the HEK cells, or alternatively, TMEM16F is a required upstream regulator of a separate Scramblase (not expressed in the HEK cells).

The results of our investigation show that TMEM16F regulates the calcium entry and scrambling activity of platelets, and that these functions of TMEM16F are independent of each other. Figure 12 illustrates the role of TMEM16F in platelets. Upon platelet activation by thrombin and collagen, calcium is released from the stores. The subsequent increase in intracellular calcium activates TMEM16F, thereby allowing for extracellular calcium influx to the cytosol. These high concentrations of calcium allow for the remodeling of the membrane cytoskeleton by calpain and

subsequent microparticle formation. Our data show that a defect in TMEM16F impairs the Store-Operated Calcium Entry. In platelets, the SOCE is regulated by the calcium-sensor STIM1 (Zhang et al., 2005) that activates the calcium channel, Orai1 (Bergmeier et al., 2009). TMEM16F might get regulated by the STIM1 and play a similar role to Orai1. However, further studies are needed to establish that. Our schematic also includes the proposed role of TMEM16f as a component regulating platelet scrambling activity. We believe that TMEM16F is likely part of a larger scramblase complex. Nonetheless, more research is required to establish the identity of individual components of the scramblase complex and understand how TMEM16F interacts with the other proteins in the complex.

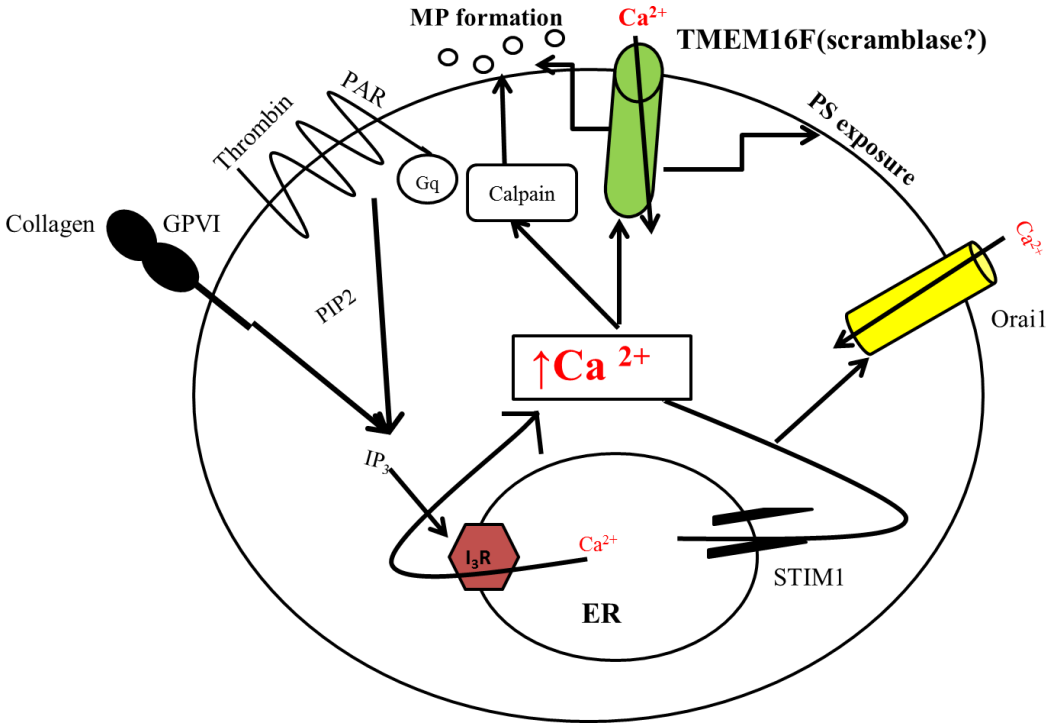


Figure 12. Schematic showing the role of TMEM16F in PS exposure, microparticle formation and calcium influx

REFERENCES

1. Abbracchio MP, Burnstock G, Boeynaems JM, Barnard EA, Boyer JL, Kennedy C, Knight GE, Fumagalli M, Gachet C, Jacobson KA, Weisman GA .International Union of Pharmacology LVIII: update on the P2Y G protein-coupled nucleotide receptors: from molecular mechanisms and pathophysiology to therapy. Pharmacol Rev. 2006; 58(3):281-341.

2. Ahmad SS, Rawala-Sheik R, Ashby B, Walsh PN. Platelet receptor-mediated factor X activation by factor IXa. High-affinity factor IXa receptors induced by factor VIII are deficient on platelets in Scott syndrome. J Clin Invest. 1989; 4(3):824-8.

3. Alberts B, Johnson A, Lewis J, et al. Molecular Biology of the Cell. 2th edition. New York: Garland Science; 1989.The Lipid Bilayer. The phospholipids of the lipid bilayer have polar heads and non-polar tails made of fatty acids

4. Almaça J, Tian Y, Aldehni F, Ousingsawat J, Kongsuphol P, Rock JR, Harfe BD, Schreiber R, Kunzelmann K. TMEM16 proteins produce volume-regulated chloride currents that are reduced in mice lacking TMEM16A. J Biol Chem. 2009; 284(42):28571-8.

5. Andersen H, Greenberg DL, Fujikawa K, Xu W, Chung DW, Davie EW. Protease-activated receptor 1 is the primary mediator of thrombin-stimulated platelet procoagulant activity. Proc Natl Acad Sci U S A. 1999; 96(20):11189-93.

6. Ardoin SP, Shanahan JC, Pisetsky DS. The role of microparticles in inflammation and thrombosis. Scand J Immunol. 2007; 66(2-3):159-65.

7. Bergmeier W, Oh-Hora M, McCarl CA, Roden RC, Bray PF, Feske S. R93W mutation in Orai1 causes impaired calcium influx in platelets. Blood. 2009; 113(3):675-8.

8. Brass LF, Stalker TJ, Xhu L, Woulfe DS. Signal Transduction During Platelet Plug Formation. In: Michelson AD, ed. Platelets. Elsevier Science, San Diego, 2007, pp 319-323

9. Brass LF. Thrombin and platelet activation. Chest. 2003; 124(3 Suppl):18S-25S.

10. Broeders MA, Tangelder GJ, Slaaf DW, Reneman RS, Egbrink MG. Endogenous nitric oxide and prostaglandins synergistically counteract thromboembolism in arterioles but not in venules. Arterioscler Thromb Vasc Biol. 2001; 21(1):163-9.

11. Brooks M, Etter K, Catalfamo J, Brisbin A, Bustamante C, Mezey J. A genome-wide linkage scan in German shepherd dogs localizes canine platelet procoagulant deficiency (Scott syndrome) to canine chromosome 27. Gene. 2010 15; 450(1-2):70-5.

12. Castoldi E, Collins PW, Williamson PL, Bevers EM. Compound heterozygosity for 2 novel TMEM16F mutations in a patient with Scott syndrome. Blood. 2011; 117(16):4399-400.

13. Chang CP, Zhao J, Wiedmer T, Sims PJ. Contribution of platelet microparticle formation and granule secretion to the transmembrane migration of phosphatidylserine. J Biol Chem. 1993; 268(10):7171-8.

14. Chu AJ. Tissue Factor, Blood Coagulation, and Beyond: An Overview. Int J Inflam. 2001. 2011.

15. Clark MR. Flippin' lipids. Nat Immunol. 2011; 12(5):373-5.

16. Coleman JA, Quazi F, Molday RS. Mammalian P4-ATPases and ABC transporters and their role in phospholipid transport. Biochim Biophys Acta. 2013; 1831(3):555-74.

17. Cooper GM. The Cell: A Molecular Approach, 2nd edition. Sunderland (MA): Sinauer Associates; 2000. Structure of the Plasma Membrane

18. CP, Zhao J, Wiedmer T, Sims PJ. Contribution of platelet microparticle formation and granule secretion to the transmembrane migration of phosphatidylserine. J Biol Chem. 1993; 268(10):7171-8.

19. Dachary-Prigent J, Pasquet JM, Fressinaud E, Toti F, Freyssinet JM, Nurden AT. Aminophospholipid exposure, microvesiculation and abnormal protein tyrosine phosphorylation in the platelets of a patient with Scott syndrome: a study using physiologic agonists and local anaesthetics. Br J Haematol. 1997; 99(4):959-67.

20. Daleke DL. Phospholipid Flippases. J. Biol. Chem. 2007, 282:821-825.

21. Davie EW, Ratnoff OD. Waterfall sequence for intrinsic blood clotting. Science.1964. 45(3638):1310-2.

22. Devaux PF, Herrmann A, Ohlwein N, Kozlov MM. How lipid flippases can modulate membrane structure. Biochim Biophys Acta. 2008; 1778(7-8):1591-600.

23. Ehlen HW, Chinenkova M, Moser M, Munter HM, Krause Y, Gross S, Brachvogel B, Wuelling M, Kornak U, Vortkamp A. Inactivation of anoctamin-6/TMEM16F, a regulator of phosphatidylserine scrambling in osteoblasts, leads to decreased mineral deposition in skeletal tissues. J Bone Miner Res. 2013; (2):246-59.

24. Erhardt JA, Pillarisetti K, Toomey JR. Potentiation of platelet activation through the stimulation of P2X1 receptors. J Thromb Haemost. 2003; 1(12):2626-35.

25. Ezumi Y, Shindoh K, Tsuji M, Takayama H. Physical and functional association of the Src family kinases Fyn and Lyn with the collagen receptor glycoprotein VI-Fc receptor gamma chain complex on human platelets. J Exp Med. 1998; 188(2):267-76.

26. Fadeel B, Xue D. The ins and outs of phospholipid asymmetry in the plasma membrane: roles in health and disease. Crit Rev Biochem Mol Biol. 2009; 44(5): 264–277.

27. Fox JE, Austin CD, Boyles JK, Steffen PK. Role of the membrane skeleton in preventing the shedding of procoagulant-rich microvesicles from the platelet plasma membrane. J Cell Biol. 1990; 111(2):483-93.

28. Furie B, Furie BC. Mechanisms of Thrombus Formation. N Engl J Med. 2008(359):938-49.

29. Gailani D, Renné T.The intrinsic pathway of coagulation: a target for treating thromboembolic disease? J Thromb Haemost. 2007; 5(6):1106-12.

30. Gale AJ. Current understanding of Hemostasis. 2011. Toxicol Pathol. 2011; 39(1): 273–280.

31. Galietta LJ. The TMEM16 protein family: a new class of chloride channels? Biophys J. 2009; 97(12):3047-53.

32. Galliani D, Renne T. Intrinsic Pathway of Coagulation and Arterial Thrombosis. Arteriosclerosis, Thrombosis, and Vascular Biology.2007; 27: 2507-2513. doi: 10.1161/ATVBAHA.107.155952

33. Greenberg CS, Miraglia CC, Rickles FR, Shuman MA. J Clin Invest. 1985; 75(5): 1463–70.

34. Harrison P. Platelet function analysis. Blood Reviews. 2005; 19: 111–123.

35. Hassock SR, Zhu MX, Trost C, Flockerzi V, Authi KS. Expression and role of TRPC proteins in human platelets: evidence that TRPC6 forms the store-independent calcium entry channel. Blood. 2002; 100(8):2801-11.

36. Hechler B, Gachet C.P2 receptors and platelet function. Purinergic Signal. 2011; 7(3): 293–303.

37. Hechler B, Léon C, Vial C, Vigne P, Frelin C, Cazenave JP, Gachet C. The P2Y1 receptor is necessary for adenosine 5'-diphosphate-induced platelet aggregation. Blood. 1998; 92(1):152-9.

38. Hugel B, Martínez MC, Kunzelmann C, Freyssinet JM. Membrane microparticles: two sides of the coin. Physiology (Bethesda). 2005; 20:22-27.

39. Jarvis GE, Atkinson BT, Snell DC, Watson SP. Distinct roles of GPVI and integrin α2β1 in platelet shape change and aggregation induced by different collagens. Br J Pharmacol. 2002; 137(1): 107–117.

40. Jedlitschky G, Greinacher A, Kroemer HK. Transporters in human platelets: physiologic function and impact for pharmacotherapy. Blood. 2012; 119(15):3394-402.

41. Kahn ML, Zheng YW, Huang W, Bigornia V, Zeng D, Moff S, Farese RV Jr, Tam C, Coughlin SR. A dual thrombin receptor system for platelet activation. Nature. 1998; 394(6694):690-4.

42. Kannemeier C, Shibamiya A, Nakazawa F, Trusheim H, Ruppert , Markar P et al. Extracellular RNA constitutes a natural procoagulant cofactor in blood coagulation. Proc Natl Acad Sci U S A. 2007; 104(15): 6388–6393.

43. Lewit-Bentley A, Réty S. EF-hand calcium-binding proteins. Curr Opin Struct Biol. 2000; 10(6):637-43.

44. Lhermusier T, Chap H, Payrastre B. Platelet membrane phospholipid asymmetry: from the characterization of a Scramblase activity to the identification of an essential protein mutated in Scott syndrome. J Thromb Haemost. 2011; 9(10):1883-91.

45. Louvain-Quintard VB, Bianchini EP, Calmel-Tareau C, Tagzirt M, Le Bonniec BF. Thrombin-activable factor X re-establishes an intrinsic amplification in tenase-deficient plasmas. J Biol Chem. 2005; 280(50):41352-9.

46. Mackman N, Tilley RE, Key NS. Role of the extrinsic pathway of blood coagulation in hemostasis and thrombosis. Arterioscler Thromb Vasc Biol. 2007; 27(8):1687-93.

47. Martins JR, Faria D, Kongsuphol P, Reisch B, Schreiber R, Kunzelmann K. Anoctamin 6 is an essential component of the outwardly rectifying chloride channel. Proc Natl Acad Sci U S A. 2011; 108(44):18168-72.

48. Mause SF, Weber C. Microparticles: protagonists of a novel communication network for intercellular information exchange. Circ Res. 2010 29; 107(9):1047-57.

49. McMichael M. Primary Hemostasis. Journal of Veterinary Emergency and Critical Care. 2005; 15(1): 1-8.

50. Miletich JP, Kane WH, Hofmann SL, Stanford N, Majerus PW. Deficiency of factor Xa-factor Va binding sites on the platelets of a patient with a bleeding disorder. Blood. 1979;54(5):1015-22

51. Morel O, Jesel L, Freyssinet JM, Toti F. Cellular mechanisms underlying the formation of circulating microparticles. Arterioscler Thromb Vasc Biol. 2011; 31(1):15-26.

52. Nakazawa Y, Joseph K, Kaplan AP. Inhibition of contact activation by a kininogen peptide (HKH20) derived from domain 5. Int Immunopharmacol. 2002; 2(13-14):1875-85.

53. Nayak MK, Kulkarni PP, Dash D. Regulatory role of proteasome in determination of platelet life span. J Biol Chem. 2013; 288(10):6826-34.

54. Owens AP 3rd, Mackman N. Microparticles in hemostasis and thrombosis. Circ Res. 2011; 108(10):1284-97.

55. Pasquet JM, Dachary-Prigent J, Nurden AT. Calcium influx is a determining factor of calpain activation and microparticle formation in platelets. Eur J Biochem. 1996; 239(3):647-54.

56. Rasche H. Haemostasis and thrombosis: an overview. Eur Heart J Suppl. 2001; 3 (suppl Q): Q3-Q7.

57. Rivera J, Lozano ML, Navarro-Núñez L, Vicente V. Platelet receptors and signaling in the dynamics of thrombus formation. Haematologica. 2009; 94(5):700-11.

58. Savage B, Ruggeri ZM. Platelet Thrombus Formation in Flowing Blood. In: Michelson AD, ed. Platelets. Elsevier Science, San Diego, 2007, pp 373-388

59. Savill J, Dransfield I, Gregory C, Haslett C. A blast from the past: clearance of apoptotic cells regulates immune responses. Nat Rev Immunol. 2002; 2(12):965-75.

60. Seigneuret M, Devaux PF. ATP-dependent asymmetric distribution of spin-labeled phospholipids in the erythrocyte membrane: relation to shape changes. Proc Natl Acad Sci U S A. 1984; 81(12):3751-5.

61. Shimizu T, Iehara T, Sato K, Fujii T, Sakai H, Okada Y. TMEM16F is a component of a Ca2+-activated Cl- channel but not a volume-sensitive outwardly rectifying Cl- channel. Am J Physiol Cell Physiol. 2013; 304(8):C748-59.

62. Sims PJ, Wiedmer T, Esmon CT, Weiss HJ, Shattil SJ. Assembly of the platelet prothrombinase complex is linked to vesiculation of the platelet plasma membrane. Studies in Scott syndrome: an isolated defect in platelet procoagulant activity. J Biol Chem. 1989 Oct 15;264(29):17049-57.

63. Sims PJ, Wiedmer T, Esmon CT, Weiss HJ, Shattil SJ. Assembly of the platelet prothrombinase complex is linked to vesiculation of the platelet plasma membrane. Studies in Scott syndrome: an isolated defect in platelet procoagulant activity. J Biol Chem. 1989; 264(29):17049-57.

64. Solum NO. Procoagulant expression in platelets and defects leading to clinical disorders. Arterioscler Thromb Vasc Biol. 1999; 19(12):2841-6.

65. Suzuki J, Umeda M, Sims PJ, Nagata S. Calcium-dependent phospholipid scrambling by TMEM16F. Nature. 2010; 468(7325):834-8.

66. Targos B, Barańska J, Pomorski P. Store-operated calcium entry in physiology and pathology of mammalian cells. Acta Biochim Pol. 2005; 52(2):397-409.

67. van Kruchten R, Mattheij NJ, Saunders C, Feijge MA, Swieringa F, Wolfs JL, Collins PW, Heemskerk JW, Bevers EM. Both TMEM16F-dependent and TMEM16F-independent pathways contribute to phosphatidylserine exposure in platelet apoptosis and platelet activation. Blood. 2013 Mar 7;121(10):1850-7.

68. VanWijk MJ, VanBavel E, Sturk A, Nieuwland R. Microparticles in cardiovascular diseases. Cardiovasc Res. 2003; 59(2):277-87.

69. Varga-Szabo D, Braun A, Nieswandt B. Calcium signaling in platelets. J Thromb Haemost. 2009; 7(7):1057-66.

70. Varga-Szabo D, Braun A, Nieswandt B. STIM and Orai in platelet function. Cell Calcium. 2011; 50(3):270-8.

71. Versteeg HH, Heemskerk JW, Levi M, Reitsma PH. New fundamentals in hemostasis. Physiol Rev. 2013; 93(1):327-58.

72. Watson SP, Auger JM, McCarty OJT, Pearce AC. GPVI and integrin aIIbb3 signaling in platelets. J Thromb Haemost 2005; 3:1752–62.

73. Weiss HJ, Lages B. Family Studies in Scott Syndrome. Blood. 1997; 90(1): 475-476

74. Wiedmer T, Sims PJ. Participation of protein kinases in complement C5b-9-induced shedding of platelet plasma membrane vesicles. Blood. 1991; 78:2880-2886

75. Wielders SJ, Broers J, ten Cate H, Collins PW, Bevers EM, Lindhout T. Absence of platelet-dependent fibrin formation in a patient with Scott syndrome. Thromb Haemost. 2009;102(1):76-82

76. Wolberg AS. Thrombin generation and fibrin clot structure. Blood Rev. 2007; 21(3):131-42.

77. Yang H, Kim A, David T, Palmer D, Jin T, Tien J, Huang F, Cheng T, Coughlin SR, Jan YN, Jan LY. TMEM16F forms a Ca^{2+}-activated cation channel required for lipid scrambling in platelets during blood coagulation. Cell. 2012; 151(1):111-22.

78. Zhang SL, Yu Y, Roos J, Kozak JA, Deerinck TJ, Ellisman MH, Stauderman KA, Cahalan MD. STIM1 is a Ca2+ sensor that activates CRAC channels and migrates from the Ca2+ store to the plasma membrane. Nature. 2005; 437(7060):902-5.

79. Zwaal RF, Comfurius P, Bevers EM..Scott syndrome, a bleeding disorder caused by defective scrambling of membrane phospholipids. Biochim Biophys Acta. 2004; 1636(2-3):119-28.

Appendix

ANIMAL CONSENT FORM

University of Delaware
Institutional Animal Care and Use Committee
Application to Use Animals in Research and Teaching

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

RECEIVED
SEP 16 2013
IACUC

Title of Protocol: Microparticles and platelet signaling in thrombosis	
AUP Number: 1257-2014-0	← (4 digits only — if new, leave blank)
Principal Investigator: Donna Woulfe	
Common Name: Mouse **Genus Species:** Mus musculus	

Pain Category: ***(please mark one)***

USDA PAIN CATEGORY: ***(Note change of categories from previous form)***

Category	Description
☐ B	Breeding or holding where NO research is conducted
☐ C	Procedure involving momentary or no pain or distress
X D	Procedure where pain or distress is alleviated by appropriate means (analgesics, tranquilizers, euthanasia etc.)
☐ E	Procedure where pain or distress cannot be alleviated, as this would adversely affect the procedures, results or interpretation

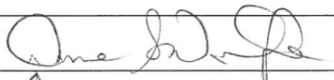
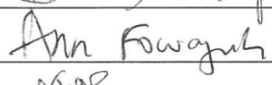
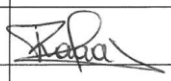
Official Use Only

IACUC Approval Signature: _____

Date of Approval: 9/15/13

NAMES OF ALL PERSONS WORKING ON THIS PROTOCOL

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

Name	Signature
1. Donna Woulfe	
2. Ann Fowajuh	
3. Aasma Khan	
4. Debora Mukaz	
5.	
6.	
7.	
8.	
9.	
10.	
11.	
12.	
13.	
14.	