Summer 8-2016

Reconstitution of Munc18-Dependent Mast Cell Degranulation

Matthew Grant Arnold
University of Southern Mississippi

RECONSTITUTION OF MUNC18-DEPENDENT

MAST CELL DEGRANULATION

by

Matthew Grant Arnold

A Thesis
Submitted to the Graduate School
and the Department of Biological Sciences
at The University of Southern Mississippi
in Partial Fulfillment of the Requirements
for the Degree of Master of Science

Approved:

_____
Dr. Hao Xu, Committee Chair
Assistant Professor, Biological Sciences

_____
Dr. YanLin Guo, Committee Member
Associate Professor, Biological Sciences

_____
Dr. Vijay Rangachari, Committee Member
Associate Professor, Chemistry and Biochemistry

_____
Dr. Karen S. Coats
Dean of the Graduate School

August 2016

Published by the Graduate School

ABSTRACT

RECONSTITUTION OF MUNC18-DEPENDENT

MAST CELL DEGRANULATION

by Matthew Grant Arnold

August 2016

Mast cells are specialized secretory leukocytes that play diverse roles in the body, mediated by release of histamine and other pharmacologically active substances. Although offering essential protection in innate and adaptive immunity, mast cells are also essential to the progression of inflammatory diseases, including allergy and asthma, (Theoharides et al., 2012). Exocytosis of pro-inflammatory mast cell mediators in response to otherwise innocuous antigens relies on evolutionarily-conserved membrane fusion machinery. These proteins, called SNAREs, are regulated by the Sec1/Munc18 (SM) protein family (Lorentz, Baumann, Vitte, & Blank, 2012). Mast cells express three mammalian Munc18 isoforms (a, b, and c), which are linked to SNARE-dependent exocytosis in numerous organisms. However, the exact roles of each of these Munc18 isoforms in mast cell degranulation have not been clearly defined. In this study, we investigated the functional relationship between Munc18 and eight sets of degranulation-relevant SNAREs using *in vitro* reconstitution. We showed that Munc18a was active in stimulating fusion mediated by VAMP2 and VAMP3, but not by VAMP7 and VAMP8. In contrast, Munc18b and Munc18c did not show any stimulatory activity. Further analysis of Munc18a function unraveled a previously unidentified role in the tethering of SNARE complexes prior to fusion, an action that is dependent on the interaction between Munc18a and N-peptide of syntaxin. Inhibition of fusion and trans-SNARE complex

formation by soluble VAMP proteins did not interfere with Munc18a-mediated tethering, indicating that Munc18a-mediated tethering occurs prior to trans-SNARE complex formation. This study therefore sheds light on new roles Munc18 proteins might play to regulate mast cell degranulation.

ACKNOWLEDGMENTS

I would like to express my gratitude to Dr. Hao Xu for his guidance, patience, and for the chance to be part of his research. His support enabled my success in research and writing my thesis. From him a learned endurance of labor, focus, promptness and management will be essential in years to come.

Additionally, I would like to acknowledge my committee members, Dr. Yanlin Guo and Dr. Vijay Rangachari, for their constructive criticisms and invaluable experience. Their critiques made this a challenging experience worth undertaking.

My sincere thanks to Dr. Shearer, Dr. Karim, Dr. Elasri, Dr. Huang, Dr. Rangachari and their respective students for various advice and sundries that helped complete this project. To Dr. Flynt and Baobin Kang I am indebted for their skilled assistance in confocal microscopy.

DEDICATION

I thank my fellow labmates for their support and coffee breaks, particularly Sushmitha Vijaya Kumar for her unrelenting optimism and fortitude.

My heartfelt thanks to Dr. Shiao Wang, who offered my first position in research, as well as Mrs. Littlejohn whose guidance began my career and graduate school.

I wholly appreciate the efforts of Mr. Burt, Mrs. Hood, and Dr. McKone in instigating my interest in science, they will never know the impact they had.

My greatest appreciation goes to my better half, Maria Basco, for her unwavering support and dedication to my own success. I could not hope for a better companion.

Finally, I would like to thank my family and friends for support and encouragement through this journey.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF ILLUSTRATIONS

LIST OF ABBREVIATIONS

BMMC	Bone marrow-derived mast cell
CTMC	Connective tissue mast cell
MMC	Mucosal mast cell
pET MBP	pET MBP His6 LIC (2Cc-T)
pMBP	pMBP-parallel1
RB150	Reconstitution Buffer
RPL	Reconstituted proteoliposome
SCF	Stem cell factor
SNAP	Synaptosomal-associated protein
SNARE	Soluble NSF Attachment Receptor protein
VAMP	Vesicle-associated membrane protein

CHAPTER I - INTRODUCTION

Mast cells

Mast cell origins and subtypes

First described by Paul Ehrlich in his 1878 dissertation (Ehrlich, 1878), mast cells originate from $CD34^+$/c-KIT^+/$CD13^+$ progenitor cells in the bone marrow, which migrate through the blood into mucosal and connective tissues where maturation occurs (Hallgren & Gurish, 2007). This is in contrast to other hematopoietic cell types which mature in the bone marrow before being released (Da Silva, Jamur, & Oliver, 2014). The cytokine stem cell factor (SCF) produced by fibroblasts is crucial to mast cell maturation, as well as their migration into the peripheral tissues (Kirshenbaum et al., 1992; Kirshenbaum, Kessler, Goff, & Metcalfe, 1991; Liu, Liu, Li, & Wu, 2010; Okayama & Kawakami, 2006; Valent et al., 1992). Mice deficient in c-KIT (SCF receptor) completely lack mast cells, pointing to c-KIT and SCF as main determinants of mast cell biogenesis *in vivo* (Galli, Tsai, & Wershil, 1993). Other factors such as nerve growth factor (NGF) (Aloe & Levi-Montalcini, 1977) and neurotrophin-3 (Lorentz et al., 2007; Metz et al., 2004) are also shown to promote mast cell maturation. However, c-kit remains highly expressed throughout the mast cell life cycle, and its interaction with SCF is considered the most essential factor in mast cell survival (Kirshenbaum et al., 1999).

While SCF serves as the pivotal factor in mast cell differentiation, the local environment further directs mast cells into phenotypically distinct subtypes, mucosal mast cells (MMCs) and connective tissue mast cells (CTMCs). In rodents, MMCs are discernible from CTMCs by their protease contents; MMCs containing only tryptase, while CTMCs contain both tryptase and chymase (Church & Levi-Schaffer, 1997).

Development of MMCs are dependent on T-cells as shown in studies with athymic nude mice (Ruitenberg & Elgersma, 1976), whereas control of CTMC development is poorly understood. Expression levels of other mediators also vary among mast cell subtypes, as MMCs are seen to secrete less histamine but more Leukotriene C_4 than CTMCs (Heavey et al., 1988), and human skin mast cells are not seen to express IL-4, 5 or 13 as in other masts cells (Gibbs et al., 2001). This distinction is not controlled genetically but rather maintained through cross-talk with the environment, as MMCs differentiation into CTMCs has been observed (Kitamura, 1989; Welle, 1997). In addition, MMCs have been seen to change protease phenotype *in vivo,* expressing tryptase and chymase in response to *Trichinella spiralis* infection (Friend et al., 1996; Friend et al., 1998). Although useful for categorizing mast cell function in the body, classification on location and protease content is not precise as both CTMCs and MMCs are found in lungs, with subtypes containing further subtypes of unique expression patterns depending on location (Andersson, Mori, Bjermer, Löfdahl, & Erjefält, 2009). Additionally, murine mast cells in the trachea express all six serine proteases, further confounding the classic definition of mast cell subtypes (Xing, Austen, Gurish, & Jones, 2011). Taken together, the functional differences of mast cell subtypes are not thoroughly understood, however it is clear that they are amenable to their residential tissue, and the diverse profile of mediators could be important to their localization and function.

Mast cell granules

The most distinguishing feature of mast cells is the large cytoplasmic granules (i.e. secretory lysosomes) which contain a panoply of mediators (Appendix A). The mass exocytosis of these granule-stored mediators is termed degranulation, and is commonly

observed in activated mast cells during allergic reactions (Amin, 2012). Among these histamine is the most well-known, but serotonin and proteases are also major constituents (Wernersson & Pejler, 2014). In accordance with the lysosomal-like properties of the granules, several lysosomal hydrolases are also present (Wernersson & Pejler, 2014). β-hexosaminidase is present in virtually all mast cells and therefore its release is frequently used as a hallmark for mast cell degranulation (Fukuishi et al., 2014).

Although appearing similar in size and staining patterns, granules are heterogeneous in their contents. One study with mouse bone marrow-derived mast cells (mBMMCs) showed a division between granules containing serotonin and cathepsin D, while others contained histamine and TNF (Wernersson & Pejler, 2014).Histamine has also been seen to be released independently of β-hexosaminidase(Baram et al., 1999). In mouse bone marrow-derived mast cells (BMMCs) at least three types of granules have been detected; type I granules containing MHC class II, β-hexosaminidase, LAMP-1, LAMP-2 and M6PR, type II containing all of the previously mentioned and serotonin, and type III containing only β-hexosaminidase and serotonin (Raposo et al., 1997). It is not clear how these distinct granules are differentially regulated for secretion.

The biogenesis of secretory granule in mast cells is poorly characterized, although it is known that granules begin from the *trans*-Golgi network with the budding of clathrin-coated vesicles to form 'pro-granules'. Fusion of these pro-granules leads to larger, immature granules, which will continue V-ATPase-meditated acidification to pH ~5.5 (Wernersson & Pejler, 2014), in a fashion similar to lysosomal acidification. A few regulators of this biogenesis have been identified, such as secretogranin III (Prasad, Yanagihara, Small-Howard, Turner, & Stokes, 2008), Rab5 (Azouz et al., 2014), and

synaptotagmin III(Grimberg, Peng, Hammel, & Sagi-Eisenberg, 2003) (Wernersson & Pejler, 2014). The mechanism of sorting the vast array of mediators into separate, heterogeneous granules is largely unclear, however, it has been reported that histamines, proteases, and lysosomal constituents are transported into granules respectively via vesicular monoamine transporter 2 (Wernersson & Pejler, 2014), electrostatic interaction with serglycin proteoglycan, and the mannose-6-phosphate dependent vesicular transport (Moon, Befus, & Kulka, 2015).

Mast Cell Pathology and Physiology

Mast cells in inflammatory disease

Mast cells are central to inflammatory diseases, especially in allergic reactions (i.e. Type I hypersensitivity/immediate hypersensitivity). According the American College of Allergy, Asthma and Immunology, allergic diseases are the 5th largest chronic disease in the US in all age groups. In the United States, food allergies in children alone are estimated to cost $24.8 billion annually (Gupta et al., 2013). For the past several years, food and skin allergies in children have increased for unknown reasons (Jackson, Howie, & Akinbami, 2013). Additionally, mast cells contribute to asthma, a chronic inflammatory lung disease with recurrent breathing problems (Bradding, Walls, & Holgate, 2006). The Centers for Disease Control and Prevention estimates that asthma affects 25 million people and cost the United States $56 billion in 2007 ().

Aside from allergies and asthma, mast cells also contribute to other common inflammatory diseases such as type IV hypersensitivity (delayed hypersensitivity) (Biedermann et al., 2000), arthritis, atopic dermatitis, psoriasis, and multiple sclerosis (Theoharides et al., 2012). Rare diseases such as mastocytosis and mast cell activation

syndrome (MCAS), where patients have an abnormal increase in mast cell number or a hyper-responsive mast cell population, lead to susceptibility to idiopathic anaphylactic shock (Metcalfe, 2008). Understanding mast cell secretion is therefore essential to developing therapeutic strategies in treatment and prevention of inflammatory diseases.

The numerous vasoactive and proinflammatory mediators released by mast cells fall into two categories, the immediately releasable, pre-formed mediators stored in secretory granules such as histamine, serotonin, TNFα, kinins and proteases, and the *de novo* synthesized mediators upon mast cell activation. Among the latter are leukotrienes, prostaglandins, and platelet-activating factor, which are synthesized from arachidonic acid liberated by phospholipases. Numerous cytokines are also synthesized *de novo* after activation, including IL-1, 2, 5, 6, 8, 9, 13 and vascular endothelial growth factor (VEGF) (Theoharides et al., 2012). Release of these mediators has shown to induce recruitment of mast cell progenitors to sites of inflammation in the lungs. These progenitors, in turn, will release their own mediators, perpetuating the cycle of inflammation (Ikeda et al., 2003).

Most mediators released influence inflammation by acting on cell receptors causing vasodilation, bronchoconstriction, immune cell chemotaxis, and/or itching sensation(reviewed in (Jutel, Akdis, & Akdis, 2009; Leurs, Smit, & Timmerman, 1995)). However, mast cell proteases play a unique dual role in regulating inflammation. In promoting inflammation, mast cell proteases can digest plasma albumin to liberate histamine-releasing peptides, stimulating mast cell degranulation and perpetuating the inflammatory process (Cochrane, Carraway, Miller, Feldberg, & Bernheim, 2003). mMCP-6 (tryptase)has shown importance in progressing allergic asthma in mice (Cui et al., 2014), perhaps due to the fact that serine proteases such as tryptase and chymase also

act on protease-activated receptors and cause further inflammation (Schechter, Brass, Lavker, & Jensen, 1998). These same proteases can also help prevent inflammation. Mouse mast cell protease 6 (mMCP-6) digests IL-6, a pro-inflammatory cytokine that is generally elevated during allograft rejection. Localized IL-6 reduction by MCP-6 is necessary for allograft tolerance (de Vries, Elgueta, Lee, & Noelle, 2010). Similarly, another protease, mMCP-4 degrades IL-33 and is protective in an allergic lung inflammation model (Waern et al., 2009; Waern, Lundequist, Pejler, & Wernersson, 2013). β-tryptase limits allergic inflammation by targeting IgE molecules after degranulation (Rauter et al., 2008). Since proteases represent 25% of mast cell protein content, they underscore a significant portion of mast cell activity (Da Silva et al., 2014).

Physiological roles of mast cells

Understandably, mast cells have become synonymous with inflammatory disease, however they also play beneficial roles in innate and adaptive immune responses, resistance to toxins, tissue repair and angiogenesis (Palker, Dong, & Leitner, 2010). Their widespread peripheral location ideally situates them as a first line of defense in regulating immune function. These functions are poorly characterized in comparison to mast cells in allergic disease, but are seemingly essential as no humans lacking mast cells have been described so far (Palker et al., 2010). The same mediators that cause inflammation act in normal mast cell function.

Mast cells are among the first cells to recognize antigens in the body. The variety of receptors such as Toll-like receptors (TLRs), nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), retinoic acid-inducible (RIG)-I-like receptors (RLRs) and complement receptors allow general recognition of foreign particles (Da

Silva et al., 2014). Interaction with these receptors causes mast cell activation, which can lead to a variety of responses dependent on the ligand. Degranulation of pro-inflammatory mediators and cytokine/chemokine production is common for clearance of bacterial, viral and parasitic infection and upregulation of immune response (Da Silva et al., 2014).

Mast cells can clear bacterial infections through either phagocytic activity or production of potent antimicrobials such as cathelicidin LL-37, β-defensins and piscidins (Silphaduang & Noga, 2001) (in fish MCs) when they are stimulated by various pathogens. Release of β- hexosaminidase has shown protection against bacterial infection, possibly through targeting of cell wall peptidoglycan (Fukuishi et al., 2014). Additionally, production of mast cell extracellular traps (MCETs), similar to neutrophils (called NETs),concentrate certain antimicrobial products such as cathepsin G and cathelicidin LL-37 and may act as a physical barrier for infection (Brinkmann et al., 2004; Thomas et al., 2014; von Köckritz-Blickwede et al., 2008). Production of chemokines will attract nearby immune cells such as eosinophils (IL-33, eotaxin/CCL11), natural killer cells (IL-8), and neutrophils (CXCL1/CXCL2, TNFα, LTB4, LTC4, and MCP-6), Th cells (IL-33) and basophils (IL-33) to sites of infection (Da Silva et al., 2014; Hsu, Neilsen, & Bryce, 2010). In particular, release of pre-stored TNFα has shown antibacterial protection against mast cell-deficient mice through neutrophil chemotaxis (Malaviya, Ikeda, Ross, & Abraham, 1996). Mast cells have also been shown to be activated by snake venom, which gets degraded subsequently by proteases (Theoharides et al., 2012).

Mast cells mediators coordinate the adaptive immune response through interactions with dendritic cells and T cells and direct their migration to lymph nodes (Galli, Nakae, & Tsai, 2005; Theoharides et al., 2012). Certain mast cell subtypes highly expressing FcεRI and MHCII can function as antigen-presenting cells (Theoharides et al., 2012). Mast cells interact with T cells and superactivate them through TNF. In turn, T cells can activate mast cell degranulation and IL-8 release (Theoharides et al., 2012). Less is known about mast cells in viral infection, although they have shown secretion of anti-inflammatory cytokines such as IL-10 and TGF-β limit the magnitude of immune responses (Da Silva et al., 2014). production of IFN-1 initiates immune responses to viral infection (Da Silva et al., 2014).

Mast cells are also crucial for the repair of damaged tissues through several distinct pathways. Mediators such as nerve growth factor (NGF), platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), fibroblast growth factor-2, histamine and tryptase induce epithelial and fibroblast proliferation. PAF, leukotrienes, IL-1 and IL-8 from mast cells can activate platelet aggregation to seal injured areas. Secretion of heparin, tryptase and t-plasminogen activator regulate fibrolytic mechanisms providing appropriate perfusion and nutrition necessary for repair. Released mast cell mediators also cause chemotaxis of leukocytes, which will clear infection from the area. Mast cells then stimulate proliferation of endothelial cells, fibroblasts and keratinocytes allowing angiogenesis, generation of new extracellular matrix, and wound contraction (Da Silva et al., 2014).

Mast cell activation

Mast cells can be activated by a variety of stimuli and release distinct patterns of mediators, dependent on the type and intensity of the stimuli (Hsu et al., 2010). Allergen-induced IgE-receptor-mediated degranulation is most recognized and best studied. In contrast, receptors for cytokines and pathogen-associated molecular patterns (PAMPs) underscore a different type of response that sometimes lacks the characteristic of classical degranulation.

IgE-mediated degranulation. Allergen-induced mast cell activation triggers three processes: 1) degranulation of prestored mediators, 2) lipid metabolism for leukotriene production, and 3) transcription factor activation for de novo synthesis of cytokines. Initiation of the allergic reaction begins with surface receptor FcεRI, bound with IgE, aggregating through interaction with a multivalent antigen. Mast cells constitutively express the high-affinity IgE receptor FcεRI, as binding monomeric IgE is known to induce pro-survival signals in the cell (Kalesnikoff et al., 2001). Due to the normally low serum concentrations of IgE, the high affinity FcεRI is needed for efficient recruitment of these molecules. This receptor is also highly expressed in basophils but minimally expressed in eosinophils, Langerhans cells, monocytes, and platelets (Owen, Punt, Stranford, & Jones, 2013).

The FcεRI receptor is a heterotetramer consisting of a single α and β chain, and two disulfide-linked γ chains. The α chain contains two N-terminal domains that are homologous with immunoglobulin-fold structures, thus binding extracellular IgE, while the β and γ polypeptides contain intracellular ITAMs (immunoreceptor tyrosine-based activation motifs) responsible for the initiation of signaling pathways. The FcεRI

complex is located on lipid rafts, a region typically devoid of transmembrane phosphatases (Simons & Toomre, 2000). Aggregation of IgE-FcεRI through multivalent antigen excludes phosphatases, allowing phospho-signaling to occur.

To begin the signaling cascade, Src-family kinases (Lyn, Fyn, and Syk) phosphorylate ITAMs located on FcεRI subunits. Phosphorylated ITAMs allow recruitment and activation of additional Src-family kinases through SH2 domains. Activated kinases subsequently phosphorylate ITAMs of adaptor proteins LAT and NTAL, which serve as platforms for recruitment of various proteins(Rivera & Gilfillan, 2006).

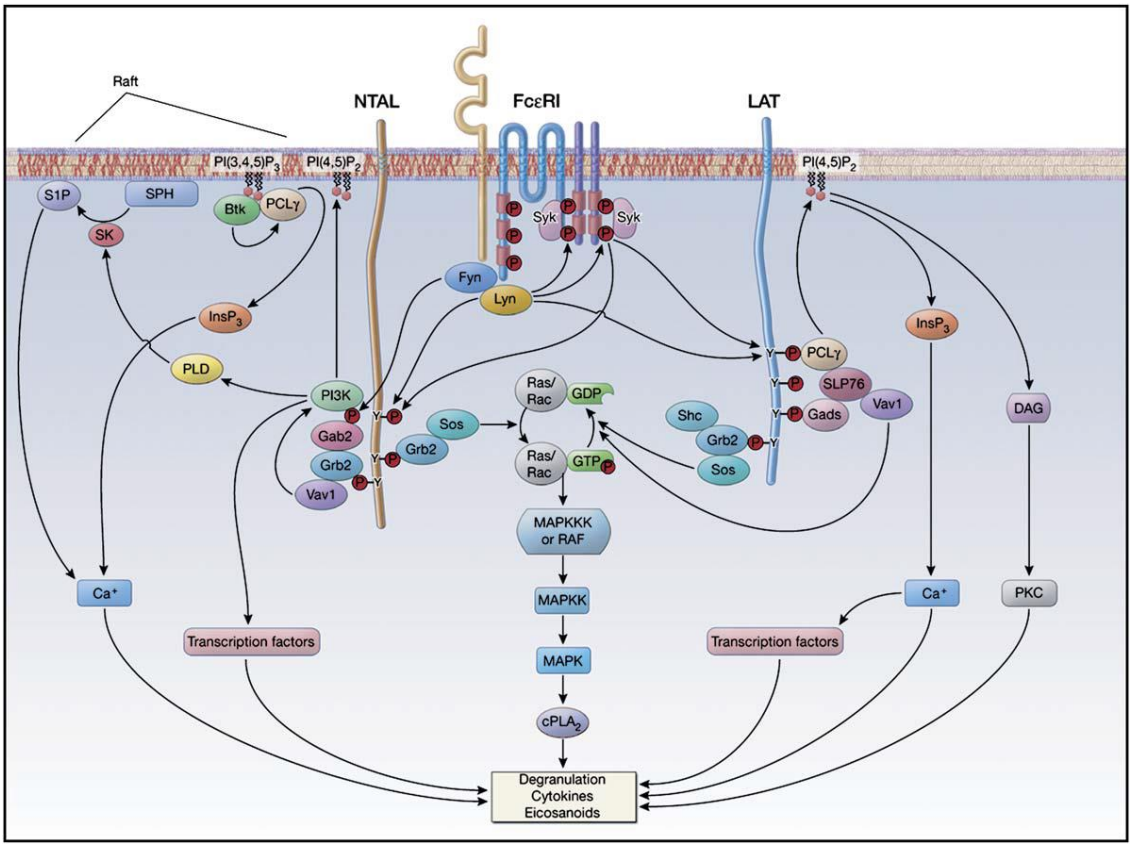

Figure 1. IgE-mediated signaling pathway in mast cells.

Simplified diagram showing IgE-mediated signal pathway in mast cells. Original picture obtained from Rivera et al., 2006 (Rivera & Gilfillan, 2006)

LAT will recruit phospholipase C to create IP_3 and DAG to increase intracellular calcium and activate protein kinase C (PKC). NTAL activates PI_3K, also contributing to calcium mobilization. Calcium release will stimulate degranulation of prestored mediators through binding to calcium sensing synaptotagmin II found on granule membranes (Baram et al., 1999). PKC activation stimulates the MAPK pathway, leading to cytokine production and leukotriene production (Rivera & Gilfillan, 2006). PKC has been shown to phosphorylate proteins associated with membrane fusion on granules, though it is currently unknown if this occurs in mast cells as well (Genç, Kochubey, Toonen, Verhage, & Schneggenburger, 2014).

Allergen-independent activation. Allergens are not the only method for inducing mast cell response. A variety of systems are used to modulate mediator release, sometimes without the characteristic degranulation. Their strategic distribution throughout the body and various physiological functions require their recognition of different signals coupled with appropriate mediator release or production. The majority of allergen-independent mechanisms are involved in mast cell immune function. Accordingly, mast cells recognize pathogens through common molecular motifs through TLRs, NLRs, and RLRs.

Activation through TLRs can selectively regulate mast cell mediator release. Studies with murine mast cells have shown expression of TLR 1-4 and 6-9, while human mast cells have expressed TLR1-7, 9 and 10 with a great degree of variation (reviewed in (Sandig & Bulfone-Paus, 2012)). Interaction of TLR 4 with LPS induces TNFα, IL-5, 10, and 13 production without inducing degranulation. TLR 3, 7, 9 activation have seen TNF, IFN and IL-6 production, also without degranulation (Theoharides et al., 2012). TLR 9

activation induces to IL-6 production, but again is not observed to affect degranulation (Ikeda et al., 2003). On the other hand, TLR 2 with staphylococcal peptidoglycan has seen induction of degranulation and histamine release. In some studies peptidoglycan and/or LPS have induced mast cell migration through unknown mechanisms. There is a high degree of variability in mast cell response to TLR ligands, due to differences in species and origin of the cell types. It can be appreciated that mast cells have different responses to antigens in context of their natural microenvironment.

Complement fragment C3a in the presence of aggregated IgG has shown increased degranulation. Free immunoglobulin light chains have shown to mediate hypersensitivity-like reactions. Anti-microbial β-defensins have been shown to activate mast cells (Theoharides et al., 2012). Mast cells are also observed to degranulate in response to complement fragments, neuropeptides (such as substance P), and certain toxins (Wernersson & Pejler, 2014). Mast cells mediators also in turn regulate degranulation. Promotion of degranulation is seen through release of corticotropin-releasing hormone, neurotensin, reactive oxygen species, stem cell factor, substance P, tryptase, thymic stromal lymphopoietin and IL-1, 9 and 33. Osteopontin is also seen to augment IgE-mediated degranulation (Nagasaka et al., 2008). Inhibitors of degranulation are chondroitin, heparin, IL-10, nitric oxide, TGFβ, and UCP2 (Theoharides et al., 2012).

SNARE-mediated Mast Cell Exocytosis

SNARE proteins are the key components to membrane fusion events and intracellular trafficking in eukaryotic cells. The transfer of newly synthesized proteins in vesicles to membrane-bound compartments requires unification of the lipid bilayers to allow delivery of vesicle contents to the target localization. This process is mediated in a

large part by SNARE proteins through formation of a *trans*-SNARE complex between the apposing membrane bilayers. SNARE proteins overcome the repulsive forces between lipid bilayers and allow selective recognition of different compartments through SNARE pairing. To distinguish one compartment from another, a variety of SNARE proteins are expressed which fall under families of Q-SNAREs and R-SNAREs. Crystal structure data from the SNARE complexes shows a characteristic central ionic layer (i.e. zero layer) consisting of the side chains of an arginine (R) and three glutamine (Q) residues (Fasshauer, Sutton, Brunger, & Jahn, 1998). SNARE proteins contributing a glutamine or an arginine to the SNARE complex are termed Q-SNAREs or R-SNAREs, respectively. In exocytosis the common Q-SNAREs are isoforms of syntaxins and SNAP-25, while R-SNAREs are usually VAMP isoforms. The *trans*-SNARE complex consists of a four helical coiled-coil bundle. SNARE complexes forming at the plasma membrane typically contain a single helix each from syntaxin and VAMP, and two helices from a SNAP-25-like protein (reviewed in (McNew, 2008)). Each of these helices contain a 60-70 aa SNARE motif that combine to form the highly stable tetrameric structure that is required for membrane fusion (Sutton, Fasshauer, Jahn, & Brunger, 1998). For the most part, SNARE proteins are anchored to their respective membranes through a C-terminal transmembrane domain. SNAP-25-like molecules, however, do not have a transmembrane domain but instead are anchored through palmitoylation of several cysteines (Fukasawa, Varlamov, Eng, Söllner, & Rothman, 2004). Additionally, several syntaxin family proteins have N-terminal regulatory regions termed the Habc region. This three-helical region folds back onto the SNARE motif of the protein, creating a ‘closed

conformation' that prevents assembly of the SNARE complex. Syntaxin must adopt an open conformation in order for fusion to occur.

Munc18 proteins

SNARE proteins constitute the minimal requirement for membrane fusion, however *in vitro* these interactions have been shown to be highly promiscuous and non-selective (Izawa, Onoue, Furukawa, & Mima, 2012). There are several accessory proteins known to regulate SNARE interactions, perhaps most important is the multi-functional Sec1-Munc18 (SM) protein family(reviewed in (Hong & Lev, 2014)). Different SM proteins are required for maintaining specificity in fusion reactions through the cell at different localizations. The Munc18 proteins coordinate membrane fusion reactions at the plasma membrane, including neurotransmitter release in neurons, insulin-regulated GLUT4 translocation, and mast cell degranulation. Munc18 proteins play vital roles for the cell, Munc18a deletion in mice causes complete paralysis and death soon after birth (Verhage et al., 2000), mutations in Munc18b cause life-threatening immunodeficiencies in natural killer and cytotoxic T cells (Spessott et al., 2015), and Munc18c deletion in mice results in death in utero or within 6 hours after birth (Kanda et al., 2005).

Due to its importance in neurotransmission, Munc18a is the most studied, and conclusions regarding the function and mechanism of SM proteins are based heavily on Munc18a. Munc18 proteins are thought to play three distinct roles in modulating intracellular trafficking: 1) chaperoning plasmalemmal syntaxins through the cell to the plasma membrane in a closed conformation, 2) activating specific *trans*-SNARE complexes for fusion and 3) docking of large dense-core vesicles to the plasma membrane (Han et al., 2009). Binary interactions between Munc18 and syntaxin proteins

are thought to prevent assembly of the *trans*-SNARE complex, thus playing an inhibitory role in fusion, while binding to the *trans*-SNARE complex accelerates membrane fusion. Munc18 proteins have two binding modes responsible for these distinct functions. Mode 1 is the binary interaction with the syntaxin protein in closed conformation; whereas Mode 2 stimulates the *trans*-SNARE complex and relies on a 'hydrophobic pocket' of Munc18 to bind the syntaxin N-terminal peptide, with additional contacts made with the VAMP protein in the complex. Munc18 stimulation of the *trans*-SNARE complex has been shown to be largely VAMP-dependent, and syntaxin-independent as the N-terminal peptide bears little selectivity for Munc18 recruitment among syntaxin isoforms (Hu et al., 2011). Transition between Munc18 binding modes is suggested to be regulated by the Munc13 proteins and post-translational modifications (Aran, Bryant, & Gould, 2011; Barclay et al., 2003; Liu et al., 2007; Ma, Li, Xu, & Rizo, 2011).

Membrane fusion machinery in mast cells

Mast cells express a variety of SNARE proteins, although their significance in degranulation is still under debate. Q-SNARE syntaxin3 is localized to secretory granules (Hibi, Hirashima, & Nakanishi, 2000; Tadokoro, Kurimoto, Nakanishi, & Hirashima, 2007), while syntaxin4 and SNAP-23 localize to the plasma membrane (Guo, Turner, & Castle, 1998; Tadokoro et al., 2007). R-SNAREs VAMP2, 3, 7 and 8 are localized on different vesicles in the cytoplasm. VAMP2 and VAMP3 have been shown to co-localize with small vesicles and multi-vesicular bodies (Paumet et al., 2000; Tiwari et al., 2008), while VAMP7 and VAMP8 localize to the larger vesicles, containing histamine and β-hexosaminidase, respectively (Puri & Roche, 2008; Sander et al., 2008). While most studies show that VAMP8 and syntaxin4 are the major SNAREs in mast cell

degranulation (Paumet et al., 2000), it is highly likely that the other SNAREs are involved. As stated earlier, mast cell granules are heterogeneous in their contents, thus studies utilizing a single marker for degranulation are most likely overlooking evidence. The abundance of possible exocytic SNARE complexes could be important in allowing selective mediator release following mast cell activation.

Mast cells also express all three mammalian isoforms of Munc18 (Nigam et al., 2005), which could have different specificities in SNARE interactions (Yu et al., 2013). Munc18b is shown to be the dominant isoform expressed in both bone marrow-derived mast cells (BMMCs) and rat basophilic leukemia cells (RBL-2H3), comprising 64% of the Munc18 isoforms; while Munc18a and Munc18c are less abundant with 21% and 15%, respectively (Nigam et al., 2005). Both Munc18a and Munc18b have been shown to regulate degranulation in RBL-2H3 cells (Bin, Jung, Piggott, & Sugita, 2013). Hydrophobic pocket mutations in Munc18a/b effectively inhibit degranulation as much as knock-down mutants implicating binding Mode 2 and stimulation of the *trans*-SNARE complex as the primary function of Munc18 isoforms in mast cell degranulation (Bin et al., 2013). Surprisingly, overexpression of either wild-type Munc18a or Munc18b efficiently rescued degranulation in double-knockdown mutants, suggesting redundancy in either the Munc18 proteins or the SNARE proteins on granules (Bin et al., 2013). Little is known about the role of Munc18c in mast cells, however overexpression has shown no difference in externalization of phosphatidylserine found on secretory lysosomes (Martin-Verdeaux et al., 2003).

While Munc18-mediated stimulation of *trans*-SNARE complexes may be essential for mast cell degranulation, there is no indication of which SNARE-complexes

are being recognized by Munc18 proteins. Additionally, there is very little known about the binary interactions between Munc18 and syntaxin isoforms in mast cells (Binding Mode 1). Munc18b has been shown to interact with syntaxin3 and not syntaxin4 (Tadokoro et al., 2007), and Munc18c with only syntaxin4 (Martin-Verdeaux et al., 2003). It is unknown whether Munc18a interacts with either in mast cells. It is unclear whether the syntaxin N-terminal peptide plays a role in the binding of different Munc18 isoforms, as well as whether or not Munc18 binding to these syntaxins causes inhibition of *trans*-SNARE complex formation.

Specific Aims

Due to the heterogeneous nature of mast cells and their granules, and the abundance of possible SNARE complexes mediating fusion it is difficult to assess the functional interaction between Munc18 isoforms and exocytic SNAREs directly during mast cell exocytosis. A powerful alternative is to studying their property in reconstitution, comparing their functional (stimulation of various SNARE-mediated fusion reactions (as shown in Figure 2), promoting *trans*-SNARE complex formation, etc) and their biochemical activities (interaction with syntaxin proteins). These studies aim to determine the relevance of each Munc18 isoform in mast cell exocytosis and which SNARE-complex interactions take place. The results from the studies will present a more clearly defined picture of how Munc18 proteins regulate mast cell exocytosis, which will facilitate future identification of targeted drug therapies for control of mast cell pathology, perhaps without major affects to normal physiological function.

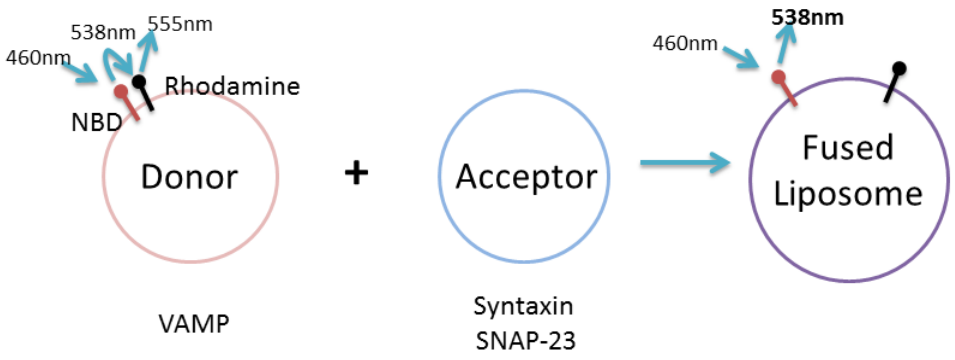

Figure 2. Illustration of RPL Fusion Assay

Donor RPLs contain fluorescence-labeled lipids where NBD fluorescence is quenched by Rhodamine. SNARE-medaited fusion of Donor with Acceptor RPLs (non-fluorescently-labeled) allows dilution and detection of NBD fluorescence.

Central Hypothesis: Munc18 isoforms differentially regulate mast cell exocytosis through interactions with isoform-specific SNARE complexes.

Aim 1: Determine fusogenic SNARE complexes present in mast cells.

Aim 2: Determine selective stimulation of various SNARE complexes by different Munc18 isoforms.

Aim 3: Investigate role of Munc18 proteins in events preceding the final membrane fusion.

CHAPTER II – MATERIALS AND METHODS

Expression and Purification of Recombinant Proteins

SNARE proteins for proteoliposome preparation

All constructs for recombinant SNARE expression were made using cDNA templates from *Rattus norvegicus* unless otherwise noted. Genes encoding VAMP2, 3 and 8, syntaxin3, syntaxin4 and SNAP-23 were inserted into the pMBP-parallel1 vector (Sheffield, Garrard, & Derewenda, 1999) using *NcoI* and *EcoRI* restriction sites for an N-terminal, TEV-cleavable MBP tag. Alternatively, VAMP7 was cloned into pET MBP His_6 vector using the LIC v3 tag sites, and expressed with a C-terminal, TEV-cleavable MBP tag. Wildtype syntaxin3 and syntaxin4 were similarly cloned into pET MBP to generate produce native N-termini on recombinant proteins. Syntaxin3ΔN aa28-289 (as previously described in (Peng, Guetg, Abellan, & Fussenegger, 2010)) and syntaxin4ΔN aa37-298 (as previously described in (Aran et al., 2009)) were cloned into pET MBP vector using respective primers with the addition of methionine at the N-terminus. pET MBP His_6 LIC cloning vector (2Cc-T) was a kind gift from Scott Gradia (Addgene plasmid # 37237).

Proteins were expressed using *E. coli* expression strains Rosetta2 for pMBP expression vectors or Rosetta2(DE3) for pET MBP expression vectors. Cells for protein expression were induced at OD_{600} = 1.00 with 0.5 mM IPTG and grown in 1 L Terrific Broth at 37°C for 4 hr. Collected cell pellets were resuspended in 20mL Buffer A containing 50 mM HEPES-KOH, pH 7.5, 500 mM KCl, 10% Glycerol, 1 mM DTT supplemented with 5 mM Benzamide, 1 mM PMSF and Protease Inhibitor Cocktail (0.62mg/mL leupeptin, 4mg/mL pepstatin A, and 24.4mg/mL pefabloc-SC). Cells were lysed

through French Press at 900 psi twice. For syntaxin3 and syntaxin4 purification, lysates were co-incubated with 40 mM CHAPS for 1hr at 4°C, while for VAMP purification, lysates were co-incubated with 100 mM β-octoglucoside. Proteins were purified with 5 mL Amylose Resin (New England Biolabs) pre-equilibrated with Buffer A supplemented with the respective detergent. Resin-bound protein and then washed with 20 mL of Buffer A containing respective detergent and eluted with the addition of 10mM Maltose to detergent-containing Buffer A. Protein concentrations were determined by standard Bradford assay before elutes were frozen in liquid N_2 and stored at -70°C.

Table 1

Primers used for cloning

Primer	Sequence	Vector
5' Munc18a *EcoRI*	CGGAATTCATGGCCCCCATTGGCC	pMBP-parallel1 N-terminal MBP tag
3' Munc18a *SalI*	ACGCGTCGACTTAACTGCTTATTTCTTCGTCTG	
5' Munc18b *BamHI*	ATGGGATCCATGGCGCCCTTGGGGC	pFastBac B N-terminal His_6 tag
3' Munc18b *SalI*	ACGCGTCGACTCAGGGCAGGGCTATGTCCTC	
5' VAMP3 *NcoI*	AAACCATGGGCATGTCTACAGGGGTGCCTTC	pMBP-parallel1 N-terminal MBP tag
3' VAMP3 *EcoRI*	GCGAATTCTTAAGAGACACACCACACAATG	
5' VAMP2 *NcoI*	TGCCATGGGAATGTCGGCTACCGCTGC	pMBP-parallel1 N-terminal MBP tag
3' VAMP2CD *EcoRI*	GCGAATTCTTACTTGAGGTTTTTCCACC	
5' VAMP8 *NcoI*	TGCCATGGAGGCCAGTGGGAG	pMBP-parallel1 N-terminal MBP tag
3' VAMP8CD *EcoRI*	GCGAATTCTTACATCTTCACATTCTTCCACC	
5' Syntaxin3 LIC v3	TTTAAGAAGGAGATATAGTTCATGAAGGACCGACTGGAG	pET MBP His6 LIC C-terminal MBP/His_6 tag
3' Syntaxin3 LIC v3	GGATTGGAAGTAGAGGTTCTCTTTCAGCCCAACGGAC	
5' Syntaxin3ΔN LIC v3	TTTAAGAAGGAGATATAGTTCATGAATACAGCGTTCATGGATGAG	
5' Syntaxin4 LIC v3	TTTAAGAAGGAGATATAGTTCATGCGCGACAGGACC	pET MBP His6 LIC C-terminal MBP/His_6 tag
3' Syntaxin4 LIC v3	GGATTGGAAGTAGAGGTTCTCTCCAACGGTTATGGTGATG	
5' Syntaxin4ΔN LIC v3	TTTAAGAAGGAGATATAGTTCATGCCGGACGACGAGTTCTTCCAG	

VAMP cytoplasmic domains

Constructs of recombinant VAMP2 and VAMP8 cytoplasmic domains (CD) were generated omitting the C-terminal transmembrane domain, expressing VAMP2 aa1-94 (as previously described in (Poirier et al., 1998)) and VAMP8 aa1-75 as determined by the TMpred program (Hofmann & Stoffel, 1993) and annotations found in NCBI. Cloning and protein purification were performed in the same way as full-length proteins

(above), except detergents were omitted during the purification process. MBP-VAMP CDs were subsequently dialyzed into RB150 (20mM HEPES-NaOH, pH 7.4, 150mM NaCl, 10% Glycerol) prior to snap-freezing in liquid N_2 and storage at -70°C.

Munc18 proteins

cDNA encoding rat Munc18a and Munc18c were obtained from ThermoScientific, while Munc18b template was generously provided by T. Südhof. Templates were amplified with respective primers listed in **Table1** and cloned into pMBP-parallel1 using *EcoRI* and *SalI* restriction sites. Munc18a was expressed using *E. coli* Rosetta2 induced at OD_{600} = 1.00 with 0.2 mM IPTG, and grown in 1 L Terrific Broth at 22°C overnight. Cell pellets were resuspended in 20 mL buffer B containing 50 mM Tris-Cl, pH 8.0, 500 mM KCl and 5 mM EDTA supplemented with 5 mM Benzamidine, 1 mM PMSF and Protease Inhibitor Cocktail. Purification via Amylose Resin proceeded as stated for VAMP CD purification. Eluted MBP-Munc18a was dialyzed into RB150 prior to snap-freezing in liquid N_2 and storage at -70°C.

For binding assays, His_6-Munc18a was expressed from plasmid pPROEX-HTb-rMunc18a, a kind gift from Axel Brunger (Zhang et al., 2015) in Rosetta2(DE3). Rosetta2(DE3)/pPROEX -HTb-rMunc18a cells were grown in 1 L Terrific Broth at 37°C to an OD600= 1.2, where expression was induced with 0.5 mM IPTG. Cultures were then incubated for 4 hr at 37°C before cell pellets were harvested by centrifugation and resuspended in 20 mL of Buffer C (50 mM HEPES-KOH, pH 7.5, 500 mM KCl, 10% glycerol, 1 mM DTT) containing 10 mM imidazole, 1 mM PMSF, and 1x PIC. Following French Press at 900 psi, 1% TritonX-100 was added to the lysate for 30 min at 4°C and lysates clarified by ultracentrifugation with a Beckman Type 70 Ti rotor (4 °C,

30 min, 18,500 rpm). The supernatants were applied to 4 mL of Ni-NTA resin (Qiagen) pre-equilibrated with Buffer C, and nutated for 2 hr at 4°C. Resins were then washed with 20 mL of respective buffers containing 50 mM imidazole, followed by protein elution in 20 mL of respective buffers containing 300 mM imidazole. His_6-Munc18a was dialyzed 1,000,000-fold in RB150 overnight at 4°C, concentrated using 30k MWCO Microsep™ Advanced Centrifugal Device, snap-frozen in small aliquots in liquid N_2, and stored at – 70°C. Protein concentration was determined by the standard Bradford assay.

Purifications of MBP-tagged Munc18b and Munc18c were attempted similarly to Munc18a but did not yield soluble proteins. As an alternative, Munc18c (mouse) was expressed from pFastBac-HT-JS-Munc18c (a kind gift from Dr. Jingshi Shen), then were expressed in *Sf9* insect cell line (gift from Dr. Fengwei Bai; in collaboration with Dr. Hao Xu). Likewise, Munc18b (rat) template was amplified by PCR and inserted into pFastBac-HT-JS (gift from Dr. Jingshi Shen) and expressed utilizing *Sf9* expression system. Recombinant proteins were purified using Ni-NTA resin from the lysates of transfected *Sf9* cells according to established protocols (Xu, Arnold, & Kumar, 2015). In brief, Buffer C containing protease inhibitors and 20 mM imidazole was used to resuspend the cell pellet, after which 1% TritonX-100 was added. Following a 30 min incubation at 4°C, the lysates were homogenized with Dounce homogenizer (20 times), and then clarified by ultracentrifugation with a Beckman Type 70 Ti rotor (4 °C, 30 min, 18,500 rpm). The supernatants were applied to 4 mL of Ni-NTA resin (Qiagen) pre-equilibrated with Buffer C containing 10 mM imidazole, and nutated for 2 hr at 4°C. Resins were then washed with 20 mL of respective buffers containing 20 mM imidazole, followed by protein elution in 20 mL of respective buffers containing 200 mM imidazole.

Recombinant proteins were dialyzed 1,000,000-fold in RB150 overnight at 4°C, concentrated using 30k MWCO Microsep™ Advanced Centrifugal Device, snap-frozen in small aliquots in liquid N_2, and stored at – 70°C. Protein concentrations were determined by the standard Bradford assay.

Reconstituted Proteoliposome Preparation

Fluorescent lipids were obtained from Invitrogen. Non-fluorescent lipids were from Avanti Polar Lipids, Inc. Donor proteoliposomes contain 60% POPC (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine), 17% POPE (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine), 10% DOPS (1,2-dioleoyl-sn-glycero-3-phosphoserine), 10% cholesterol, 1.5% NBD-DHPE [N-(7-Nitrobenz-2-Oxa-1,3-Diazol-4-yl)-1,2-Dihexadecanoyl-sn-Glycero-3-Phosphoethanolamine], and 1.5% rhodamine DHPE(Lissamine™ Rhodamine B 1,2-Dihexadecanoyl-sn-Glycero-3-Phosphoethanolamine), whereas acceptor proteoliposomes contain 60% POPC, 19% POPE, 10% DOPS, 10% cholesterol and 1% Dansyl DHPE [N-(5-Dimethylaminonaphthalene-1-Sulfonyl)-1,2-Dihexadecanoyl-sn-Glycero-3-Phosphoethanolamine]. Proteoliposomes were prepared under N_2 gas to prevent lipid oxidation. Lipid films and proteins were resuspended in RB500 with 40mM CHAPs and subsequently dialyzed into RB150 overnight. RPLs (reconstituted proteoliposomes) were then harvested through flotation in a Histodenz density gradient as previously described (Shen, Tareste, Paumet, Rothman, & Melia, 2007). SNARE proteins were kept at similar densities as reconstitution studies of neuronal transmission (Hickey & Wickner, 2010; Ma et al., 2011; Shen et al., 2007), with protein: lipid ratios at or below 1:200 for R-SNARE bearing donor RPLs and at or below 1:500 for Q-SNARE bearing acceptor

RPLs. Concentrations of harvested RPLs were determined by quantification of Dansyl-DHPE or Rhodamine on acceptor and donor RPLs, respectively (Hickey & Wickner, 2010). RPLs were stored in 10 µL aliquots snap-frozen in liquid N_2 and stored at -70°C.

RPL Fusion Assay

Fusion reactions were mixed in 20 µL volumes containing 400 µM Q-SNARE RPLs (based on lipid concentration) and 50 µM R-SNARE RPLs in RB150incubated at 4°C overnight. Where Munc18 proteins were used, TEV protease was added in a 2:1 molar ratio to ensure cleavage of the affinity tag in the reaction. Standard fusion reactions contained 2 µM Munc18a. To prevent trans-SNARE complex formation, Q-RPLs were pre-incubated with 4 µM VAMP8CD for 1 hr at 4°C before addition of Munc18a and R-RPLs. Reactions were added to 384 black Corning microplates and RPL fusion monitored at 37°C for dequenching of the NBD (λ_{ex}= 460 nm, λ_{em}= 538 nm, λcutoff= 515 nm) on R-SNARE RPLs. Fluorescence values are given as fold change from the initial fluorescence (Ft/F0). Where used, lipid mixing rate was determined by the 5-15 min slope.

RPL Clustering Assay

Standard fusion reactions, containing 4µM Munc18a, were diluted 40-fold in ice-cold RB150. 4µL were placed on a microscope slide with a 22mm coverslip. A Zeiss confocal fluorescence microscope with a 63x objective was used to collect four images at random through Rhodamine fluorescence. Images were analyzed with ImageJ software (National Institute of Health) to determine the pixel size of particles after setting the lower threshold limit to 50 and upper threshold limit to 255, as previously described

(Hickey & Wickner, 2010). Pixel sizes were plotted on a probability graph using KaleidaGraph software.

His_6-tag Pulldown Assay

A 20 nMol (2.5 µg) sample of His_6-Munc18a was co-incubated with a 30 nMol of syntaxin incorporated into RPLs in a 30 µL volume overnight at 4°C. Samples were then co-incubated with 10 µL Ni-NTA resin (Qiagen) pre-equilibrated in binding buffer (20 mM HEPES, pH 7.4, 150 mM NaCl, 10% Glycerol, and 20 mM Imidazole) in a 200 µL volume for 1hr at 4°C. Unbound protein was removed by collecting resin with centrifugation at 1000 × g for 1 min at 4°C, washing resin with 200 µL binding buffer three times. After washing bound proteins were solubilized with 20 µL SDS-PAGE sample buffer, boiled at 95°C for 5 min, loading 10 µL onto a 12% SDS-PAGE gel for analysis.

CHAPTER III - RESULTS

Mast cell SNAREs forms at least six fusogenic trans-SNARE complexes

In a first step to investigate the mechanism of mast cell exocytosis, preliminary experiments were conducted to determine which combinations of the seven SNARE proteins implicated in degranulation formed fusogenic *trans*-SNARE complex. To this end, recombinant SNARE proteins (4 R-SNAREs, and 3 Q-SNAREs) were purified through affinity chromatography and incorporated into liposomes as shown in Figure 3A. R-SNAREs VAMP2, 3, 7, and 8 (lanes 1 to 4) were incorporated into donor liposomes in relatively equal amounts, whereas comparable levels of Q-SNAREs syntaxin3 and syntaxin4 were reconstituted along with SNAP-23 (lanes 5 to 6) into acceptor liposomes. Additionally, protein-free acceptor liposomes (lane 7) were reconstituted as negative controls for SNARE-mediated fusion.

Resulting RPLs were assayed for lipid mixing through monitoring the FRET (fluorescence resonance energy transfer) between NBD-PE and Rhodamine-PE on the donor RPLs. At high concentrations in the donor liposomes, the NBD fluorescence is quenched by the neighboring rhodamine. However, as the result of lipid mixing with acceptor RPLs lacking bear rhodamine-PE or NBD-PE, this quenching effect becomes relieved via dilution, resulting in detectable increase of NBD signal.

The eight possible combinations of mast cell SNAREs were assayed for lipid mixing (Figure 3B to 3E). Of these, six *trans*-SNARE complexes appear to be fusogenic: i) syntaxin3/SNAP-23/VAMP2, ii) syntaxin4/SNAP-23/VAMP2, iii) syntaxin3/SNAP-23/VAMP3, iv) syntaxin4/SNAP-23/VAMP3, v) syntaxin3/SNAP-23/VAMP8, and vi) syntaxin4/SNAP-23/VAMP8 (Figure 3B, C and E). Reactions containing syntaxin4

and/or VAMP8 appear to be the most significant. In contrast, VAMP7 reactions (Figure 3D) show similarity to reactions containing protein-free acceptor RPLs, indicating a lack of membrane fusion.

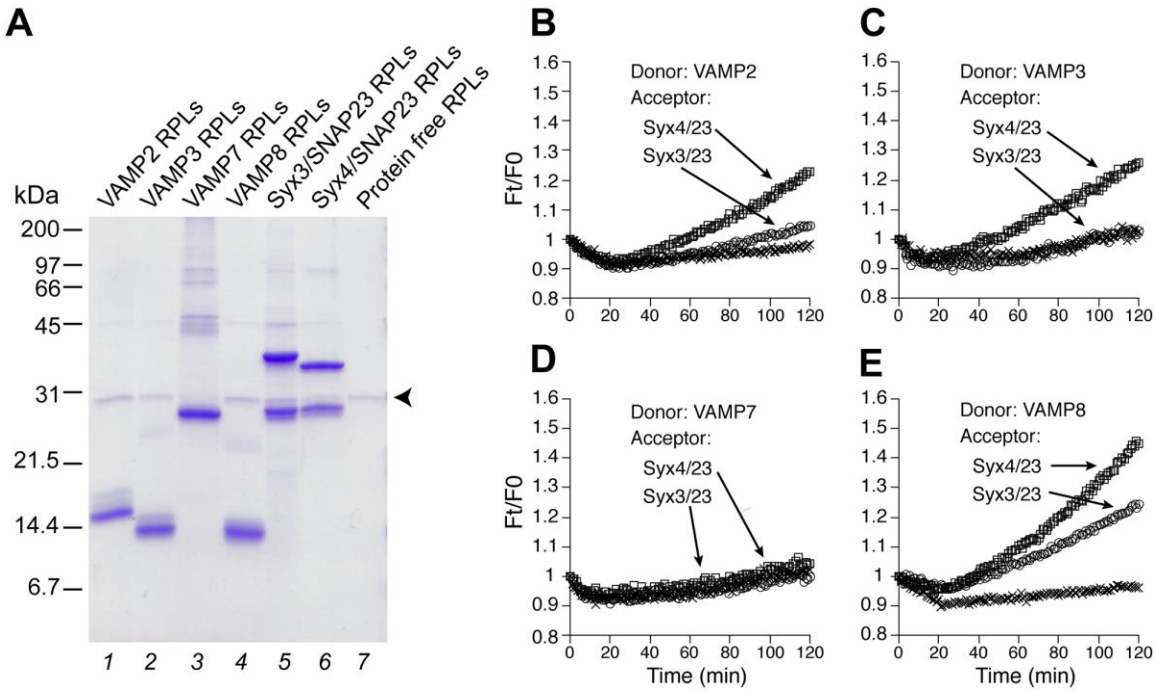

Figure 3. SNARE-bearing RPLs implicated in mast cell exocytosis

(A) Coomassie blue-stained SDS-PAGE gel showing incorporation of mast cell SNAREs into recovered liposomes. RPLs are loaded by calculating 20nmol total lipid. His_6-TEV protease, used during reconstitution for affinity tag cleavage, is visible in small amounts in all RPLs (indicated by arrowhead). (B to E) Standard fusion reactions showing potential fusogenic SNARE complexes. Fluorescence of NBD on donor RPLs was monitored every minute for 2 hr. Data is represented as Ft/F_0, Ft being fluorescence at a respective time point and F_0 being initial fluorescence. Data represented by 'x' are control reactions with non-fusogenic protein-free acceptors (not readily visible in C and D). These results are representative of more than three biological replicates.

Munc18a Selectively Stimulates Membrane Fusion Mediated by Four SNARE-Complexes

To clarify the role Munc18 isoforms play in mast cell degranulation, recombinant Munc18a, b and c were purified to test for any effect on each SNARE complex. After purification, affinity-tags at the N-terminus of Munc18 proteins were removed by TEV

protease during or prior to the RPL fusion reaction. Significant stimulation of fusion activity was observed when testing Munc18a in reactions containing R-SNAREs VAMP2 (Figure 4, lanes 2 and 3) or VAMP3 (lanes 5 and 6) with either Q-SNARE acceptor RPLs. Replacing acceptor RPLs with protein-free liposomes showed no stimulation of fusion (lanes 1 and 4), indicating that the presence of SNARE proteins on apposing membranes is critical to Munc18a-dependent function. In contrast, reactions containing R-SNAREs VAMP7 (lanes 7-9) or VAMP8 (lanes 10-12) showed no significant change in activity with the addition of Munc18a. The specificity of Munc18a for VAMP2, but not VAMP8, has been observed during neurotransmission when VAMP2 residues were replaced with corresponding VAMP8 residues, resulting in diminished exocytosis *in vivo* (Shen et al., 2007).

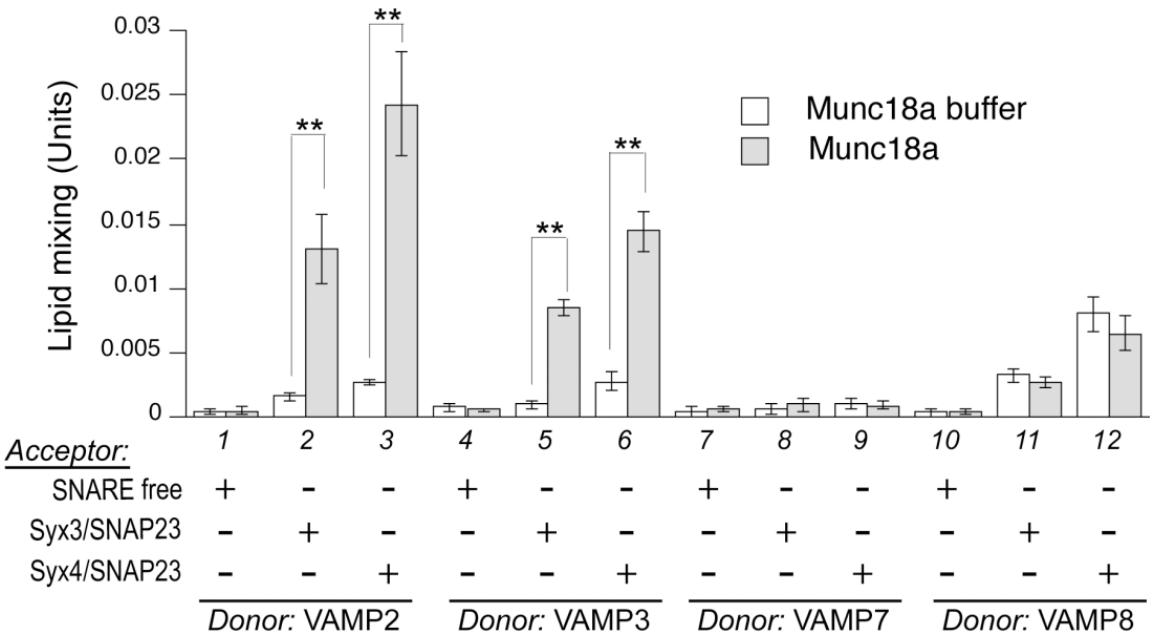

Figure 4. Munc18a selective stimulation of degranulation-relevant SNARE complexes.

Liposomes bearing mast cell SNAREs were co-incubated overnight at 4°C with 2μM Munc18a or its control buffer, prior to fluorescence monitoring at 37°C. Lipid mixing was determined by the maximal rate of fluorescence increase from 5-20 minutes. Error

bars shown represent standard deviation and p-values determined through Student's t-test (** indicates p<0.01). Results were compiled from three independent experiments.

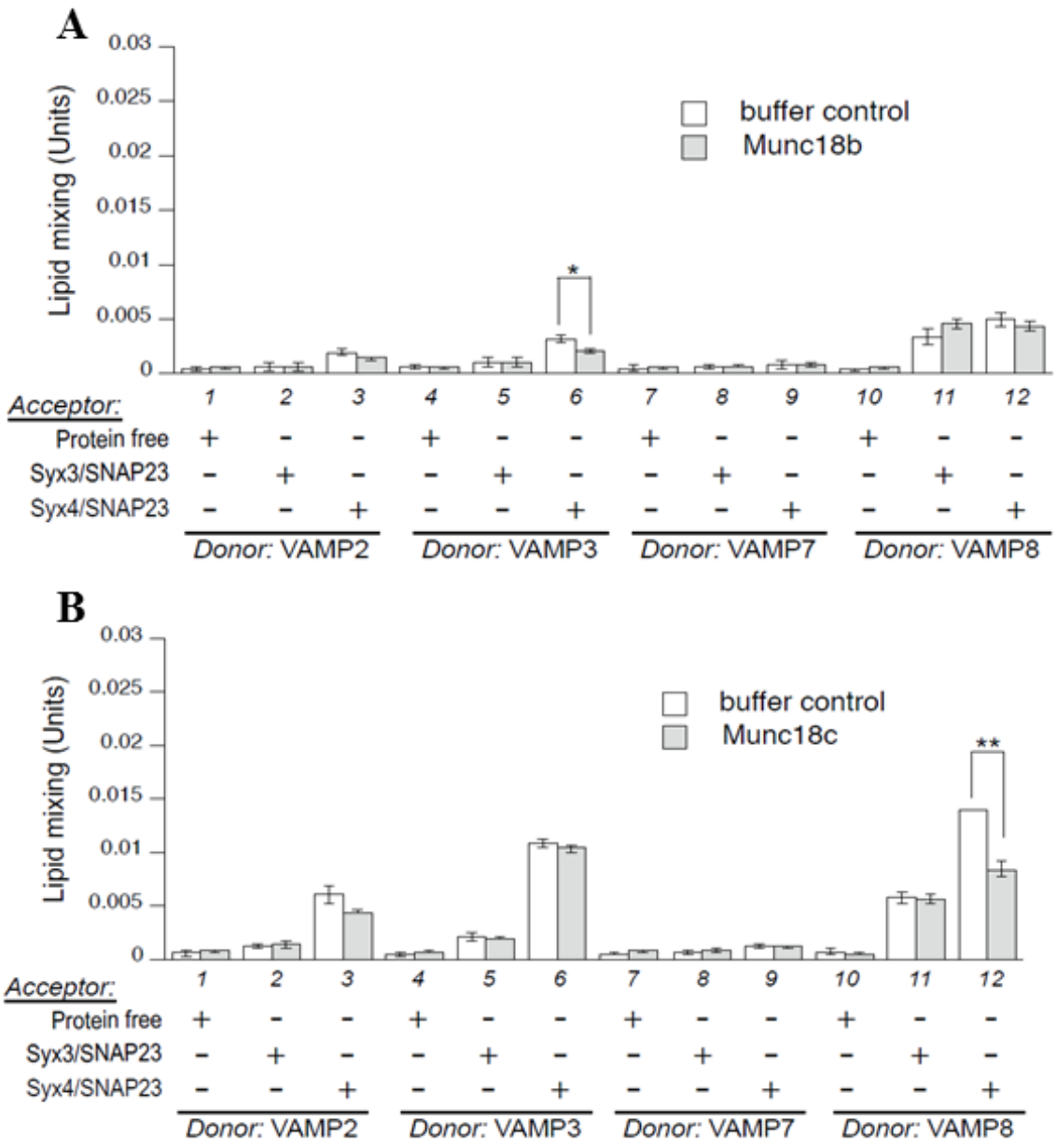

Figure 5. Munc18b/c exhibit no stimulation of mast cell SNARE complexes.

Lipsomes bearing mast cell SNAREs were co-incubated overnight at 4°C with 2μM Munc18b, Munc18c or respective control buffers, prior to fluorescence monitoring at 37°C. Lipid mixing was determined by the maximal rate of fluorescence increase from 5-20 minutes. Error bars shown represent standard deviation and p-values determined through Student's t-test (* indicates p<0.05, ** indicates p<0.01). Results were compiled from three independent experiments.

Munc18b and Munc18c Do Not Stimulate Lipid Mixing Reactions

In contrast to Munc18a, it seems that Munc18b and Munc18c are unable to stimulate fusion between mast cell SNARE-complexes (Figure **5**). Pull-down assays with cognate syntaxins were performed to examine the possibility that recombinant Munc18b and Munc18c were expressed as misfolded, inactive proteins. Surprisingly, these results showed both these proteins as active in binding to their cognate SNAREs; Munc18b associated with both syntaxin3 and syntaxin4, while Munc18c showed a strong interaction with syntaxin4, but not syntaxin3 (Figure 6). These results show that these proteins remain biochemically active despite inability to promote membrane fusion. However, binary interaction between Munc18 and syntaxins can be viewed as Binding Mode 1, and not Binding Mode 2 which is responsible for fusion stimulation.

The results of the pull-down assays (Figure 6) led us to question the syntaxin N-terminal peptide on our recombinant protein. The N-terminal peptide in syntaxin plays an important role in Munc18-syntaxin Binding Mode 2, responsible for fusion enhancement. We wondered if the presence of an additional 4 amino acid residues at the N-terminus of recombinant syntaxin4 after affinity tag removal might have interfered with Munc18b and Munc18c's stimulation of SNARE-mediated fusion. To examine this possibility, C-terminally tagged Syntaxin4-MBP with a native N-terminus was incorporated into acceptor RPLs in place of the MBP-Syntaxin4 used in previous experiments. However, these data showed no difference from previous reactions shown in Figure 5 where no stimulation of fusion was seen (data not shown). Having tested the above mentioned possibilities, we propose that post-translational modifications to Munc18b and Munc18c

may be critical to activate their pro-fusion function and enable Binding Mode 2 (see discussion).

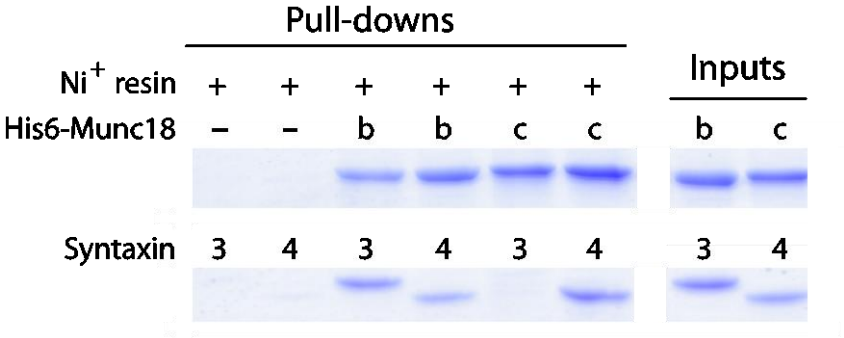

Figure 6. Recombinant Munc18b and Munc18c retain ability to bind syntaxin

5µg His_6-Munc18b or His_6-Munc18c were pre-incubated with Ni-NTA resin prior to incubation with 2.5µg syntaxin-bearing RPLs overnight at 4°C. Resins were washed with RB150 thrice to remove unbound protein, then boiled in 2x Laemmli buffer before loading on an SDS-PAGE gel.

Munc18a Selectively Promotes Liposome Clustering in an R-SNARE-Dependent Fashion

Although Munc18a has been known tointeract with the trans-SNARE complex in neurotransmission (Shen et al., 2007), the exact roles of Munc18a in mast cell degranulation in unclear. To explore if Munc18a has an early role in the fusion cascade, we first examined Munc18a's potential function in *trans*-SNARE complex formation (i.e. trans-SNARE zippering). To this end, mast cell R-SNAREs VAMP2 and VAMP8 were chosen for the study as they respond differently to Munc18a in lipid-mixing reactions. Syntaxin4/SNAP-23 acceptor RPLs are depicted as representatives for Q-SNAREs as similar results were obtained when syntaxin3/SNAP-23 RPLs were used. An observable feature of trans-SNARE zippering between apposing membranes would be clustering of RPLs, visible under confocal microscopy through excitation of rhodamine found on donor RPLs. A comparison of liposome size distribution would allow assessment of

clustering. The addition of Munc18a to syntaxin4/SNAP-23 + VAMP2 reactions (Figure 7B) causes a noticeable increase in observed particle size, while reactions containing protein free acceptors (Figure 7A) or VAMP8-bearing donors (Figure 7C) were not influenced by Munc18a's presence.

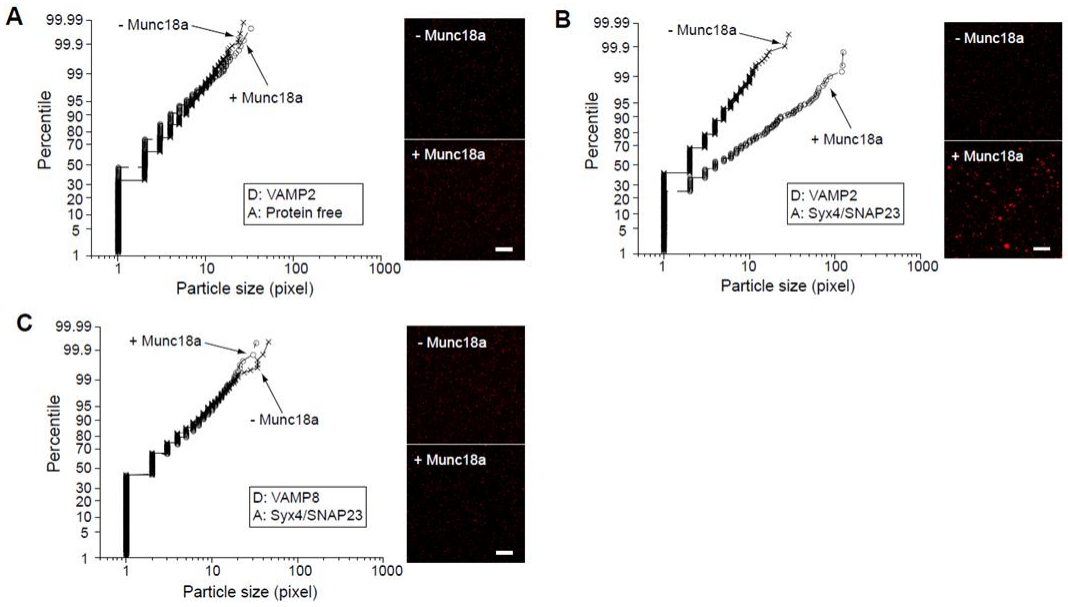

Figure 7. Munc18a clusteres SNARE-bearing RPLs when VAMP2 is present

Lipsomes were mixed according to standard fusion reaction conditions, with either 4μM Munc18a or buffer. After overnight incubation at 4°C samples were diluted 1:40 in RB150 before confocal microscopy. Four images were collected randomly from each sample and analyzed for accumulative distribution of particle sizes (A to C). Scale bars represent 20 μm. Images are representative of three experiments.

Munc18a Relies on Syntaxin N-terminus for RPL Clustering

Munc18 proteins function via interactions with their cognate SNAREs. Our results indicate a clear R-SNARE specificity to Munc18a's stimulation (Figure 4) and trans-SNARE zippering functions (Figure 7). However, while the importance of syntaxin N-terminal peptide has been noted in fusion (Shen, Rathore, Khandan, & Rothman, 2010), it is unclear whether this plays a role in trans-SNARE zippering. To address this, recombinant syntaxin4ΔN proteins were purified and incorporated into acceptor

liposomes along with His_6-SNAP-23. Reactions with VAMP2 show syntaxin4ΔN/SNAP-23 maintains a similar pixel size distribution regardless of the presence of Munc18a (Figure 8B), indicating a clear necessity for this peptide in *trans*-SNARE complex formation. Furthermore, pulldown assays showed that His_6-Munc18a failed to interact with both syntaxin3ΔN and syntaxin4ΔN, while full-length syntaxins were successfully detected (Figure 8A). Additionally, lipid-mixing assays with syntaxin4ΔN failed to show Munc18a-dependent stimulation, in contrast to the wildtype syntaxin4 (Figure 8C).

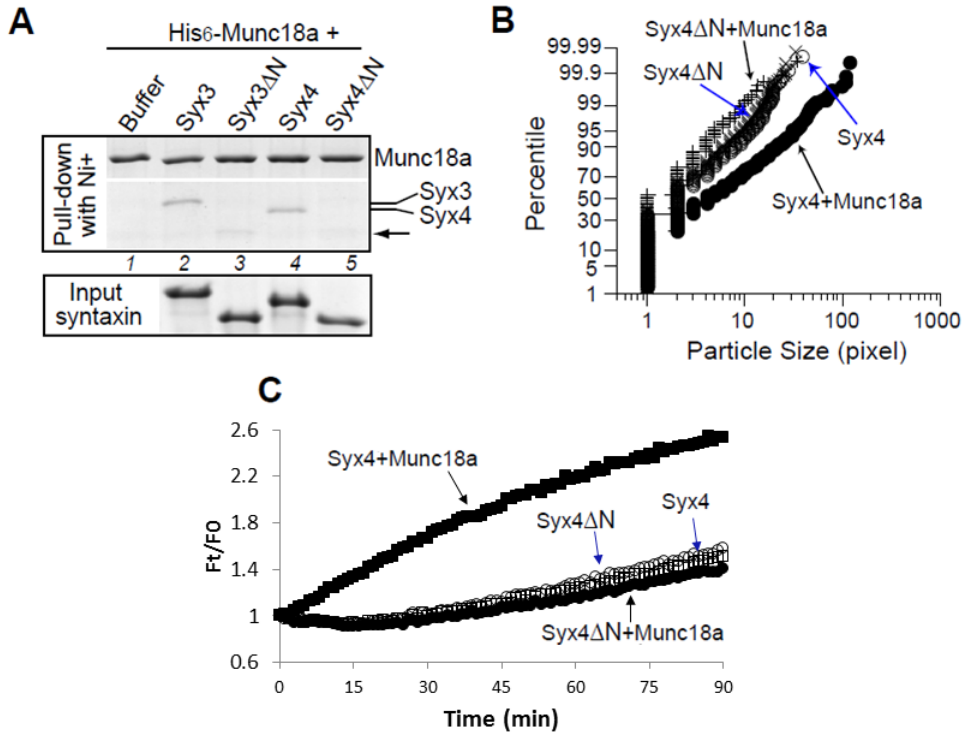

Figure 8. Syntaxin4 N-terminus required for Munc18a-mediated tethering

Conditions for confocal microscopy are as previously described. A) His_6-Munc18a pulldown of syntaxin RPLs. B) Accumulative distribution of particle sizes under confocal microscopy. Images are representative of three experiments.

Munc18a Facilities RPL Clustering through Binding Syntaxin and VAMP2 Prior to Trans-SNARE Complex Formation

Having uncovered the biochemical requirement of cognate SNAREs in Munc18a tethering, we decided to further investigate whether Munc18a's mechanism preluded trans-SNARE complex formation. Since Munc18a was previously shown to have no affinity for VAMP8 (Figure 4), soluble fragments of VAMP cytoplasmic domain (VAMPcd) were purified and co-incubated with syntaxin4/SNAP-23 liposomes to create an inert, tetrametric SNARE complex, incapable of fusion with VAMP2-bearing liposomes (as shown in Figure 9A). Blocking trans-SNARE complex formation between syntaxin4/SNAP-23 and VAMP2 would prevent RPL clustering, unless Munc18a could bind both simultaneously and hold them together. In fusion reactions, Munc18a-dependent stimulation with VAMP2 RPLs is effectively inhibited when syntaxin4/SNAP-23 RPLs are pre-blocked with VAMP8 cytoplasmic domain (VAMP8cd) (Figure 9B). In contrast, clustering of VAMP2 liposomes is still observed in the presence of Munc18a (Figure 9C). Unexpectedly, an identical effect is seen when VAMP2cd is used to block trans-SNARE complex formation; VAMP2 being a cognate SNARE of Munc18a. This indicates that Munc18a may have a preference for full-length, membrane-anchored VAMP2 over soluble cytoplasmic domains, possibly through altered conformation state, as previously suggested (Aran et al., 2009). Munc18a-dependent RPL clustering excluding membrane fusion indicates that Munc18a is able to bind the syntaxin N-terminal peptide and VAMP2 without the formation of the *trans*-SNARE complex, as previously suggested (Yu et al., 2013). This is the strongest evidence to date that Munc18a aids tethering in a step distinct from *trans*-SNARE complex formation.

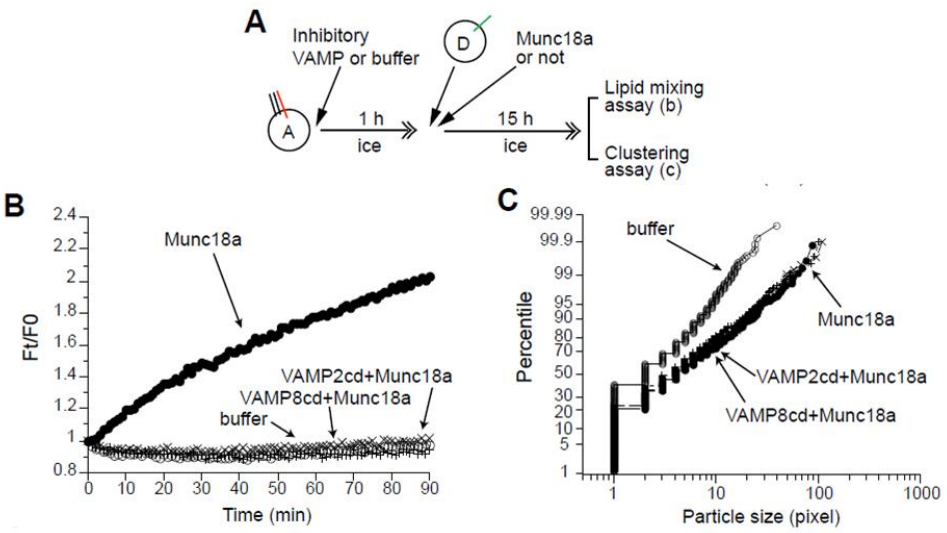

Figure 9. Soluble R-SNAREs prevent fusion but not Munc18a-mediated tethering

Conditions for confocal microscopy are as previously described. A) Diagram of reaction procedure, B) Fusion assay showing VAMP8cd and VAMP2cd inhibition of Munc18a-dependent stimulation, C) Accumulative distribution of particle sizes under confocal microscopy. Images are representative of three experiments.

CHAPTER IV – DISCUSSION

While Munc18 proteins play a vital role in regulating SNARE-mediated fusion in all eukaryotic cells, limited studies have been done in immune cells, particularly mast cells. Arguably, mast cells have enormous potential for thorough understanding of these proteins due to expression of all three mammalian isoforms and numerous exocytic SNAREs that serve as potential cognate receptors. In this study, it was found that Munc18a was the only isoform active in stimulating membrane fusion in mast cell SNAREs. Inquiries into its role in trans-SNARE zippering revealed a similar R-SNARE-dependent selectivity that was seen in lipid mixing. Despite being R-SNARE-dependent, Munc18a-mediated trans-SNARE zippering was dependent on the N-terminal peptide from Q-SNARE syntaxin. Finally, our data suggests Munc18a facilitates RPL clustering through initiation of trans-SNARE complex formation, underpinning a new role for Munc18a in non-neuronal fusion events.

The existence of numerous exocytic SNAREs in mast cells provides opportunity for multiple avenues for exocytosis. However, there have been major obstacles in the identification of functional SNARE complexes in mast cell degranulation. Although syntaxin4/SNAP-23/VAMP8 is considered to be the prime candidate for histamine release, syntaxin4 has also been seen to interact with VAMP2 and VAMP3 (Paumet et al., 2000). Syntaxin3 has only been seen to interact with VAMP7 (Puri, Kruhlak, Whiteheart, & Roche, 2003) and is speculated to regulate chemokine release in human mast cells (Frank, Thon, Bischoff, & Lorentz, 2011), possibly under the influence of Munc18b (Tadokoro et al., 2007). Our *in vitro* membrane fusion assay shows that all SNAREs except VAMP7 are capable of forming fusogenic complexes (Figure 3). Why

certain complexes are favored above others *in vivo* requires further investigation, but our data begins to present a clearer understanding of SNARE-mediated exocytosis in mast cells.

VAMP7 shows little activity in fusion assays (Figure 3D), although it may still have a role in mast cell exocytosis, as antibodies against VAMP7 reduced histamine release by 50% (Sander et al., 2008). VAMP7 is unique among VAMP isoforms as it contains an N-terminal self-inhibitory Longin domain that hinders its ability to enter a trans-SNARE complex. Phosphorylation of Y45 has been shown to relieve this inhibition in fibroblasts (Burgo et al., 2013), though it is currently unknown if a similar mechanism is used in mast cells. Due to the native self-inhibiting conformation, a mutant ΔLongin-VAMP7 could potentially be used to investigate the nature of VAMP7 in future *in vitro* fusion assays, although current efforts to purify the deletion mutant have not been successful (data not shown).

Cell-based secretion studies have shown that Munc18a had a positive role in promoting mast cell β-hexosaminidase release (Bin et al., 2013). While VAMP8 remains the prime R-SNARE involved in β-hexosaminidase release (Paumet et al., 2000), our data shows that Munc18a is unable to stimulate VAMP8-containing complexes (Figure 4). Previous studies show that VAMP8-knockout mast cells have a severe deficiency in β-hexosaminidase release, however, its inhibition is not complete (Puri & Roche, 2008; Tiwari et al., 2008). In the absence of VAMP8, an increasing amount of VAMP2 and VAMP3 were found associated with SNAP-23 (Tiwari et al., 2009), Taken together, I speculate that VAMP2 or VAMP3 play an supplemental role in β-hexosaminidase release in the event of compromised VAMP8. This release may be through a small, piecemeal

exocytosis rather than the dramatic degranulation typically observed. The observation that Munc18a regulates β-hexosaminidase release (Bin et al., 2013), but only has the capacity to regulate VAMP2 or VAMP3-based reactions warrants further investigation into these SNAREs as potential VAMP8-independent pathways for mast cell mediator release.

The data shows that recombinant Munc18a is active in stimulating fusion (Figure 4), whereas recombinant Munc18b and Munc18c do not share this activity (Figure 5). Despite the inability of Munc18b and Munc18c to stimulate fusion, they retain the ability to bind syntaxins (Figure 6), showing they remain in a biochemically active protein conformation. This may potentially indicate an important divergence in regulation of Munc18 protein function. It has been shown in numerous studies that phosphorylation can play an important role in modulating Munc18 activity inside the cell. Phosphorylation of Munc18a at Ser_{306} and Ser_{313} by PKC has been reported in chromaffin cells (Barclay et al., 2003; Fujita et al., 1996) and is critical to neurotransmission (Genç et al., 2014). Additionally, phosphorylation of Thr_{574} by CDK5 has also been reported in chromaffin cells to be essential for norepinephrine release (Fletcher et al., 1999). Similarly, it has been shown that Munc18b requires CDK5-dependent phosphorylation of Thr_{572} to promote assembly of syntaxin3/SNAP-25/VAMP8 complexes in gastric parietal cells *in vivo* (Liu et al., 2007). For Munc18c, phosphorylation of Y_{219} and Y_{521} by insulin receptor in adipose cells stimulates syntaxin4/SNAP-23/VAMP2 complexes critical for GLUT4 exocytosis (Aran et al., 2011; Jewell et al., 2011; Kioumourtzoglou, Gould, & Bryant, 2014), while native Munc18c inhibits fusion through binding of syntaxin4 (Brandie et al., 2008). A possible explanation to the discrepancy between Munc18a, and

Munc18b and Munc18c, is that phosphorylation of Munc18b and Munc18c may be necessary to observe fusion stimulation, whereas Munc18a is active in its native form. This divergence between Munc18a, and Munc18b and Munc18c, could allow modification of exocytic activity in the absence of a phosphorylation cascade *in vivo*. This could avoid a complete shutdown of exocytosis in the cell.

Munc18b has been regarded as the major regulator of mast cell degranulation. Whether or not Munc18b stimulates the same or different SNARE complexes as Munc18a remains to be determined, but VAMP8 is strongly indicated in vivo in other cell types(Al Hawas et al., 2012). CDK5-dependent phosphorylation of Munc18b, as observed in gastric parietal cells (Liu et al., 2007), is currently the best known mechanism for Munc18b activation. Further studies could utilize a phosphomimetic Munc18b T572D mutant to possibly observe stimulation of membrane fusion in reconstitution.

Munc18c's role in mast cells is completely unknown, with only a single study reporting that Munc18c overexpression did not affect phosphoserine externalization, a marker for degranulation (Martin-Verdeaux et al., 2003). Previous data suggests unphosphorylated Munc18c acts as a negative fusion regulator in translocation of GLUT4-containing vesicles (Brandie et al., 2008). There are no identified kinases responsible for Munc18c phosphorylation in mast cells, which would be required to activate it.

Previous *in vitro* data has shown Munc18a selectively stimulating syntaxin1/SNAP-25 fusion with VAMP2, 3 and 4, but not syntaxin4/SNAP-23 with VAMP2 or VAMP8 (Shen et al., 2007). In contrast, our data shows that Munc18a is able

to stimulate both syntaxin3 and syntaxin4-containing complexes (Figure 4), aligning with previous work stating that the N-terminal peptide of plasmalemmal syntaxins is not particularly selective (Hu et al., 2011). Additionally, recent data has shown Munc18c actively stimulating RPL fusion (Yu et al., 2013), whereas our data has shown no activity (Figure 5). However, Munc18c has been seen to play an inhibitory role in RPL fusion using SNAREs from GLUT4-vesicle translocation (syntaxin4, SNAP-23, and VAMP2) instead of negative (Brandie et al., 2008). The reason for these differences is unknown but could be related to the different protein expression and purification methods or differences in RPL lipid composition. Preliminary results indicate salt concentration in fusion reactions could play a role in determining Munc18c's activity (data not shown).

Munc18a's ability to selectively promote RPL clustering (Figure **7**) mirrors studies on related SM protein Vps33p, a subunit of the HOPS complex. HOPS plays a vital role in tethering membranes by binding regulatory lipids and membrane-associated Ypt7p (Hickey & Wickner, 2010). This tethering is independent of SNARE proteins, but by bringing apposing membranes into close proximity, it indirectly promotes trans-SNARE complex formation. HOPS is also instrumental in protecting the preformed trans-SNARE complex from disassembly via NSF and αSNAP (Xu, Jun, Thompson, Yates, & Wickner, 2010). Whether Munc18a fulfills the protective role in mast cell degranulation remains to be investigated.

The requirement for syntaxin N-terminal peptide in conjunction with Munc18a in fusion has been reported but the precise role of this interaction is yet to be established (Munson & Bryant, 2009). Our data shows that Munc18a is unable to tether SNARE complexes or bind syntaxins in the absence of syntaxin N-terminal peptide (Figure 8). In

addition, both full-length syntaxin3 and syntaxin4 are capable of recruiting Munc18a, aligning with previous work stating that the N-terminal peptide of plasmalemmal syntaxins is not particularly selective (Hu et al., 2011; Shen et al., 2010). Showing that Munc18a tethers SNARE-bearing liposomes prior to trans-SNARE complex formation (Figure 9) reveals an important aspect of Munc18 proteins that has been overlooked. Whether this applies to other SM proteins will be an interesting topic of future research.

APPENDIX A – Mast cell mediators

Table A1. *Mast cell mediators*

Pre-stored Mediators	Physiological Effect
Biogenic amines	
Histamine	Vasodilation, angiogenesis, mitogenesis, pain
Serotonin	Vasoconstriction, pain
Chemokines	
CXCL1[a], IL-8 (CXCL8), MCP-1 (CCL2), MCP-3 (CCL7), MCP-4, RANTES (CCL5), Eotaxin (CCL11)	Chemoattraction and tissue infiltration of Leukocytes
Enzymes	
Arylsulfatases	Lipid/Proteoglycan hydrolysis
Carboxypeptidase A	Peptide Processing
Chymase (mMCP4)	Tissue damage, pain, angiotensin II synthesis
Kinogenases	Synthesis of vasodilatory kinins, pain
Phospholipases	Arachidonic acid generation
Trypase (mMCP6)	Tissue damage, activation of PAR, inflammation, pain
Matrix metalloproteinases	Tissue damage, modification of cytokines/chemokines
Peptides	
Angiogenin	Neovascularization
Corticotropin-releasing hormone	Inflammation, vasodilation
Endorphins	Analgesia
Endothelin	Sepsis
Kinins (bradykinin)	Inflammation, pain, vasodilation
Leptin	Food intake regulation
Renin	Angiotensin synthesis
Somatostatin	Anti-inflammatory (?)
Substance P	Inflammation, pain
Urocortin	Inflammation, vasodilation
VEGF	Neovascularization, vasodilation
Vasoactive intestinal peptide	Vasodilation, mast cell activation
Proteoglycans	
Chondroitin sulfate	Cartilage synthesis, anti-inflammatory
Heparin	Angiogenesis, nerve growth, factor stabilization
Hyaluronic acid	Connective tissue, nerve growth, factor stabilization

Table A1 (continued).

De Novo Synthesized		Physiological Effect
Cytokines		
	IL-1, 2, 3, 4, 5, 6, 8[b], 9, 10, 12[a], 13, 16, 18, 33[a]	Inflammation, leukocyte migration, pain
	IFNα, IFNβ, IGNγ, MIF, TGFβ, TNF-α[b], MIP-1α (CCL3), MIP-1β (CCL4)[a]	Inflammation, leukocyte proliferation/activation
Growth Factors		
	SCF, GM-CSF, β-FGF, Neutrophin-3, NGF, PDGF, TGFβ, VEGF	Growth of variety of cells
Phospholipid Metabolites		
	Leukotriene B_4	Leukocyte chemotaxis
	Leukotriene C_4	Vasoconstriction, pain
	Platelet activating Factor	Platelet activation, vasodilation
	Prostaglandin D_2	Bronchoconstriction, pain
Others		
	Nitric Oxide	Vasodilation

Original table derived from (Theoharides et al., 2012) with minor modifications. [a] mediator not listed in original table, [b]mediators seen prestored and also newly synthesized.

REFERENCES

Al Hawas, R., Ren, Q., Ye, S., Karim, Z. A., Filipovich, A. H., & Whiteheart, S. W. (2012). Munc18b/STXBP2 is required for platelet secretion. *Blood, 120*(12), 2493-2500.

Aloe, L., & Levi-Montalcini, R. (1977). Mast cells increase in tissues of neonatal rats injected with the nerve growth factor. *Brain Research, 133*(2), 358-366.

Amin, K. (2012). The role of mast cells in allergic inflammation. *Respiratory Medicine, 106*(1), 9-14.

Andersson, C. K., Mori, M., Bjermer, L., Löfdahl, C. G., & Erjefält, J. S. (2009). Novel site-specific mast cell subpopulations in the human lung. *Thorax, 64*(4), 297-305.

Aran, V., Brandie, F., Boyd, A., Kantidakis, T., Rideout, E., Kelly, S., . . . Bryant, N. (2009). Characterization of two distinct binding modes between syntaxin 4 and Munc18c. *Biochemical Journal, 419*, 655-660.

Aran, V., Bryant, N. J., & Gould, G. W. (2011). Tyrosine phosphorylation of Munc18c on residue 521 abrogates binding to Syntaxin 4. *BMC Biochemistry, 12*(1), 19.

Azouz, N. P., Zur, N., Efergan, A., Ohbayashi, N., Fukuda, M., Amihai, D., . . . Sagi-Eisenberg, R. (2014). Rab5 is a novel regulator of mast cell secretory granules: impact on size, cargo, and exocytosis. *The Journal of Immunology, 192*(9), 4043-4053.

Baram, D., Adachi, R., Medalia, O., Tuvim, M., Dickey, B. F., Mekori, Y. A., & Sagi-Eisenberg, R. (1999). Synaptotagmin II negatively regulates Ca2+-triggered exocytosis of lysosomes in mast cells. *The Journal of Experimental Medicine, 189*(10), 1649-1658.

Barclay, J. W., Craig, T. J., Fisher, R. J., Ciufo, L. F., Evans, G. J., Morgan, A., & Burgoyne, R. D. (2003). Phosphorylation of Munc18 by protein kinase C regulates the kinetics of exocytosis. *Journal of Biological Chemistry, 278*(12), 10538-10545.

Biedermann, T., Kneilling, M., Mailhammer, R., Maier, K., Sander, C. A., Kollias, G., . . . Röcken, M. (2000). Mast cells control neutrophil recruitment during T cell–mediated delayed-type hypersensitivity reactions through tumor necrosis factor and macrophage inflammatory protein 2. *The Journal of Experimental Medicine, 192*(10), 1441-1452.

Bin, N.-R., Jung, C. H., Piggott, C., & Sugita, S. (2013). Crucial role of the hydrophobic pocket region of Munc18 protein in mast cell degranulation. *Proceedings of the National Academy of Sciences, 110*(12), 4610-4615.

Bradding, P., Walls, A. F., & Holgate, S. T. (2006). The role of the mast cell in the pathophysiology of asthma. *Journal of Allergy and Clinical Immunology, 117*(6), 1277-1284.

Brandie, F. M., Aran, V., Verma, A., McNew, J. A., Bryant, N. J., & Gould, G. W. (2008). Negative regulation of syntaxin4/SNAP-23/VAMP2-mediated membrane fusion by Munc18c in vitro. *Public Library of Science One, 3*(12), e4074.

Brinkmann, V., Reichard, U., Goosmann, C., Fauler, B., Uhlemann, Y., Weiss, D. S., . . . Zychlinsky, A. (2004). Neutrophil extracellular traps kill bacteria. *Science, 303*(5663), 1532-1535.

Burgo, A., Casano, A. M., Kuster, A., Arold, S. T., Wang, G., Nola, S., . . . Galli, T. (2013). Increased activity of the Vesicular Soluble N-Ethylmaleimide-sensitive

Factor Attachment Protein Receptor TI-VAMP/VAMP7 by Tyrosine Phosphorylation in the Longin Domain. *Journal of Biological Chemistry, 288*(17), 11960-11972.

Church, M. K., & Levi-Schaffer, F. (1997). The human mast cell. *Journal of Allergy and Clinical Immunology*, *99*(2), 155-160.

Cochrane, D. E., Carraway, R. E., Miller, L. A., Feldberg, R. S., & Bernheim, H. (2003). Histamine releasing peptide (HRP) has proinflammatory effects and is present at sites of inflammation. *Biochemical Pharmacology, 66*(2), 331-342.

Cui, Y., Dahlin, J. S., Feinstein, R., Bankova, L. G., Xing, W., Shin, K., . . . Hallgren, J. (2014). Mouse mast cell protease-6 and MHC are involved in the development of experimental asthma. *The Journal of Immunology, 193*(10), 4783-4789.

Da Silva, E. Z. M., Jamur, M. C., & Oliver, C. (2014). Mast Cell Function A New Vision of an Old Cell. *Journal of Histochemistry & Cytochemistry, 62*(10), 698-738.

de Vries, V., Elgueta, R., Lee, D., & Noelle, R. (2010). *Mast cell protease 6 is required for allograft tolerance.* Paper presented at the Transplantation proceedings.

Ehrlich, P. (1878). *Beiträge für Theorie und Praxis der histologischen Färbung* (Unpublished doctoral dissertation). Leipzig University, Germany.

Fasshauer, D., Sutton, R. B., Brunger, A. T., & Jahn, R. (1998). Conserved structural features of the synaptic fusion complex: SNARE proteins reclassified as Q-and R-SNAREs. *Proceedings of the National Academy of Sciences, 95*(26), 15781-15786.

Fletcher, A. I., Shuang, R., Giovannucci, D. R., Zhang, L., Bittner, M. A., & Stuenkel, E. L. (1999). Regulation of exocytosis by cyclin-dependent kinase 5 via phosphorylation of Munc18. *Journal of Biological Chemistry, 274*(7), 4027-4035.

Frank, S. P., Thon, K. P., Bischoff, S. C., & Lorentz, A. (2011). SNAP-23 and syntaxin-3 are required for chemokine release by mature human mast cells. *Molecular Immunology, 49*(1), 353-358.

Friend, D. S., Ghildyal, N., Austen, K. F., Gurish, M. F., Matsumoto, R., & Stevens, R. L. (1996). Mast cells that reside at different locations in the jejunum of mice infected with Trichinella spiralis exhibit sequential changes in their granule ultrastructure and chymase phenotype. *The Journal of Cell Biology, 135*(1), 279-290.

Friend, D. S., Ghildyal, N., Gurish, M. F., Hunt, J., Hu, X., Austen, K. F., & Stevens, R. L. (1998). Reversible expression of tryptases and chymases in the jejunal mast cells of mice infected with Trichinella spiralis. *The Journal of Immunology, 160*(11), 5537-5545.

Fujita, Y., Sasaki, T., Fukui, K., Kotani, H., Kimura, T., Hata, Y., . . . Takai, Y. (1996). Phosphorylation of Munc-18/n-Sec1/rbSec1 by protein kinase C Its implication in regulating the interaction of Munc-18/n-Sec1/rbSec1 with syntaxin. *Journal of Biological Chemistry, 271*(13), 7265-7268.

Fukasawa, M., Varlamov, O., Eng, W. S., Söllner, T. H., & Rothman, J. E. (2004). Localization and activity of the SNARE Ykt6 determined by its regulatory domain and palmitoylation. *Proceedings of the National Academy of Sciences of the United States of America, 101*(14), 4815-4820.

Fukuishi, N., Murakami, S., Ohno, A., Yamanaka, N., Matsui, N., Fukutsuji, K., . . . Akagi, M. (2014). Does β-hexosaminidase function only as a degranulation indicator in mast cells? The primary role of β-hexosaminidase in mast cell granules. *The Journal of Immunology, 193*(4), 1886-1894.

Galli, S. J., Nakae, S., & Tsai, M. (2005). Mast cells in the development of adaptive immune responses. *Nature Immunology, 6*(2), 135-142.

Galli, S. J., Tsai, M., & Wershil, B. (1993). The c-kit receptor, stem cell factor, and mast cells. What each is teaching us about the others. *The American Journal of Pathology, 142*(4), 965.

Genç, Ö., Kochubey, O., Toonen, R. F., Verhage, M., & Schneggenburger, R. (2014). Munc18-1 is a dynamically regulated PKC target during short-term enhancement of transmitter release. *Elife, 3*, e01715.

Gibbs, B. F., Wierecky, J., Welker, P., Henz, B., Wolff, H., & Grabbe, J. (2001). Human skin mast cells rapidly release preformed and newly generated TNF-α and IL-8 following stimulation with anti-IgE and other secretagogues. *Experimental Dermatology, 10*(5), 312-320.

Grimberg, E., Peng, Z., Hammel, I., & Sagi-Eisenberg, R. (2003). Synaptotagmin III is a critical factor for the formation of the perinuclear endocytic recycling compartment and determination of secretory granules size. *Journal of Cell Science, 116*(1), 145-154.

Guo, Z., Turner, C., & Castle, D. (1998). Relocation of the t-SNARE SNAP-23 from lamellipodia-like cell surface projections regulates compound exocytosis in mast cells. *Cell, 94*(4), 537-548.

Gupta, R., Holdford, D., Bilaver, L., Dyer, A., Holl, J. L., & Meltzer, D. (2013). The economic impact of childhood food allergy in the United States. *JAMA Pediatrics, 167*(11), 1026-1031.

Hallgren, J., & Gurish, M. F. (2007). Pathways of murine mast cell development and trafficking: tracking the roots and routes of the mast cell. *Immunological Reviews, 217*(1), 8-18.

Han, L., Jiang, T., Han, G. A., Malintan, N. T., Xie, L., Wang, L., . . . Meunier, F. A. (2009). Rescue of Munc18-1 and-2 double knockdown reveals the essential functions of interaction between Munc18 and closed syntaxin in PC12 cells. *Molecular Biology of the Cell, 20*(23), 4962-4975.

Heavey, D. J., Ernst, P., Stevens, R., Befus, A., Bienenstock, J., & Austen, K. (1988). Generation of leukotriene C4, leukotriene B4, and prostaglandin D2 by immunologically activated rat intestinal mucosa mast cells. *The Journal of Immunology, 140*(6), 1953-1957.

Hibi, T., Hirashima, N., & Nakanishi, M. (2000). Rat basophilic leukemia cells express syntaxin-3 and VAMP-7 in granule membranes. *Biochemical and Biophysical Research Communications, 271*(1), 36-41.

Hickey, C. M., & Wickner, W. (2010). HOPS initiates vacuole docking by tethering membranes before trans-SNARE complex assembly. *Molecular Biology of the Cell, 21*(13), 2297-2305.

Hofmann, K., & Stoffel, W. (1993). TMbase—a database of membrane spanning protein segments. *Biological Chemistry Hoppe-Seyler, 374*, 166.

Hong, W., & Lev, S. (2014). Tethering the assembly of SNARE complexes. *Trends in Cell Biology, 24*(1), 35-43.

Hsu, C.-L., Neilsen, C. V., & Bryce, P. J. (2010). IL-33 is produced by mast cells and regulates IgE-dependent inflammation. *Public Library of Science One, 5*(8), e11944.

Hu, S.H., Christie, M. P., Saez, N. J., Latham, C. F., Jarrott, R., Lua, L. H., . . . Martin, J. L. (2011). Possible roles for Munc18-1 domain 3a and Syntaxin1 N-peptide and C-terminal anchor in SNARE complex formation. *Proceedings of the National Academy of Sciences, 108*(3), 1040-1045.

Ikeda, R. K., Miller, M., Nayar, J., Walker, L., Cho, J. Y., McElwain, K., . . . Broide, D. H. (2003). Accumulation of peribronchial mast cells in a mouse model of ovalbumin allergen induced chronic airway inflammation: modulation by immunostimulatory DNA sequences. *The Journal of Immunology, 171*(9), 4860-4867.

Izawa, R., Onoue, T., Furukawa, N., & Mima, J. (2012). Distinct contributions of vacuolar Qabc-and R-SNARE proteins to membrane fusion specificity. *Journal of Biological Chemistry, 287*(5), 3445-3453.

Jackson, K. D., Howie, L., & Akinbami, L. J. (2013). Trends in allergic conditions among children: United States, 1997-2011. *NCHS data brief, 121*, 1-8.

Jewell, J. L., Oh, E., Ramalingam, L., Kalwat, M. A., Tagliabracci, V. S., Tackett, L., . . . Thurmond, D. C. (2011). Munc18c phosphorylation by the insulin receptor links cell signaling directly to SNARE exocytosis. *The Journal of Cell Biology, 193*(1), 185-199.

Jutel, M., Akdis, M., & Akdis, C. (2009). Histamine, histamine receptors and their role in immune pathology. *Clinical & Experimental Allergy*, *39*(12), 1786-1800.

Kalesnikoff, J., Huber, M., Lam, V., Damen, J. E., Zhang, J., Siraganian, R. P., & Krystal, G. (2001). Monomeric IgE stimulates signaling pathways in mast cells that lead to cytokine production and cell survival. *Immunity*, *14*(6), 801-811.

Kanda, H., Tamori, Y., Shinoda, H., Yoshikawa, M., Sakaue, M., Udagawa, J., . . . Kasuga, M. (2005). Adipocytes from Munc18c-null mice show increased sensitivity to insulin-stimulated GLUT4 externalization. *Journal of Clinical Investigation, 115*(2), 291-301.

Kioumourtzoglou, D., Gould, G. W., & Bryant, N. J. (2014). Insulin stimulates syntaxin4 SNARE complex assembly via a novel regulatory mechanism. *Molecular and Cellular Biology*, *34*(7), 1271-1279.

Kirshenbaum, A., Goff, J., Kessler, S. W., Mican, J., Zsebo, K., & Metcalfe, D. (1992). Effect of IL-3 and stem cell factor on the appearance of human basophils and mast cells from CD34+ pluripotent progenitor cells. *The Journal of Immunology, 148*(3), 772-777.

Kirshenbaum, A. S., Goff, J. P., Semere, T., Foster, B., Scott, L. M., & Metcalfe, D. D. (1999). Demonstration that human mast cells arise from a progenitor cell population that is CD34+, c-kit+, and expresses aminopeptidase N (CD13). *Blood*, *94*(7), 2333-2342.

Kirshenbaum, A. S., Kessler, S. W., Goff, J. P., & Metcalfe, D. D. (1991). Demonstration of the origin of human mast cells from CD34+ bone marrow progenitor cells. *The Journal of Immunology*, *146*(5), 1410-1415.

Kitamura, Y. (1989). Heterogeneity of mast cells and phenotypic change between subpopulations. *Annual Review of Immunology, 7*(1), 59-76.

Leurs, R., Smit, M., & Timmerman, H. (1995). Molecular pharmacological aspects of histamine receptors. *Pharmacology & Therapeutics, 66*(3), 413-463.

Liu, C., Liu, Z., Li, Z., & Wu, Y. (2010). Molecular regulation of mast cell development and maturation. *Molecular Biology Reports, 37*(4), 1993-2001.

Liu, Y., Ding, X., Wang, D., Deng, H., Feng, M., Wang, M., . . . Aikhionbare, F. (2007). A mechanism of Munc18b–syntaxin 3–SANP25 complex assembly in regulated epithelial secretion. *FEBS Letters, 581*(22), 4318-4324.

Lorentz, A., Baumann, A., Vitte, J., & Blank, U. (2012). The SNARE machinery in mast cell secretion. *Frontiers in Immunology, 3*(143).

Lorentz, A., Hoppe, J., Worthmann, H., Gebhardt, T., Hesse, U., Bienenstock, J., & Bischoff, S. (2007). Neurotrophin-3, but not nerve growth factor, promotes survival of human intestinal mast cells. *Neurogastroenterology & Motility, 19*(4), 301-308.

Ma, C., Li, W., Xu, Y., & Rizo, J. (2011). Munc13 mediates the transition from the closed syntaxin–Munc18 complex to the SNARE complex. *Nature Structural & Molecular Biology, 18*(5), 542-549.

Malaviya, R., Ikeda, T., Ross, E., & Abraham, S. N. (1996). Mast cell modulation of neutrophil influx and bacterial clearance at sites of infection through TNF-α. *Nature*, 381(6577), 77-80.

Martin-Verdeaux, S., Pombo, I., Iannascoli, B., Roa, M., Varin-Blank, N., Rivera, J., & Blank, U. (2003). Evidence of a role for Munc18-2 and microtubules in mast cell granule exocytosis. *Journal of Cell Science, 116*(2), 325-334.

McNew, J. A. (2008). Regulation of SNARE-mediated membrane fusion during exocytosis. *Chemical Reviews, 108*(5), 1669-1686.

Metcalfe, D. D. (2008). Mast cells and mastocytosis. *Blood, 112*(4), 946-956.

Metz, M., Botchkarev, V. A., Botchkareva, N. V., Welker, P., Tobin, D. J., Knop, J., . . . Paus, R. (2004). Neurotrophin-3 regulates mast cell functions in neonatal mouse skin. *Experimental Dermatology, 13*(5), 273-281.

Moon, T. C., Befus, A. D., & Kulka, M. (2015). Mast cell mediators: their differential release and the secretory pathways involved. *Secretion of Cytokines and Chemokines by Innate Immune Cells*, 45.

Munson, M., & Bryant, N. J. (2009). A role for the syntaxin N-terminus. *Biochemical Journal, 418*(1), e1-e3.

Nagasaka, A., Matsue, H., Matsushima, H., Aoki, R., Nakamura, Y., Kambe, N., . . . Shimada, S. (2008). Osteopontin is produced by mast cells and affects IgE-mediated degranulation and migration of mast cells. *European Journal of Immunology, 38*(2), 489-499.

Nigam, R., Sepulveda, J., Tuvim, M., Petrova, Y., Adachi, R., Dickey, B. F., & Agrawal, A. (2005). Expression and transcriptional regulation of Munc18 isoforms in Mast Cells. *Biochimica et Biophysica Acta (BBA)-Gene Structure and Expression, 1728*(1), 77-83.

Office of Surveillance, Epidemiology and Laboratory Services (2011, May 3, 2011). Asthma in the US. Retrieved from http://www.cdc.gov/vitalsigns/asthma/

Okayama, Y., & Kawakami, T. (2006). Development, migration, and survival of mast cells. *Immunologic Research, 34*(2), 97-115.

Owen, J. A., Punt, J., Stranford, S. A., & Jones, P. P. (2013). *Kuby immunology*. New York, NY: WH Freeman.

Palker, T. J., Dong, G., & Leitner, W. W. (2010). Mast cells in innate and adaptive immunity to infection. *European Journal of Immunology, 40*(1), 13-18.

Paumet, F., Le Mao, J., Martin, S., Galli, T., David, B., Blank, U., & Roa, M. (2000). Soluble NSF Attachment Protein Receptors (SNAREs) in RBL-2H3 Mast Cells: Functional Role of Syntaxin 4 in Exocytosis and Identification of a Vesicle-Associated Membrane Protein 8-Containing Secretory Compartment. *The Journal of Immunology, 164*(11), 5850-5857.

Peng, R., Guetg, C., Abellan, E., & Fussenegger, M. (2010). Munc18b regulates core SNARE complex assembly and constitutive exocytosis by interacting with the N-peptide and the closed-conformation C-terminus of syntaxin 3. *Biochemical Journal, 431*, 353-361.

Poirier, M. A., Xiao, W., Macosko, J. C., Chan, C., Shin, Y.-K., & Bennett, M. K. (1998). The synaptic SNARE complex is a parallel four-stranded helical bundle. *Nature Structural & Molecular Biology, 5*(9), 765-769.

Prasad, P., Yanagihara, A. A., Small-Howard, A. L., Turner, H., & Stokes, A. J. (2008). Secretogranin III directs secretory vesicle biogenesis in mast cells in a manner

dependent upon interaction with chromogranin A. *The Journal of Immunology, 181*(7), 5024-5034.

Puri, N., Kruhlak, M. J., Whiteheart, S. W., & Roche, P. A. (2003). Mast cell degranulation requires N-ethylmaleimide-sensitive factor-mediated SNARE disassembly. *The Journal of Immunology, 171*(10), 5345-5352.

Puri, N., & Roche, P. A. (2008). Mast cells possess distinct secretory granule subsets whose exocytosis is regulated by different SNARE isoforms. *PNAS, 105*(7), 2580-2585.

Raposo, G., Tenza, D., Mecheri, S., Peronet, R., Bonnerot, C., & Desaymard, C. (1997). Accumulation of major histocompatibility complex class II molecules in mast cell secretory granules and their release upon degranulation. *Molecular Biology of the Cell, 8*(12), 2631-2645.

Rauter, I., Krauth, M.-T., Westritschnig, K., Horak, F., Flicker, S., Gieras, A., . . . Huss-Marp, J. (2008). Mast cell–derived proteases control allergic inflammation through cleavage of IgE. *Journal of Allergy and Clinical Immunology, 121*(1), 197-202.

Rivera, J., & Gilfillan, A. M. (2006). Molecular regulation of mast cell activation. *Journal of Allergy and Clinical Immunology, 117*(6), 1214-1225.

Ruitenberg, E., & Elgersma, A. (1976). Absence of intestinal mast cell response in congenitally athymic mice during Trichinella spiralis infection. *Nature, 264*, 258-260.

Sander, L. E., Frank, S. P. C., Bloat, S., Blank, U., Galli, T., Bigalke, H., . . . Lorentz, A. (2008). Vesicle associated membrane protein (VAMP)-7 and VAMP-8, but not

VAMP-2 or VAMP-3, are required for activation-induced degranulation of mature human mast cells. *European Journal of Immunology, 38*, 855-863.

Sandig, H., & Bulfone-Paus, S. (2012). TLR signaling in mast cells: common and unique features. *Frontiers in Immunology, 3*.

Schechter, N. M., Brass, L. F., Lavker, R. M., & Jensen, P. J. (1998). Reaction of mast cell proteases tryptase and chymase with protease activated receptors (PARs) on keratinocytes and fibroblasts. *Journal of Cellular Physiology, 176*(2), 365-373.

Sheffield, P., Garrard, S., & Derewenda, Z. (1999). Overcoming expression and purification problems of RhoGDI using a family of "parallel" expression vectors. *Protein Expression and Purification, 15*(1), 34-39.

Shen, J., Rathore, S. S., Khandan, L., & Rothman, J. E. (2010). SNARE bundle and syntaxin N-peptide constitute a minimal complement for Munc18-1 activation of membrane fusion. *The Journal of Cell Biology, 190*(1), 55-63.

Shen, J., Tareste, D. C., Paumet, F., Rothman, J. E., & Melia, T. J. (2007). Selective activation of cognate SNAREpins by Sec1/Munc18 proteins. *Cell, 128*(1), 183-195.

Silphaduang, U., & Noga, E. J. (2001). Antimicrobials: peptide antibiotics in mast cells of fish. *Nature, 414*(6861), 268-269.

Simons, K., & Toomre, D. (2000). Lipid rafts and signal transduction. *Nature Reviews Molecular Cell Biology, 1*(1), 31-39.

Spessott, W., Sanmillan, M., McCormick, M., Patel, N., Villanueva, J., Zhang, K., . . . Giraudo, C. (2015). Hemophagocytic lymphohistiocytosis caused by dominant

negative mutations in STXBP2 that inhibit SNARE-mediated membrane fusion. *Blood*, *125*(10), 1566-1577.

Sutton, R. B., Fasshauer, D., Jahn, R., & Brunger, A. T. (1998). Crystal structure of a SNARE complex involved in synaptic exocytosis at 2.4 Å resolution. *Nature, 395*(6700), 347-353.

Tadokoro, S., Kurimoto, T., Nakanishi, M., & Hirashima, N. (2007). Munc18-2 regulates exocytotic membrane fusion positively interacting with syntaxin-3 in RBL-2H3 cells. *Molecular Immunology*, *44*(13), 3427-3433.

Theoharides, T. C., Alysandratos, K. D., Angelidou, A., Delivanis, D. A., Sismanopoulos, N., Zhang, B., . . . Miniati, A. (2012). Mast cells and inflammation. *Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease, 1822*(1), 21-33.

Thomas, M. P., Whangbo, J., McCrossan, G., Deutsch, A. J., Martinod, K., Walch, M., & Lieberman, J. (2014). Leukocyte protease binding to nucleic acids promotes nuclear localization and cleavage of nucleic acid binding proteins. *The Journal of Immunology*, *192*(11), 5390-5397.

Tiwari, N., Wang, C. C., Brochetta, C., Ke, G., Vita, F., Qi, Z., . . . Blank, a. U. (2008). VAMP-8 segregates mast cell-preformed mediator exocytosis from cytokine trafficking pathways. *Blood*, *111*(7), 3665-3674.

Tiwari, N., Wang, C., Brochetta, C., Scandiuzzi, L., Hong, W., & Blank, U. (2009). Increased formation of VAMP-3-containing SNARE complexes in mast cells from VAMP-8 deficient cells. *Inflammation Research, 58*, 13-14.

Valent, P., Spanblochl, E., Sperr, W. R., Sillaber, C., Zsebo, K. M., Agis, H., . . . Lechner, K. (1992). Induction of differentiation of human mast cells from bone marrow and peripheral blood mononuclear cells by recombinant human stem cell factor/kit-ligand in long-term culture. *Blood, 80*(9), 2237-2245.

Verhage, M., Maia, A. S., Plomp, J. J., Brussaard, A. B., Heeroma, J. H., Vermeer, H., . . . Geuze, H. J. (2000). Synaptic assembly of the brain in the absence of neurotransmitter secretion. *Science, 287*(5454), 864-869.

von Köckritz-Blickwede, M., Goldmann, O., Thulin, P., Heinemann, K., Norrby-Teglund, A., Rohde, M., & Medina, E. (2008). Phagocytosis-independent antimicrobial activity of mast cells by means of extracellular trap formation. *Blood, 111*(6), 3070-3080.

Waern, I., Jonasson, S., Hjoberg, J., Bucht, A., Åbrink, M., Pejler, G., & Wernersson, S. (2009). Mouse mast cell protease 4 is the major chymase in murine airways and has a protective role in allergic airway inflammation. *The Journal of Immunology, 183*(10), 6369-6376.

Waern, I., Lundequist, A., Pejler, G., & Wernersson, S. (2013). Mast cell chymase modulates IL-33 levels and controls allergic sensitization in dust-mite induced airway inflammation. *Mucosal Immunology, 6*(5), 911-920.

Welle, M. (1997). Development, significance, and heterogeneity of mast cells with particular regard to the mast cell-specific proteases chymase and tryptase. *Journal of Leukocyte Biology, 61*(3), 233-245.

Wernersson, S., & Pejler, G. (2014). Mast cell secretory granules: armed for battle. *Nature Reviews Immunology, 14*(7), 478-494.

Xing, W., Austen, K. F., Gurish, M. F., & Jones, T. G. (2011). Protease phenotype of constitutive connective tissue and of induced mucosal mast cells in mice is regulated by the tissue. *Proceedings of the National Academy of Sciences, 108*(34), 14210-14215.

Xu, H., Arnold, M. G., & Kumar, S. V. (2015). Differential Effects of Munc18s on Multiple Degranulation-Relevant Trans-SNARE Complexes. *Public Library of Science One, 10*(9), e0138683.

Xu, H., Jun, Y., Thompson, J., Yates, J., & Wickner, W. (2010). HOPS prevents the disassembly of the Trans-SNARE complexes by Sec17p/Sec18p during membrane fusion. *European Molecular Biology Organization Journal, 29,* 1948-1960.

Yu, H., Rathore, S. S., Lopez, J. A., Davis, E. M., James, D. E., Martin, J. L., & Shen, J. (2013). Comparative studies of Munc18c and Munc18-1 reveal conserved and divergent mechanisms of Sec1/Munc18 proteins. *Proceedings of the National Academy of Sciences*, 3271–3280.

Zhang, Y., Diao, J., Colbert, K. N., Lai, Y., Pfuetzner, R. A., Padolina, M. S., . . . Choi, U. B. (2015). Munc18a does not alter fusion rates mediated by neuronal SNAREs, synaptotagmin, and complexin. *Journal of Biological Chemistry, 290*(16), 10518-10534.