Unraveling the Molecular Mechanisms of Munc18 and Munc13 in Mast Cell Exocytosis

by

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Department of Physiology
University of Toronto

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Doctor of Philosophy

Department of Physiology
University of Toronto

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Abstract

Exocytosis is a fundamental cellular process in which contents stored in vesicles are released out of cells via fusion of membranes. Exocytosis of secretory granules from immune cells such as cytotoxic T-lymphocytes, natural killer cells and mast cells play crucial physiological roles in protecting individuals from pathogens. The exocytosis is known to be mediated by Soluble N-ethylmaleimide sensitive factor Attachment protein REceptor (SNARE) complex where intertwined helix bundles provide energy to fuse lipid membranes. Furthermore, this process is regulated by indispensable proteins such as Munc18 and Munc13 through physically interacting with SNARE proteins. Disruption of proteins that are involved in the secretory granule exocytosis causes serious immune disorders including familial hemophagocytic lymphohistiocytosis. However, compared to vastly investigated mechanisms of neuronal exocytosis, natures of proteins and their precise molecular mechanisms involved in immune cell exocytosis remain elusive. It has been revealed that the immune cells employ Ca^{2+}-triggered SNARE-mediated exocytosis, but how such processes are being regulated in the immune cells needs to be investigated thoroughly. Using Rat Basophilic Leukemia-2H3 (RBL-2H3) mast cells, the structure and interactive properties of Munc18 and Munc13 in respect to mast cell exocytosis
were examined. Through knockdown and rescue approaches, results indicate that Munc18 protein is crucial for exocytosis of mast cells partly through regulating its cognate syntaxin partners such as syntaxin-3 and -11 for their protein level and trafficking. Independent knockdowns of syntaxin-3 and -11 demonstrate that syntaxin-3 is the key cognate syntaxin whose level and intracellular localization are regulated by Munc18. In addition, our data demonstrate that Munc13-4 plays an essential role in mast cell degranulation; mutational studies revealed that point mutations in C2 domains of Munc13-4 dramatically alter Ca\(^{2+}\)-sensitivity of degranulation, whereas absence of Munc13-4 or multiple mutations in C2 domains result in almost complete loss of exocytosis from mast cells. Moreover, Munc13-4 mediates Ca\(^{2+}\)-dependent regulation of fusion pore opening of single granule fusion events. Taken together, we postulate that both Munc18 and Munc13-4 are essential for mast cell exocytosis albeit their functions, therefore molecular mechanisms, are exerted in different stages of exocytosis.
Acknowledgments

First and foremost, I would like to express my sincere appreciation to my supervisor, Dr. Shuzo Sugita, who has guided me throughout my graduate studies. For the last 6 years, I have been blessed to work with such a great teacher, a mentor and a person. He has demonstrated encourage and persistence as well as curiosity when approaching experiments. It was a truly fun and enjoyable time that I had spent with Dr. Sugita which I will never forget.

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<th>Description</th>
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<tbody>
<tr>
<td>α-SNAP</td>
<td>α-soluble NSF-attachment proteins</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>(H³)NE</td>
<td>Tritium Labeled Norepinephrine</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CAPS1</td>
<td>Calcium-dependent activator protein for secretion 1</td>
</tr>
<tr>
<td>CMV</td>
<td>Cytomegalovirus</td>
</tr>
<tr>
<td>CTL</td>
<td>Cytotoxic T-lymphocyte</td>
</tr>
<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DCV</td>
<td>Dense core vesicle</td>
</tr>
<tr>
<td>DKD</td>
<td>Double knockdown</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle medium</td>
</tr>
<tr>
<td>dsRNA</td>
<td>double stranded Ribonucleic acid</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>EIEE</td>
<td>Early infantile epileptic encephalopathy</td>
</tr>
<tr>
<td>EmGFP</td>
<td>Emerald green fluorescent protein</td>
</tr>
<tr>
<td>EPSC</td>
<td>Excitatory postsynaptic current</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FHL</td>
<td>Familial Hemophagocytic Lymphohistiocytosis</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------------------------------</td>
</tr>
<tr>
<td>GABA</td>
<td>γ-aminobutyric acid</td>
</tr>
<tr>
<td>GLUT4</td>
<td>Glucose transporter 4</td>
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<tr>
<td>GST</td>
<td>Glutathione S-transferase</td>
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<tr>
<td>HA</td>
<td>Hemagglutinin</td>
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<td>Human embryonic kidney-293 FT</td>
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<tr>
<td>IP₃</td>
<td>Inositol 1,4,5-trisphosphate</td>
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<tr>
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<td>Isothermal Titration Calorimetry</td>
</tr>
<tr>
<td>IRES</td>
<td>Internal ribosome entry site</td>
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<td>KCl</td>
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<td>KD</td>
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<td>Potassium glutamate</td>
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<tr>
<td>KO</td>
<td>Knockout</td>
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<tr>
<td>MC</td>
<td>Mast cell</td>
</tr>
<tr>
<td>MHD</td>
<td>Munc13 homology domain</td>
</tr>
<tr>
<td>NE</td>
<td>Norepinephrine</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer cell</td>
</tr>
<tr>
<td>NMJ</td>
<td>Neuromuscular junction</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
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<tr>
<td>NPY</td>
<td>Neuropeptide Y</td>
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xvi
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>NSF</td>
<td>N-ethylmaleimide sensitive factor</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PC12</td>
<td>Pheochromocytoma 12</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>PI(4)P</td>
<td>Phosphatidylinositol 4-phosphate</td>
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<td>PI(4)P5-</td>
<td>Phosphatidylinositol 4-phosphate 5-</td>
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<tr>
<td>PIP2</td>
<td>Phosphatidylinositol-4,5-biphosphate</td>
</tr>
<tr>
<td>PITP</td>
<td>Phosphatidylinositol transfer protein</td>
</tr>
<tr>
<td>PLC</td>
<td>Phospholipase C</td>
</tr>
<tr>
<td>PMA</td>
<td>Phorbol-12-myristate-13-acetate</td>
</tr>
<tr>
<td>PSS</td>
<td>Physiological Saline Solution</td>
</tr>
<tr>
<td>RBL-2H3</td>
<td>Rat basophilic leukemia-2H3</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SG</td>
<td>Secretory granule</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SERCA</td>
<td>Sarco/endoplasmic reticulum Ca(^{2+})-ATPase</td>
</tr>
<tr>
<td>shRNA</td>
<td>Short hairpin ribonucleic acid</td>
</tr>
<tr>
<td>SM</td>
<td>Sec1/Munc18</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>-------------</td>
<td>------------</td>
</tr>
<tr>
<td>SNAP</td>
<td>Synaptosomal-associated protein</td>
</tr>
<tr>
<td>SNARE</td>
<td>Soluble NSF Attachment protein receptor</td>
</tr>
<tr>
<td>SNM</td>
<td>Silent nucleotide mutation</td>
</tr>
<tr>
<td>Syt</td>
<td>Synaptotagmin</td>
</tr>
<tr>
<td>TIRFM</td>
<td>Total internal reflection fluorescence microscopy</td>
</tr>
<tr>
<td>UBC</td>
<td>Ubiquitin Ligase C promoter</td>
</tr>
<tr>
<td>VAMP</td>
<td>Vesicle associated membrane protein</td>
</tr>
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Chapter 1: Introduction

1.1 Exocytosis

Exocytosis is a fundamental cellular process that plays a critical role in communications between cells and organs. Exocytosis refers to a process where a vesicle fuses with the plasma membrane which then releases vesicular contents to exert various functions. The functions of exocytosis include but not least, the secretion of neurotransmitters from neurons mediating synaptic transmission, neuronal secretion of peptides and hormones that regulate various mental states, secretion of insulin from pancreatic cells to regulate blood glucose level and blood cell exocytosis for mediating immune responses. The fusion of secretory vesicles with the plasma membrane can occur in virtually all cells in the form of constitutive exocytosis (Burgess and Kelly, 1987). Constitutive exocytosis takes place without an external signal and is required for the insertion of new cell membrane as well as plasma membrane proteins and extracellular matrix components. However, in many cell types, the exocytosis is tightly regulated such that the release can take place only upon a physiological signal such as Ca$^{2+}$. This type of exocytosis allows the controlled release of vesicle contents or regulated insertion of new membrane components only when it is needed. It is ideal for cells where the release needs to be rapid and precise such as neurons and neuroendocrine cells as well as immune cells where their contents need to be secreted in large quantities to mediate efficient functions. Therefore, through the release of vesicle contents, exocytosis ensures the maintenance of physiological functions of an organism.
1.2 Physiology of mast cell exocytosis

Mast cells are resident cells in connective and mucosal tissues playing crucial roles in innate immunity (Wedemeyer et al., 2000). They have large and dense granules containing key immune mediators such as histamine, heparin and chemokines/cytokines as well as proteases such as trypase and chymase. Mast cells release their contents in response to IgE antibody, which binds to FcεRI receptors located on the surface of the cell. Crossing linking of IgE antibody by its antigen causes an intracellular signaling cascade which ultimately results in an increase of the cytoplasmic Ca$^{2+}$ level. Subsequently, the exocytotic process is triggered and the mast cell secretes immune mediators from secretory/lysosomal granules (Stassen et al., 2002). Upon release, these substances cause reddening, vasoconstriction, matrix destruction and tissue remodeling which are hallmarks of inflammatory responses. Importantly, released chemokines/cytokines can recruit other immune players such as other leukocytes thereby allowing long-term protection of the host against particular pathogens. Therefore, the mast cell is an important player which bridges between innate and adaptive immunities (Wedemeyer et al., 2000). Interestingly, mast cells have been implicated with development and progression of atherosclerosis and hypertension (Nagata et al., 2000; Nakano et al., 1997). It was reported that in the site of atherosclerotic lesions, there was an increased number of mast cell populations found. Chymase, a serine protease, which is released from mast cells not only degrades extracellular matrix, but it can generate active angiotensin II, TGF-β1, IL-1β, and endothelin which are all essential players in atherogenesis. Especially, angiotensin II is a very potent substance to increase blood pressure therefore causing hypertension (Ju et al., 2001). Therefore, understanding the precise mechanisms of mast cell exocytosis will not only provide fundamental knowledge about exocytosis in general, but potential therapeutic interventions of immunological
and cardiovascular diseases. More information about the nature of mast cell degranulation and molecular players involved in this process to be covered in section 1.7.
1.3 **Stages of secretory granule exocytosis**

In order for secretory cells to release their contents by exocytosis, the materials must be encapsulated in some organelle-like structures. In general, secretory granules are used for this purpose. The highly condensed granule matrix, which gives rise to dense core visualized by electron microscopy (EM), allows efficient storage of large amounts of secretory material. In neurons and endocrine cells, the granules are a product of biosynthesis that is initiated at the trans-Golgi network (TGN) (Arvan and Castle, 1987). It is thought that membrane deformation for buddings may result from the aggregation of secretory proteins in the TGN. Nascent granules bud from TGN to form immature secretory granules and subsequent maturation of granules occurs. In neurons and neuroendocrine cells, this involves both fusion with other immature secretory granules and removal of mis-sorted material via budding off from the nascent granules (Tooze and Huttner, 1990).

Biogenesis of granules in hematopoietic cells is also initiated at the TGN, as nascent progranules bud off from TGN. These progranules are small and uniform in size which they undergo successive rounds of fusion with other granules to become immature granules (Combs, 1966; Hammel et al., 1985). Interestingly, in contrast to the biosynthetic pathway of granules in neurons and neuroendocrine cells, granules in various hematopoietic cells share several properties with lysosomes (Blott and Griffiths, 2002). For example, in addition to possessing lysosomal hydrolases, such secretory granules are accessible via endocytic pathways and dense-core formation can occur in multi-vesicular bodies (Reed et al., 2000). This suggests that a convergence of biosynthetic and endocytic membrane traffic analogous to lysosomal biogenesis is required for secretory granule formation in these blood-residing cells. Although the mature granules in many hematopoietic cells seem to be highly homogenous and appear to be
morphologically similar, there is an evidence which suggests that granules are heterogeneous in their composition and morphology. For example, the mast cells have certain granules containing serotonin and cathepsin D, whereas others contain histamine and TNF (Puri and Roche, 2008). Moreover, this various composition of the granules gives arise to heterogeneity in ultrastructural morphologies of granules in the mast cells. Therefore, although the mast cells share a similar organelle structure like neurons and neuroendocrine cells in which they utilize for exocytosis, it is important to appreciate that granules in the mast cells possess unique aspects that may employ distinct mechanisms.

Exocytosis involves a series of steps that include docking of secretory vesicles at specialized release sites; in neurons, it is known as active zones (Sugita, 2008). At active zones, priming steps occur which render the vesicles ready for Ca^{2+} triggered fusion. Since extensive studies have been conducted focusing on neuronal exocytosis, most of the information presented in this section will be based on synaptic vesicles and/or dense-core vehicles (DCV) of neurons and neuroendocrine cells (Figure 1.1). In addition, functions and mechanisms of proteins that are involved in each step of exocytosis will be briefly mentioned. More detailed information on the players will be presented in later sections of the Introduction.
Figure 1.1 Stages of Ca\textsuperscript{2+}-dependent dense-core vesicle exocytosis. Stages of regulated dense-core vesicle (DCV) cycle leading to DCV secretion. The proteins implicated in each stage of the DCV cycle are listed below of each stage. Adapted and modified from Sugita, 2008.
1.3.1 Docking

Once mature vesicles are formed, they proceed to become docked with the plasma membrane. Docking has been defined in two ways: Morphological docking refers when vesicles appear to be within a close proximity from the plasma membrane by EM analysis. Biochemical docking refers to a physical connection between the vesicle and plasma membranes via a protein or protein complex (Lin and Scheller, 2000).

In mouse chromaffin cells, cleavage of syntaxins by botulinum toxin C1 or null mutation of Munc18-1 produces a robust reduction of docked vesicles, without affecting the total number of vesicles (de Wit et al., 2006; Toonen et al., 2006; Voets et al., 2001). In pancreatic β cells of syntaxin-1A null mutant mice, fewer insulin granules were found docked to the plasma membrane (Ohara-Imaizumi et al., 2007). Deficient in Munc18-1 protein level reduces docking in neuroendocrine cells (Voets et al., 2001). Unc-18 null C. elegans neuromuscular junctions (NMJ) display a remarkable reduction in docking (Weimer et al., 2003). However, syntaxin deficient synapses were reported to show unaltered docking profiles in chemically fixed flies (Broadie et al., 1995) and cultured mouse hippocampal synapses (de Wit et al., 2006), but in nematodes, using rapid freezing, a robust reduction in vesicles with a docking was observed in syntaxin orthologue, Unc-64-deficient animals (Hammarlund et al., 2007). Moreover, overexpression of Rab3A in mouse chromaffin cells or pheochromocytoma 12 (PC12) cells increased docking (van Weering et al., 2007). Similarly, Rab27A and Rab27B appear to mediate docking in different neurosecretory cells (Gomi et al., 2007). But null mutation of Rab3A or of all four Rab3 genes in mice does not seem to influence docking (Geppert et al., 1994a). Also, in C. elegans Rab3 mutant synapses, vesicles were distributed laterally at synapses without a clear
docking defect (Nonet et al., 1997). Because of the difference in fixation methods and docking phenotype criteria, it is currently unclear the potential candidates mediating docking of vesicles.

1.3.2 Priming

Once the vesicles are properly docked, then they undergo a priming step where vesicles are prepared to be “fusion-competent” upon arrival of Ca\(^{2+}\). In mammalian systems, priming step can occur in either an adenosine triphosphate (ATP)-dependent or -independent manner (Sugita, 2008). ATP-dependent priming was characterized in the cracked PC12 cell assay and in chromaffin cells as the cytosol- and ATP-requiring step preceding Ca\(^{2+}\)-dependent fusion (Holz et al., 1989). Two of the important cytosolic factors identified during priming step are N-ethylmaleimide sensitive factor (NSF) and phosphatidylinositol transfer proteins (PITPs). The ATPase NSF was first discovered in yeast as an essential factor in endoplasmic reticulum (ER) to Golgi vesicle transport. It has *Drosophila, C. elegans*, and mammalian orthologous and actually acts at multiple trafficking steps, including synaptic vesicle exocytosis (Barnard et al., 1997; Eakle et al., 1988; Graham and Emr, 1991; Pallanck et al., 1995; Wilson et al., 1989). In the cracked PC12 cell assay, NSF stimulates secretion only when added during the priming stage and have no effect on the fusion stage, suggesting that NSF’s essential role in priming of vesicles (Banerjee et al., 1996).

PITPs also participate in the priming stage of regulated exocytosis. These proteins bind phospholipids and transfer them between membrane compartments (Hay et al., 1995; Hay and Martin, 1993). During priming, they act by binding phosphatidylinositol 4-phosphate (PI(4)P) and presenting it to phosphatidylinositol 4-phosphate 5- (PI(4)P5)-kinase to generate phosphatidylinositol-4,5-biphosphate (PIP\(_2\)). PIP\(_2\) is then essential for subsequent events leading to membrane fusion. Importantly, PIP\(_2\) is believed to bind to synaptotagmin-1 (syt-1) (Südhof,
2013) and calcium-dependent activator protein for secretion 1 (CAPS1) (Grishanin et al., 2004), both of which are implicated in Ca\(^{2+}\)-dependent membrane fusion. Thus, generation and regulation of PIP\(_2\) appear to be the key events in a priming stage before membrane fusion is executed.

Although priming processes require ATP, it has become clear that ATP-independent components are also important for priming. Munc13 proteins (Munc13-1, -2 and -3) are considered to be the major priming proteins for neurotransmitter release from neurons. Through yeast two-hybrid screening, C-terminal residues of Munc13-1 were found to bind syntaxin-1 (Betz et al., 1997; Brose et al., 2000). Munc13-1 and Munc18-1 bind an overlapping domain of syntaxin-1, suggesting that Munc13-1 and Munc18-1 can compete for binding to syntaxin-1 (Ma et al., 2011). Munc18-1 binds to a closed conformation of syntaxin-1, but the syntaxin-1 that forms a SNARE complex has to adopt an open conformation (Dulubova et al., 1999). Thus, Munc13-1 has been considered to be the key protein that displaces Munc18-1 from syntaxin-1 and facilitates the formation of the SNARE complex (Ma et al., 2011).

CAPS1 is another protein that has been suggested to be involved in priming. CAPS1 contains a Munc13-homology domain (MHD), implying that the function of CAPS1 may be related to that of Munc13 (Ann et al., 1997). CAPS1 was originally purified as a cytosolic factor critical for Ca\(^{2+}\)-dependent triggering of DCV secretion in permeabilized PC12 cells (Walent et al., 1992). Since then, CAPS1 has been thought to be a triggering factor for exocytosis. However, more recent work suggested that CAPS1 does not directly trigger membrane fusion, but rather acts at a rate-limiting, priming step (Grishanin et al., 2004). CAPS1 contains a Pleckstrin homology (PH) domain that specifically binds to PIP\(_2\) in Ca\(^{2+}\)-dependent manner. Through this Ca\(^{2+}\)-dependent interaction with PIP\(_2\), CAPS1 appears to regulate the number of
DCVs that undergo exocytosis. Detailed analysis of *C. elegans* mutants of Unc-31, an orthologue of CAPS1, also supported its function in DCV exocytosis (Speese et al., 2007). Thus, CAPS1 is indeed involved in priming of DCV exocytosis in mammalian and *C. elegans*.

Munc18 has also been implicated to be involved in a priming stage. For many years, the molecular mechanism of Sec1/Munc18 (SM) proteins has been enigmatic because it binds syntaxin-1 in a closed conformation, in which syntaxin cannot form SNARE complexes (Misura et al., 2000). However, complete abolishment of neurotransmitter release upon deletion of Munc18 (Verhage et al., 2000) suggests that SNARE-mediated exocytosis absolutely requires Munc18. Recently it was demonstrated that Munc18-3, which bears high structural similarity to Munc18-1, can bind to the N-peptide of their cognate syntaxins with a binding site on the hydrophobic surface of the SM protein (Christie et al., 2012). This binding mode would enable the syntaxins to remain open, and the SNARE assembly not being inhibited. Moreover, it has been recognized that SM proteins, including Munc18, generally bind to their respective syntaxins using this spatially distinct binding sites (Shen et al., 2007). In line with this view, a syntaxin mutant where it adopts a constitutively open conformation (LE mutant) is known to bind Munc18 and still not inhibit formation of SNARE complexes (Dulubova et al., 1999). Indeed, the LE mutant results in enhanced spontaneous exocytosis, supporting that under resting conditions it is more reactive with respect to SNARE binding. Thus, it seems that binding of Munc18-1 to the N-peptide of syntaxin-1 is an integral part of the priming pathway in which Munc18 guides syntaxin towards productive SNARE complex formation.

1.3.3 Ca$^{2+}$-triggered membrane fusion

In regulated exocytosis, influx of Ca$^{2+}$ into the cytoplasm triggers a fusion of docked and primed vesicles. The increase in Ca$^{2+}$ can trigger exocytosis extremely rapidly, within 200 µs in
the squid giant axon (Llinás et al., 1981). Despite the fact that the dependence on Ca\(^{2+}\) has been recognized for decades, the identity of the Ca\(^{2+}\) sensor remains elusive. The best candidate of Ca\(^{2+}\) sensor studied in most detail has been synaptotagmin-1 (syt-1), a protein first found on purified synaptic vesicles. Here, brief information on syt-1 will be presented and more details will be discussed in section 1.6 of the Introduction. Syt-1 clearly plays a role in synaptic transmission, as evidenced by experiments employing protein overexpression or injection of peptides, antibodies, or antisense RNA into neurons (Südhof, 2013). Syt-1 knockouts have been produced in *C. elegans*, *Drosophila*, and mice. The *C. elegans* knockout displays severe defects in locomotion, feeding, and defecation, yet is capable of making coordinated motor movements, indicating that synaptic transmission is impaired but still present (Nonet et al., 1993). *Drosophila* mutants display an increased amount of spontaneous, Ca\(^{2+}\)-independent exocytosis at the NMJ but a decreased amount of evoked, Ca\(^{2+}\)-dependent secretion (Broadie et al., 1994; Littleton et al., 1994). The mouse knockout also displays a decreased amount of fast-evoked neurotransmitter release, but a slower component of secretion remains intact (Geppert et al., 1994b). Interestingly, mammals have many different isoforms of syt-1, some of which do not even bind Ca\(^{2+}\), which argues for a non-calcium sensing function of syts. In addition, there are Doc2 (double C2 domain), Unc-13, Rabphilin, and RIM (Rab3-interacting molecule) which are like syt, synaptic proteins containing Ca\(^{2+}\) binding C2 domains. Also, CAPS1 binds PIP\(_2\) synthesized during the priming stage; interestingly, it then switches its binding to different phospholipids in the presence of Ca\(^{2+}\) thus may help to promote lipid bilayer fusion (Ann et al., 1997). Although in vitro SNARE complex assembly itself is not Ca\(^{2+}\) sensitive, it has been found that Ca\(^{2+}\)-sensing proteins may directly alter SNARE proteins and their assembly. In next section, details on SNARE proteins will be introduced.
1.4 The SNARE protein complex

NSF (N-ethylmaleimide sensitive factor) is a cytosolic homohexameric ATPase which was found to be an essential component of fusion between transport vesicles and Golgi stack in a cell-free system (Malhotra et al., 1988). A following study revealed that the NSF works together with cytoplasmic factors, α, β, γ-SNAPs (α, β, γ -soluble NSF-attachment proteins). These two proteins were then found to be critical for driving intracellular membrane fusions (Clary et al., 1990). Subsequently, three synapse-associated proteins, VAMP-2, syntaxin-1 and SNAP-25 were isolated via binding to NSF/α- and γ-SNAP fusion proteins and these proteins were classified as receptors for SNAPs (Söllner et al., 1993). Since the 1990s when SNARE (Soluble N-ethylmaleimide-sensitive factor attachment protein receptor) proteins were first discovered, vast progress has identified SNAREs as key elements in membrane fusion (Weber et al., 1998). SNARE proteins that are localized on the membranes of opposing compartments drive membrane fusion. It is thought that formation of a four helix bundle provide enough free energy to overcome the energetical barrier imposed on bringing membranes together (Südhof and Rothman, 2009). Then, once the SNARE complex assembly has successfully fused the membranes, NSF comes and dissociates the helical bundle using ATP thereby allowing the recycling of now separated SNARE proteins (Lin and Scheller, 2000).

1.4.1 General structure of SNARE proteins

SNARE proteins are a superfamily with a considerable number of proteins in various organisms; 25 members in Saccharomyces cerevisiae, 36 members in homo sapiens and 54 members in Arabidopsis thaliana (Jahn and Scheller, 2006). All of these proteins contain a characteristic of domain called a SNARE motif, which is an evolutionarily conserved 60-70 amino acids (Figure 1.2). At their C-terminal ends, most SNARE proteins have a single
transmembrane domain that is connected to the SNARE motif by a short linker. Many SNARE proteins have independently folded domains that are positioned N-terminal to the SNARE motif (Rossi et al., 2004). Some subsets of SNARE proteins lack transmembrane domains, but most of these SNARE proteins contain hydrophobic post-translational modifications that mediate anchoring to the membrane. These SNARE proteins include a small group that is represented by the neuronal SNARE SNAP-25 (25-kDa synaptosome associated protein), which contains two different SNARE motifs that are joined by a flexible linker which is palmitoylated. In the S. cerevisiae SNARE, Ykt6, the transmembrane domain is replaced by a CAAX box that is farnesylated (McNew et al., 1997). Intriguingly, SNARE proteins that carry transmembrane domains can also be palmitoylated, which has been suggested to protect the proteins from ubiquitination and subsequent degradation (Valdez-Taubas and Pelham, 2005).
Figure 1.2 Domain and three dimensional structure of SNARE proteins and complex. Adapted and modified from Rizo and Xu, 2015.
1.4.1 SNARE motif

The SNARE complex formation is mediated by the SNARE motifs, and the core complexes are formed by coiled-coils of four intertwined α-helices, with each helix being provided by a different SNARE motif of SNARE proteins. The centre of the bundle contains 16 stacked layers of interacting side chains. These layers are largely hydrophobic, except for a central zero layer that contains three conserved glutamine residues and one conserved arginine residue. Depending on the identity of amino acids at the zero layer, the SNARE motifs, therefore, SNARE proteins are classified into Qa-, Qb-, Qc- and R-SNAREs (Bock et al., 2001; Fasshauer et al., 1998). In the most conventional view, Qa represents syntaxin proteins, Qbc represents SNAP proteins and R represents VAMP or synaptobrevin proteins. Functional SNARE complexes that drive membrane fusion are hetero-oligomeric, four parallel helices requiring one of each of the Qa-, Qb-, Qc- and R-SNAREs (Figure 1.3). Interestingly, due to their amphiphilic nature, SNARE motifs can also associate in other combinations that result in helical bundles that are less stable than core complexes. These include a Qaaaa complex (an antiparallel four-helix bundle), a Qabab complex (a parallel four-helix bundle), a Qaabc complex (a parallel four-helix bundle with some disordered regions) and, surprisingly, an antiparallel QabcR complex (Margittai et al., 2001; Misura et al., 2002; Misura et al., 2001a; Misura et al., 2001b; Weninger et al., 2003; Zhang et al., 2002a). These complexes might not have the correct membrane topology or they might not contribute sufficient energy to drive membrane fusion. Therefore, these wrong SNARE complex may be dissociated then be re-formed by regulatory mechanisms.
Figure 1.3 Structure of the neuronal SNARE complex. Structure of the four-helical bundle of the cis-SNARE (postfusion) core complex, with the syntaxin-1a coil in red, VAMP2 in blue, and the two SNAP-25 coils in green. (A) A cross section of a typical hydrophobic layer (-5), with ball and stick structures representing the indicated amino acids. (B) The ionic layer with its three glutamines, Q226 from syntaxin, Q53 from SNAP-25 N-terminal coil, and Q174 from SNAP-25 C-terminal coil, and the arginine R56 of VAMP. The dotted lines indicate the three hydrogen bonds formed between these residues in the wild-type complex. (C) Examples of electrostatic surface interactions (dotted lines) between the SNARE coils. Adapted and modified from Scales et al., 2001
1.4.2 N-terminal domains

Unlike the conserved SNARE motifs, there are different types of independently folded N-terminal domain. Syntaxin and some SNAP-25, have N-terminal antiparallel three-helix bundles (Dietrich et al., 2003; Misura et al., 2002) (Figure 1.2). These bundles can vary in length and are connected to the SNARE motif by a flexible linker. By contrast, the N-terminal domains of many VAMP proteins have profilin-like folds, which are sometimes referred to as longin domains and are also found in proteins that are unrelated to SNARE proteins (Rossi et al., 2004). Some N-terminal domains reversibly associate with the SNARE motif of the same SNARE protein to form a closed conformation (Dulubova et al., 1999), which prevents the SNARE motif from forming a SNARE complex. The N-terminal domains might function as recruitment platforms for the binding of other proteins such as SM proteins (detailed information about N-terminal interaction with Munc18 will be presented in section 1.5).

1.4.3 Trans-SNARE complexes

A central zippering hypothesis of SNARE function suggests that for fusion to proceed, SNARE proteins must assemble in a trans configuration, with at least one SNARE that has a transmembrane domain being contributed by each of the fusing membranes (Nichols et al., 1997). Assembly starts at the N-terminal end of the SNARE motifs and then proceeds in a zipper-like fashion towards the C-terminal membrane anchors. As a result, mechanical force is exerted on the membranes, which overcomes the energy barrier for fusion. The trans-SNARE complexes are subject to regulation by other proteins. In neuronal exocytosis, syt-1 is essential for fast, Ca\(^{2+}\)-triggered fusion (Fernández-Chacón et al., 2001). Following Ca\(^{2+}\) binding, syt-1 is thought to promote fusion, although it is still unclear to what extent this is caused by SNARE or phospholipid binding by the C2 domains. A second example of SNARE-binding proteins are the
complexins. Complexins are small helical proteins that bind to the surface of SNARE complexes, and they are also involved in the Ca\textsuperscript{2+}-dependent triggering step of neuronal exocytosis (Reim et al., 2001). Intriguingly, a structure of a complexin-SNARE complex indicates that complexins can bind to partially assembled SNARE complexes (Xue et al., 2007).

### 1.4.4 Fusion

During fusion, opposing membranes that are in contact proceed through a series of intermediates. An intuitive and physically well founded model is provided by the stalk hypothesis which describes fusion as an ordered sequence of lipid non-bilayer transition states (Jahn and Grubmüller, 2002) (Figure 1.4). The SNARE complex assembly exerts a mechanical force on membranes, which directly causes fusion. The model implies that the linkers between the transmembrane domains and the helical bundle of the SNARE motifs are stiff. Therefore, straining these linkers puts energy into membranes, bending them and/or disturbing the hydrophilic-hydrophobic boundary. As a result, the opposing membranes are not only pressed against each other but they are also deformed, which facilitates the formation of fusion stalks. SNARE-mediated fusion in both native membranes and proteoliposomes can be arrested at a hemifusion state (Reese et al., 2005; Xu et al., 2005). Hemifusion is experimentally defined as a state in which the lipids of the proximal leaflets are already exchanging, but an aqueous connection between the structures that are undergoing fusion has not yet formed. Although this hypothesis is intuitively making sense, it is unclear how many SNARE complexes are needed for a single fusion event. The fusion pore contains a diameter of only a few nanometers. It is unclear how 3 to 15 estimated SNARE complex can fit in this physically constrained space (Montecucco et al., 2005). In summary, a large body of evidence supports the view that SNARE proteins
function as fusion catalysts that not only provide the energy for the fusion reaction, but that also bring fusion to completion.
Figure 1.4 Hypothetical transition states in SNARE-mediated fusion according to the stalk hypothesis. Hemifusion is defined as the state in which the outer membrane leaflets are already continuous, but no aqueous connection has formed. Qa-SNARE represents syntaxin-1, Qbc-SNARE represents SNAP-25 and R-SNARE represents synaptobrevin. Adapted and modified from Jahn and Scheller, 2006.
1.5 The Munc proteins

The strong impairments of neurotransmitter release observed upon genetic deletion of the neuronal SNARE proteins support the notion that they play a crucial role in membrane fusion. However, such studies also found that although Ca\(^{2+}\)-evoked release was severely impaired, some levels of spontaneous or sucrose-induced release remained in the absence of VAMP and SNAP-25 (Broadie et al., 1995). A possible explanation for these results is that VAMP and SNAP-25 are particularly critical for evoked release and can be functionally compensated in other forms of release, but this remains to be proven. In contrast, genetic ablation of Sec1/Munc18 (SM) proteins have resulted in complete abolishment of both evoked and spontaneous release in mouse neurons (Verhage et al., 2000). Similarly, the lack of Munc13-1 and -2 showed severe impairment of both forms of excitatory postsynaptic currents (EPSCs) in mouse hippocampal neurons (Varoqueaux et al., 2002). Therefore, a key to fully understand the SNARE mediated exocytosis is to appreciate the function of other proteins that also play crucial roles in the secretion process.

1.5.1 Munc18

Reconstitution of the SNARE proteins into artificial lipid bilayers has demonstrated that they are sufficient to drive membrane fusion, but it was slow (Weber et al., 1998). This implies that SNARE complex-mediated membrane fusion relies on other regulators which coordinate the topology and function of SNARE complexes into efficient fusion machinery. Like the SNAREs, Sec1/Munc18 (SM) proteins are a family of evolutionary conserved proteins (60-70 kDa) that are indispensable for fusion in all species (Figure 1.5). SM proteins are thought to execute membrane fusion by interacting with their cognate SNARE partners, particularly syntaxin homologues. A universal requirement for the SM protein family in membrane fusion has been
recognized for many years through genetic studies in a wide variety of species, including *Saccharomyces cerevisiae*, *C. elegans*, *Drosophila melanogaster* and mice. Each of these organisms contain between four to seven SM proteins, which are believed to be involved in trafficking and fusion of different intracellular membrane compartments. In mammals, seven SM genes have been identified: Munc18-1, Munc18-2, Munc18-3, mVps45p, mVps33Ap, mVps33Bp, and mSly1p (Toonen and Verhage, 2003). Munc18-1, -2 and -3 are three mammalian isoforms of the first identified SM protein, Unc-18 in *C. elegans*. Munc18-1 is expressed primarily in neurons and neuroendocrine cells; Munc18-2 is widely expressed except in the brain; and Munc18-3 is expressed ubiquitously (Hata and Südhof, 1995; Riento et al., 1998; Tellam et al., 1995). Munc18-1 was initially found to tightly bind syntaxin-1 when first isolated (Hata et al., 1993). Since then, the specificity of binding between Munc18 and various syntaxin isoforms has been studied extensively. Munc18-1 and -2 can bind to syntaxin-1A, -1B, -2, -3, and -11, whereas Munc18-3 binds to syntaxin-2 and -4 (Hata and Südhof, 1995; Holz et al., 1989; Katagiri et al., 1995; Latham et al., 2006; Riento et al., 1998; Tamori et al., 1998; Tellam et al., 1995).

The importance of SM proteins on SNARE-mediated exocytosis was highlighted by the fact that the absence of the SM proteins leads to a block in membrane fusion (Südhof and Rothman, 2009), including a complete abolishment of neurotransmitter release in synapses of Munc18-1 KO mice (Verhage et al., 2000). However, the general function of Munc18-1 and other SM proteins remains enigmatic. Although SM proteins are as essential for fusion as the SNARE proteins, a crucial question that remains unanswered is whether SM proteins are regulators of SNARE function or are intrinsic components of the fusion apparatus as essential as the SNARE proteins. The difficulty in understanding SM function arises because they perform multiple roles and SM/SNARE interactions are complex. The crystal structure of the syntaxin-
1/Munc18-1 complex showed that Munc18-1 contains three domains that form an arch shape with a central cavity, and that closed syntaxin-1 binds to the central cavity (Misura et al., 2000). However, other studies demonstrated that Sec1p, the SM protein in yeast, binds to the SNARE complex rather than to Sso1p, syntaxin orthologue in yeast (Carr et al., 1999). Furthermore, the syntaxins that function in the endoplasmic reticulum, the Golgi complex, the trans-Golgi network, and early endosomes of yeast and mammals bind tightly through their N-peptide to their cognate SM proteins (Sly1pand Vps45p) without any requirement for the H_{abc} domain or the closed conformation (Bracher and Weissenhorn, 2002; Yamaguchi et al., 2002). It was also found that besides binding to closed syntaxin-1, Munc18-1 also exhibits binding to the SNARE complex in an interaction that involves the syntaxin-1 N-peptide (Dulubova et al., 2007; Khvotchev et al., 2007; Shen et al., 2007). Further data from other systems also revealed binding of SM proteins to their cognate SNARE complexes (Carpp et al., 2006; Collins et al., 2005; Latham et al., 2006). Therefore, these studies suggest that the functions of Munc18-1 and the SNARE proteins in membrane fusion are coupled by at least two different binding modes, 1) binary interaction with closed syntaxins and 2) an interaction with the SNARE complex through N-peptide of syntaxin.
Figure 1.5 Domain diagram of Munc18-1 and crystal structure of Munc18-1-closed syntaxin-1 complex. Three domains of Munc18-1 are coloured differently and labelled. Dotted curves represent the sequences linking N-peptide with the H_{abc} domain of syntaxin-1. Adapted and modified from Rizo and Xu, 2015.
1.5.1.1 Munc18-Syntaxin

The binary Munc18-1/closed syntaxin-1 interaction has originally been demonstrated that Munc18-1 binding stabilizes the closed conformation of syntaxin-1 and this strongly hinders the SNARE complex assembly (Burkhardt et al., 2008; Ma et al., 2011). A study of knockin mice bearing a LE mutation that constitutively opens syntaxin-1 has found an impairment of Munc18-1 binding to closed syntaxin-1 (Dulubova et al., 1999), but facilitates binding to the SNARE complexes. Nevertheless, some studies have indicated that Munc18-1 and closed syntaxin-1 travel together to the plasma membrane, which may prevent syntaxin-1 from being trapped in the Golgi complex and forming ectopic SNARE complex (Medine et al., 2007; Rowe et al., 1999). Mutations which disrupted such binary interaction indeed hampered trafficking of syntaxin-1 to the plasma membrane (Han et al., 2011; Han et al., 2009). Also, this interaction has been implicated in a role in docking of DCV of PC12 cells, presumably due to absence of closed syntaxin-1 at the plasma membrane (Han et al., 2011).

1.5.1.2 Munc18-SNARE complex

In addition to cognate syntaxin partners, Munc18-1 also exhibits binding to the whole SNARE complex. This notion is supported by physiological data (Deák et al., 2009; Khvotchev et al., 2007; Rathore et al., 2010) and reconstitution studies showing that Munc18-1 enhances the SNARE-dependent lipid mixing in a manner that depends on Munc18-1/SNARE-complex binding (Rodkey et al., 2008; Shen et al., 2007). From lipid mixing assay, Munc18-1 stimulates docking and lipid mixing between the VAMP and syntaxin-1-SNAP-25-containing vesicles, and a mutation in the syntaxin-1 N-peptide abrogates the stimulation of lipid mixing but not of docking (Tareste et al., 2008). By interacting to hydrophobic pocket of Munc18-1, the syntaxin-1 N-peptide has been thought to be crucial for Munc18-1 binding to the SNARE complex and for
stimulation of lipid mixing by Munc18-1 (Burkhardt et al., 2008). This was further supported by a study which revealed that a synthetic N-terminal peptide of syntaxin-1 inhibited secretion from neurons by competitively binding to Munc18-1 (Khvotchev et al., 2007). Also, in C. elegans, the F113R mutant in Unc-18 that is known to disrupt binding to the N-terminus of Unc-64 (syntaxin-1 homologue) could not rescue its null phenotype, locomotion defects, supporting the essential contribution of this binding mode in vivo (Johnson et al., 2009). However, there are also studies that suggest limited function of N-peptide binding to Munc18-1 in the exocytosis. For example, specific mutations on the hydrophobic pocket region of Munc18-1 (F115E and E132A), which abrogated binding to the SNARE complex through N-peptide of syntaxin-1, resulted in decent level of rescue on defective secretion phenotypes of Munc18-1/2 DKD PC12 cells (Han et al., 2009; Malintan et al., 2009). Moreover, these mutants showed full rescue on protein level and localization of syntaxin-1 thus implying that the interaction of N-peptide syntaxin-1 toward the hydrophobic pocket of Munc18-1 is not essential for the regulatory function of Munc18-1 on syntaxin-1. In addition, L130K mutation in hydrophobic pocket region of Munc18-1, which reduced binding to the SNARE complex, was still able to rescue the reduced synaptic vesicle docking and impaired synaptic transmission of hippocampal neurons to that of WT (Meijer et al., 2012). Although some data suggest that the syntaxin-1 N-terminal region alone is responsible for binding of Munc18-1 to the SNARE complex (Burkhardt et al., 2008), other studies suggest that Munc18-1 also interacts with the SNARE four-helix bundle (Dulubova et al., 2007; Shen et al., 2007) and with the SNARE motifs of the syntaxin-1/SNAP-25 heterodimer (Weninger et al., 2008). These results as well as reconstitution data suggest that the SNARE four-helix bundle also binds to the cavity of Munc18-1 where closed syntaxin-1 binds (Hashizume et al., 2009). In summary, although most of studies are converged on the essential role of Munc18, the specific functions and therefore molecular mechanisms of Munc18 are still diffused. This could be partly
due to pleiotropic functions of Munc18 and complicated binding properties toward syntaxin and the SNARE complex, as well as differences existing between various model systems and techniques. Therefore, understanding the roles of Munc18 in mast cell exocytosis could contribute to establish a common mechanism in which this protein may employ.

1.5.2 Munc13

When Munc13 proteins were identified as active-zone proteins, their sequences were found to be homologous to C. elegans Unc-13, a gene whose function was unclear but was known to be essential for coordinated movements (Brose et al., 2000). Munc13 is a large protein (~200kDa) that plays critical roles in synaptic vesicle exocytosis, as shown by the major impairments in neurotransmitter release observed in their absence (Aravamudan et al., 1999; Augustin et al., 1999; Richmond et al., 2001; Varoqueaux et al., 2002). Munc13 is a multidomain protein with dispersed C2 domains, one C1 domain, and a large central executive region called the Munc13 homology domain (MHD) (Figure 1.6). In mammals, Munc13 proteins consist of four members, Munc13-1 to Munc13-4, of which the Munc13-2 isoform exists in two splice variants, bMunc13-2 and ubMunc13-2 (Brose et al., 2000). Munc13-1, bMunc13-2 and Munc13-3 are predominantly expressed in neurons and neuroendocrine cells and have been shown to be essentially required for the priming of synaptic vesicles (Augustin et al., 1999). Furthermore, all three isoforms contain a calmodulin-binding domain and a diacylglycerol-binding C1 domain. In contrast, the more distantly related Munc13-4 lacks those N-terminal regulatory domains, but shares the two MHDs and the two C-terminal C2 domains. Munc13-4 has been expressed ubiquitously but exhibits higher expression in immune tissues. C2 domains often function as Ca^{2+}-phospholipid binding modules. Only the middle C2B domain of Munc13-1, -2, -3 constitutes a Ca^{2+}- phospholipid binding module, whereas the other C2A and C2C domains do
not bind Ca\(^{2+}\) (Lu et al., 2006; Shin et al., 2010). By contrast, both C2A and C2B domains of Munc13-4 have been shown to bind Ca\(^{2+}\) and phospholipids (Boswell et al., 2012; Feldmann et al., 2003).
Figure 1.6 Schematic diagram of Munc13 family and BAP. Asterisks indicate Ca$^{2+}$ binding residues in C2 domains of each protein.
1.5.2.1 MHD

Initial computational analyses showed that a variety of proteins contain sequences with homology to the Munc13 MHD (Basu et al., 2005; Koch et al., 2000), with significant sequence homology between the MHD and subunits from a variety of tethering complexes such as the exocyst, COG, GARP, and Dsl1p complexes, which play critical roles in trafficking at diverse membrane compartments. These results suggest that Munc13 and these tethering factors may form a family of proteins with a general role in membrane trafficking. The MHD is primarily responsible for the priming function of Munc13, although the adjacent C2C domain may also assist in this function (Basu et al., 2005; Stevens et al., 2005). Other domains of Munc13 are involved in diverse forms of regulation of neurotransmitter release. Early studies suggested that Munc13-1 fragments corresponding to the MHD sequences and the C2C domain bind to the N-terminus of syntaxin-1 (Betz et al., 1997). These data, together with the finding that the open syntaxin-1 LE mutant partially rescues the strong phenotype of unc-13 null mutants in *C. elegans* (Richmond et al., 2001), led to a model in which Munc13 opens syntaxin-1 by binding to its N-terminus. However, no binding of the Munc13-1 MHD to syntaxin-1 at low micromolar concentrations was observed. Further studies revealed that the Munc13-1 MHD binds to membrane-anchored SNARE complexes and syntaxin-1/SNAP-25 heterodimers (Guan et al., 2008; Weninger et al., 2008). The interaction of the MHD with the SNARE complex involves the SNARE four-helix bundle and is rather weak. Similarly, the MHD binds very weakly to Munc18-1 thus the interactions between the MHD, Munc18-1, and the SNARE complex may cooperate with each other (Ma et al., 2011). Moreover, the MHD also binds weakly to the SNARE motif of syntaxin-1 and even more weakly to the syntaxin-1 in a closed conformation, but no binding to the syntaxin-1 N-terminal region could be detected. These findings led to a model in which the MHD helps to open syntaxin-1 by binding to the SNARE motif from the
closed conformation and providing a template to assemble the SNARE complex, a process which could be aided by interactions of the MHD with Munc18-1 and the membranes. Indeed, further biophysical experiments demonstrated that the MHD dramatically accelerates the transition from the Munc18-1/closed syntaxin-1 complex to the Munc18-1/SNARE-complex assembly (Ma et al., 2011). However, it was found that the syntaxin-1 LE mutant does not appear to rescue release in Munc13-1/2 DKO mice (Gerber et al., 2008). Hence, the MHD very likely has another key function in addition to helping to open syntaxin-1.

Interestingly, a MHD is also found in CAPS, a protein that reconstitutes DCV exocytosis in permeabilized neuroendochrine cells (Ann et al., 1997), suggesting that CAPS play important priming roles in DCV. Indeed, this is further supported by the findings that DCV docking and priming defects observed in the absence of CAPS can be partially rescued by the open syntaxin-1 LE mutant (Hammarlund et al., 2008; Liu et al., 2010) and that CAPS also binds to the syntaxin-1 SNARE motif, stimulating the SNARE-complex formation (Daily et al., 2010; James et al., 2009). However, forced expression of Munc13 in neuroendocrine cells induces a strong enhancement in the DCV exocytosis only in the presence of at least one isoform of CAPS, and the DCV secretion defects caused by the absence of CAPS cannot be rescued by Munc13 (Liu et al., 2010; Zhou et al., 2007). Also synaptic deficits in CAPS-deficient mouse neurons could be partly rescued by phorbol ester (Jockusch et al., 2007). Hence, further research is required to understand other mechanisms driven by domains outside of MHD on the functions of CAPS and Munc13 in various types of exocytosis.

1.5.2.2 The role of C2 domains

In addition to MHD, Munc13 proteins contain other accessory domains known as C2 domains. Munc13-1, -2 and -3 contains C2A, C2B and C2C domains but only C2B domain has
been shown to bind to Ca$^{2+}$. Shin et al., 2010 has demonstrated that mutations in Ca$^{2+}$-binding domain, C2B of Munc13-1, result in impairment of short-term plasticity. This indicates that C2B domain could influence MHD activity through direct intramolecular interactions given the physical proximity between these two domains. However, the function of the Munc13 C2B domain depends on its ability to bind to PIP$_2$ in a Ca$^{2+}$-dependent manner (Shin et al., 2010). Therefore, the C2B domain could stimulate MHD activity indirectly by enhancing the plasma membrane localization of Munc13. Direct or indirect effects on the MHD may also be exerted by the Munc13 C1 domain, which mediates diacylglycerol- and phorbol ester-dependent increase of neurotransmitter release (Basu et al., 2007; Rhee et al., 2002). Short-term presynaptic plasticity is also regulated by a calmodulin-binding sequence of Munc13-1 that is N-terminal to the C1 domain (Junge et al., 2004) and binds to calmodulin via a novel mode involving two helical modules connected by a flexible linker (Rodríguez-Castañeda et al., 2010). In addition to these regulatory modes, Munc13 activity is tightly controlled by another family of active-zone proteins called RIMs. RIMs, in turn, are also essential for short- and long-term synaptic plasticity by additional mechanisms, and they moreover function as tethering factors in the active zone that co-localize synaptic vesicles, Ca$^{2+}$-channels, and other proteins besides Munc13 at release sites (Calakos et al., 2004; Castillo et al., 2002; Kaeser et al., 2011; Koushika et al., 2001; Schoch et al., 2001). The N-terminal C2A domain that is present in Munc13-1 and ubMunc13-2 binds to the zinc-finger domain present in RIM1α, RIM1β, and RIM2α (Betz et al., 2001). The Munc13 C2A domain forms a constitutive homodimer and binding of the RIM ZF domain to the Munc13 C2A domain converts it into a Munc13/RIM heterodimer (Lu et al., 2006). Deletion of RIMs causes a priming defect (Calakos et al., 2004; Koushika et al., 2001) that is rescued by an N-terminal fragment of RIM1α including its ZF domain (Kaeser et al., 2011). More strikingly, the priming defect in RIM-deficient synapses is also rescued by ubMunc13-2 bearing a point
mutation that disrupts C2A domain homodimerization, but not by WT ubMunc13-2. These results suggest that the key role of RIMs in vesicle priming involves reversal of an inhibition of Munc13 function arising from C2A domain-mediated homodimerization. The RIM/Munc13 interaction may also contribute to RIM-dependent forms of presynaptic plasticity. RIM and Rab3 are essential for multiple forms of short- and long-term plasticity (Schlüter et al., 2004; Schoch et al., 2002). Thus, the regulation of long-term plasticity by RIMs may involve the RIM-dependent regulation of the priming function of Munc13 and/or of the RIM/Munc13/Rab3 complex that tethers synaptic vesicles in close proximity to Munc13.
1.6 Synaptotagmin

The best candidate of Ca\textsuperscript{2+}-sensor of SNARE-mediated exocytosis is synaptotagmin-1 (syt-1). Syt-1 was first purified from synaptic vesicles, and the primary sequence of syt-1 revealed a single membrane spanning domain, a short intraluminal domain, and a large cytoplasmic domain consisting of tandem C2 domains, C2A and C2B, which both domains can bind to Ca\textsuperscript{2+} (Perin et al., 1991; Perin et al., 1990) (Figure 1.7). In addition to syt-1, 16 additional isoforms have been identified that are expressed widely in different tissues. The crystal structure of the C2A domain of syt-1 revealed that C2 domains are compact eight-stranded β-barrels, with two protruding loops that form the Ca\textsuperscript{2+}-binding pockets (Sutton et al., 1995). NMR studies indicate that five acidic amino acid side chains mediate binding of three Ca\textsuperscript{2+} ions in C2A domain and two Ca\textsuperscript{2+} ions in C2B domain (Ubach et al., 1998). In addition to its role as a Ca\textsuperscript{2+}-sensor, it is also involved in numerous aspects of the vesicle cycle in various organisms (Broadie et al., 1994; Nonet et al., 1993; Reist et al., 1998). In the following sections, their role as a Ca\textsuperscript{2+}-sensor will be discussed.
Figure 1.7 Domain structure and three dimensional structure of mouse synaptotagmin-1. The number of residues is indicated above each diagram. TMR, transmembrane region. The spheres represent Ca$^{2+}$. *Adapted and modified from Kasai et al., 2012.*
1.6.1 Role of synaptotagmin-1

In early studies on hippocampal neurons, it was concluded that disruption of syt-1 largely abolished the rapid synchronous component of exocytosis; while the slower asynchronous phase of release, which occurs over the next few hundred milliseconds following the rapid synchronous phase, was unaffected (Geppert et al., 1994b). In parallel with these studies in mice, NMJ of *Drosophila* larvae lacking syt-1 showed significant levels of evoked release, but this release was no longer tightly coupled, in time, with depolarization (Yoshihara and Littleton, 2002). These findings suggested that syt-1 is needed to convert the asynchronous release events into rapidly triggered synchronous events. In addition, more evidences from hippocampal neurons demonstrated that the total amount of exocytosis syt-1 KO was invariable, but the rate of release was dramatically reduced, and virtually all exocytosis became asynchronous (Nishiki and Augustine, 2004). Thus, syt-1 is essential to couple the fusion with depolarization mediating fast synchronous Ca$^{2+}$-dependent release.

1.6.2 Molecular mechanism of synaptotagmin-1

The crystal structure of the C2A domain of syt-1 revealed that C2 domains are compact eight-stranded β-barrels, with two protruding loops that form the Ca$^{2+}$-binding pockets. NMR studies indicate that five acidic amino acid side chains mediate binding of three Ca$^{2+}$ ions in C2A domain and two Ca$^{2+}$ ions in C2B domain (Fernandez et al., 2001; Ubach et al., 1998). Surprisingly, when all five Ca$^{2+}$-binding residues in C2A were mutated, there was no apparent defect in synaptic transmission (Stevens and Sullivan, 2003). In contrast, mutations in C2B domain resulted in significant loss of function. Substitution of Ca$^{2+}$ ligands (D309N or D363N) completely disrupted function (Mackler et al., 2002; Nishiki and Augustine, 2004).
It is also known that syt-1 can bind to phospholipids. Through the use of fluorescent probes, it was discovered that in response to Ca\(^{2+}\) the Ca\(^{2+}\)-binding loops of C2A partially insert into phospholipid bilayers (Bai et al., 2000; Davis et al., 1999). Also, isolated C2B can bind to lipid membranes, but this interaction is weaker than isolated C2A (Fernandez et al., 2001; Wu et al., 2003). In contrast, isolated C2B preferentially binds to membranes that harbor PIP\(_2\). This mechanism might serve to direct syt-1 toward the target membrane where PIP\(_2\) are high. PIP\(_2\) is highly concentrated in microdomains where it co-localizes with syntaxin, and these domains occur at sites where DCV exocytosis occurs in PC12 cells (Aoyagi et al., 2005). In addition, it was found that the phospholipid binding activity of C2B is markedly enhanced by the presence of an adjacent C2A domain, suggesting that C2A and C2B cooperate to bind to membranes (Bai et al., 2002; Hui et al., 2006). One idea is that each C2 domain influences the structure of the bilayer to change the lipid environment experienced by the adjacent C2 domain. Therefore, it is now established that both Ca\(^{2+}\)-binding loops of C2A and C2B simultaneously penetrate membranes. The Ca\(^{2+}\)-binding loops of C2 domains from other proteins also interact directly with the membranes, indicating that this could be a general mechanism by which C2 domains engage lipid bilayers (Kohout et al., 2003; Nalefski et al., 2001).

In brain extracts, syt-1 is associated with syntaxin as well as with assembled SNARE complexes; these are highly specific interactions, as few other proteins were detected when either syt-1 or the SNAREs were immunoprecipitated (Bennett et al., 1992; Söllner et al., 1993). Moreover, it was shown using purified recombinant proteins, that bindings of the cytoplasmic domain of syt-1 to syntaxin, SNAP-25, assembled t-SNARE heterodimers, or fully assembled SNARE complexes were enhanced by Ca\(^{2+}\) (Chapman et al., 1995; Schiavo et al., 1997; Zhang et al., 2002b). Thus, syt-1 can interact with t-SNAREs at all stages of SNARE complex assembly. Although syt-1 forms direct contacts with the t-SNAREs, syntaxin, and SNAP-25, it does not
bind to the v-SNARE synaptobrevin. A few studies have begun to map the regions of syt-1 that mediate binding to the t-SNAREs. Specifically, substitution of two lysine residues (K326, K327) on the C2B domain with neutral residues reduced binding (Rickman et al., 2004). This mutant form of syt-1 exhibited an impaired ability to support regulated exocytosis in the Drosophila NMJ and in autaptic cultures of hippocampal neurons (Li et al., 2006; Loewen et al., 2006).

Substitution of a charged residue in a membrane penetration loop of either C2 domain (R233Q in C2A and K366Q in C2B) reduced binding of syt-1 to both membranes and SNAP-25 (Wang et al., 2003). It was found that syt-1 binds to the SNARE motif of syntaxin-1 (Chapman et al., 1995; Davis et al., 1999). Importantly, this region of syntaxin-1 assembles into SNARE complexes, so binding of syt-1 could potentially regulate assembly of the fusion apparatus.

Analogous to its interaction with the membrane proximal SNARE domain of syntaxin, syt-1 binds to the C-terminal region of SNAP-25 (Zhang et al., 2002b) at the base of the SNARE complex near the vesicle and plasma membranes. This binding region is positioned so that syt-1 can interact with membranes while simultaneously driving transitions in the SNARE complex. In summary, through interactions with Ca$^{2+}$, phospholipid and the SNARE proteins, syt-1 can facilitate the SNARE complex assembly therefore the fusion in a Ca$^{2+}$-dependent manner.
1.7 Molecular mechanism of mast cell exocytosis

Mast cells contain the molecular machinery that drives membrane fusion during secretory granule exocytosis. Like neuronal exocytosis, the SNARE proteins are essential for mast cell exocytosis, however, the isoforms of these proteins are different. Unlike neuronal exocytosis where the fusion between vesicles and plasma membrane is driven by SNARE complex composed of syntaxin-1/SNAP-25 and synaptobrevin, mast cells express several different plasma membrane- and secretory granule-localized SNAREs that could be part of the exocytotic machinery. They include SNAP-23, syntaxin-2, -3, -4, and -11, and several VAMPs (VAMP-2, -3, -7, -8) (Blank, 2011). In addition, the SNARE proteins in mast cells function with accessory proteins, such as the NSF ATPase, which disassembles SNARE complexes, and Rab GTPases (Rab3A, Rab3D and Rab27A), which are generally involved in regulating exocytotic trafficking steps. Moreover, the syntaxin-binding SM family members, such as Munc18-2 and Munc18-3, are expressed in the mast cells, with the former having been implicated in secretory granule exocytosis. Taken together, the role of the accessory proteins is to increase fusion efficiency by regulating the fusion-competent state of SNARE proteins. All of these proteins must coordinately function in response to FcεRI stimulation.

1.7.1 Mast cell activation

The initial step in IgE+antigen induced mast cell activation occurs when antigen-specific IgE binds FcεRI, stabilizing and increasing the number of FcεRI receptors on the mast cell surface (Kinet, 1999). This sensitizes the mast cell to a specific antigen so that the mast cell can be activated immediately upon subsequent antigen exposure. Antigen binding to antigen-specific, FcεRI-bound, IgE cross-links FcεRI (FcεRI aggregation/cross-linking), thereby inducing various mast cell activation events via multiple signal transduction pathways within the mast cell.
(Figure 1.8). The FcεRI receptor consists of four subunits (αβγ2) - one α subunits that binds to IgE, one β subunit involved in signal amplification and FcεRI stabilization and two disulfide-bonded γ subunits acting as the main signal transducer. FcεRI aggregation leads to the activation of Lyn, a Src family protein tyrosine kinase (PTK) associated with the β subunit of FcεRI. The intracellular parts of FcεRI β and γ contain immunoreceptor tyrosine-based activation motifs (ITAMs), that consist of six conserved amino acid residues spread out over 26 amino acid sequences (Cambier, 1995). Activated Lyn phosphorylates tyrosine residues in these intracellular FcεRI β and γ subunit ITAMs. β phosphorylated ITAM recruits Lyn PTK and γ phosphorylated ITAM recruits Syk PTK. Two other Src family PTKs, Fyn and Hck, also work with Lyn and Syk to activate several signaling pathways (Parravicini et al., 2002), including the PI3K and phospholipase C (PLC) signaling pathway (Turner and Kinet, 1999).

The signal results from the receptor-mediated activation of PLC and the associated production of inositol 1,4,5-trisphosphate (IP₃) induce release of Ca²⁺ from stores in the ER and Golgi through Ca²⁺-conducting IP₃-receptors (IP3R) (Ali et al., 1990; Hoth, 1996; Hoth and Penner, 1992; Ma and Beaven, 2009). Depletion of Ca²⁺-stores activates influx of Ca²⁺ from outside through channel proteins such as Orai1 and TRPC1 (Liou et al., 2005; Soboloff et al., 2006). This influx replenishes ER Ca²⁺ stores via ATP-dependent (sarco/endoplasmic reticulum Ca²⁺-ATPase) SERCA pumps, which allows sustained elevation of cytoplasm Ca²⁺ as long as IP₃ is produced. The Ca²⁺ signal is terminated through uptake via the ER located SERCA pumps, or plasma membrane located Ca²⁺-ATPase pump and ion-exchange transporters as well as mitochondria (Ma and Beaven, 2009).
Figure 1.8 A highly simplified scheme for mast cell granule biogenesis, maturation and degranulation. Adapted and modified from Wernersson and Pejler, 2014.
1.7.2 SNARE and accessory proteins in mast cells

Mast cell exocytosis requires the SNARE complex, as blockade of their function with N-ethylmaleimide (NEM) strongly impairs degranulation (Lippert et al., 2007; Puri et al., 2003). Mast cells express a wide range of SNARE proteins. These include the t-SNAREs SNAP-23 and syntaxins-2, -3, -4, -6 and -11 (Guo et al., 1998; Paumet et al., 2000). Expressed v-SNAREs comprise the VAMP family members VAMP-2, -3, -7 and -8 (Sander et al., 2008). The first protein characterized to function in degranulation was t-SNARE SNAP-23, where introduction of blocking antibodies into permeabilized rat mast cells potently inhibited exocytosis (Vaidyanathan et al., 2001). Analysis of complex formation in mouse bone marrow derived mast cells (BMMCs) and primary human mast cells showed that complex formation between SNAP-23 and syntaxin-4 or VAMP-8 increased during FcεRI-induced stimulation (Tiwari et al., 2008). In addition, evidence for the implication of syntaxin-3 function has been presented. This t-SNARE is primarily located on secretory granules and relocates to the periphery upon stimulation (Puri and Roche, 2008). Furthermore, it is able to form complexes with other SNARE partners involved in exocytosis including SNAP-23 and VAMP-7. siRNA-mediated KD of syntaxin-3 in RBL-2H3 cells has resulted in defective degranulation, suggesting this syntaxin might be involved in the SNARE complex assembly (Brochetta et al., 2014). Regarding VAMP, VAMP-8 was initially shown to localize to early and late endosomes, confirming a close connection between endo- and exocytotic apparatus in mast cells. BMMC from VAMP8-deficient mice inhibited degranulation of histamine and β-hexosaminidase (Tiwari et al., 2008). Also, absence of VAMP-8 markedly inhibited histamine release in vivo, observed from passive anaphylaxis experiment. Deficiency of VAMP-2 and -3 or introduction of antibodies and
soluble recombinant proteins did not affect release, suggesting they may not play a role in mast cell degranulation (Sander et al., 2008).

Mast cells express the isoforms Munc18-1, Munc18-2 and Munc18-3 (Martin-Verdeaux et al., 2003; Nigam et al., 2005). Functional studies demonstrated a role for Munc18-2, which is localized on secretory granules and associates to syntaxin-2 and syntaxin-3. Ectopic expression of Munc18-2 and Munc18-2 peptides inhibited mast cell exocytosis (Martin-Verdeaux et al., 2003). Similarly, siRNA-mediated KD of Munc18-2 inhibited exocytosis, partly due to lack of proper syntaxin-3 translocation upon stimulation (Brochetta et al., 2014; Tadokoro et al., 2007). In contrast to other immune cells, no role has been demonstrated for Munc18-3, which is plasma membrane-localized and syntaxin-4-interacting isoform in mast cells.

Studies also indicated a role of the Munc13 family members in mast cell degranulation. Mutations causing a loss of Munc13-4 function expressed in hematopoietic cells impair exocytosis of cytotoxic secretory granule responsible for FHL-3 (Feldmann et al., 2003). In cytotoxic T cells, it has been suggested that Munc13-4 has two functions (Ménager et al., 2007). In an early step, it promotes attachment of exocytotic vesicles generated from Rab11/Munc13-4-positive and Rab27A-positive endosomal vesicular pools; and then it primes fusion of genuine cytotoxic secretory granules with these exocytotic vesicles to secrete their contents. Priming involves interaction of Munc13-4 with Rab27A (Elstak et al., 2011). In mast cells, both Munc13-4 and Rab27 isoforms co-localize on secretory granules, where Munc13-4 is anchored via its MHDs (Elstak et al., 2011; Neeft et al., 2005). Over-expression of Munc13-4, but not mutant Munc13-4 that lacks binding to Rab27A, enhanced degranulation, suggesting a positive regulatory function (Neeft et al., 2005).
Mast cells express several syt family members (syt-1, -2, -3 and -9). Initial siRNA knockdown study showed that deficiency of syt-2 did not affect histamine release, but rather blocked release of cathepsin D suggesting that it acts to prevent fusion of genuine lysosomes, but not secretory granules (Baram et al., 1999). However, this finding has been challenged, as BMMC grown from syt-2-deficient mice showed a severe defect in both lysosomal and β-hexosaminidase and histamine release (Tuvim et al., 2009). Syt-3 was found to function as a critical regulator of the perinuclear endocytic recycling compartment (ERC) and could possibly regulate secretory granule size (Grimberg et al., 2003), while syt-9 functions in protein export from the ERC to the cell surface (Haberman et al., 2003). These data suggest that non-neuronal syt(s) interfere with distinct steps of membrane trafficking in mast cells, along the endo- and exocytotic pathway.
1.8 Immunological diseases implicated with mast cell and immune cell degranulation

Mast cells play an important role in initiating an appropriate program of inflammation and repair in response to pathogenic invasion and tissue damages. They are important for host immunity against bacterial and virus infections. However, when the activation and degranulation of mast cells are mis-regulated, over- or less- secretion of pro-inflammatory cytokines and chemokines can lead to multiple pathophysiological conditions. In this section, two particular diseases that are manifested by the mis-regulation of mast cell activation will be discussed.

1.8.1 Allergy

Allergy is a disease where an individual experience exaggerated response of their immune system against a certain substance. People with allergic disorder such as atopic dermatitis, allergic rhinitis (hay fever), food allergy and allergic asthma can develop acute signs and symptoms of allergy within minutes of exposure to associated allergens (Galli and Tsai, 2012). A large body of evidences suggests IgE antibody and mast cells play a pivotal role in the development and progress of allergy as well as a more severe condition such as asthma where chronic exposure have led to long-term changes in affected tissues (Amin, 2012; Galli and Tsai, 2012; Tsai et al., 2005). Production of antigen-specific IgE requires that such antigens are taken up by dendritic cells, B cells or other antigen-presenting cells. Once B cells are exposed to antigens, they undergo class-switch recombination resulting in switching immunoglobulin heavy chain linking (Gauchat et al., 1993; Geha et al., 2003). This allows IgE-producing B cells to undergo clonal selection and affinity maturation and the IgE antibody production primarily occur in lymphoid germinal centres. However, patients with food allergy have higher concentrations of IgE in the gastrointestinal tract, and people with seasonal allergic rhinitis or atopic asthma have accumulation of IgE producing B cells in airway-associated lymphoid tissue (Coëffier et al.,
This antigen-specific IgE antibody has no ability to directly complement allergen and its main biological role is its ability to bind to FceRI on mast cells. In asthmatic patients, mast cells infiltrate key sites such as airway smooth muscle, airway mucous glands and bronchial epithelium (Brightling et al., 2002; Carroll et al., 2002). In an allergic person, whose tissue mast cells already have antigen-specific IgE bound, re-exposure to original antigen results in crosslinking of FceRI-bound IgE and consequent aggregation of surface receptor therefore the release of granules containing histamine, serotonin, protease and cytokines. These mediators induce a response of immediate hypersensitivity reaction. If localized to airways, it is characterized by increased vascular permeability, contraction of the airway smooth muscle and enhanced secretion of mucus. Mast cells activated to drive an early phase reaction can contribute to late phase reactions leading to chronic allergic inflammation (Galli and Tsai, 2010). The mechanisms of chronic mast cell activation in allergic conditions are not fully understood. It is assumed that mis-regulation of mast cell degranulation is a dominant factor resulting in chronic condition as once allergen exposure is established, removal of allergen has a minor effect. Mast cells can be activated by a number of diverse stimuli. Studies have shown mice which genetically lack FceRIα have diminished airway inflammation indicating activation of mast cells, through antigen-free monomeric IgE, can generate chronic condition (Tsai et al., 2005). In addition to locally concentrated IgE, many other factors can modify mast cell function. These include adenosine, sphingosine-1 phosphate and certain chemokines and cytokines such as IL-4, IL-33 that can tune the activations of mast cells in allergic conditions (Beer et al., 2007; Ochi et al., 2000; Olivera and Rivera, 2011).
1.8.2 Familial Hemophagocytic lymphohistiocytosis

The release of immunogenic chemicals by degranulation is not only essential in mast cells but also in other types of immune system such as cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells (Hong, 2005; Topham and Hewitt, 2009). Both cells recognize infected cells of the body and release their mediators such as perforin and other proteases to trigger lysis or apoptosis to eliminate from the host. It was found that proteins as well as Ca\(^{2+}\)-dependent degranulation processes that occur in mast cells also exist in CTLs and NK cells. Importantly, Munc13-4, syntaxin-11 and Munc18-2, which are the proteins that are involved in exocytosis of these cells have been implicated as causal genes of familial hemophagocytic lymphohistiocytosis (FHL) types 3, 4 and 5, respectively (Cetica et al., 2010; Côte et al., 2009; Dabrazhynetskaya et al., 2012; Feldmann et al., 2003; Hackmann et al., 2013; Hellewell et al., 2014; Meeths et al., 2010; Pagel et al., 2012; Yamamoto et al., 2004; zur Stadt et al., 2009). FHL is a genetic-based heterogeneous autosomal recessive life-threatening immune disease characterized by uncontrolled hyper-inflammation by supra-activation of macrophages and lymphocytes. It has been found that CTLs and NK cells isolated from FHL-3, -4, and -5 patients exhibited impaired cytotoxic granule exocytosis indicating the importance of those proteins in degranulation of immune cells. From studies in humans, it is clear that the principal underlying defect is impaired T and NK cell cytotoxicity, even though the actual numbers of these effector cells are normal (Jordan et al., 2004). NK cells and CTLs play a major role in the immune response to invading pathogens, predominantly viruses. They secrete soluble mediators such as interferon (IFN)-\(\gamma\), which enhances immunity, interferes with viral replication and is also a potent macrophage activator. CTLs also mediate direct killing of infected cells and antigen-presenting cells, including macrophages, pre-dominantly via the release of cytotoxic granule (Ménasché et al., 2005). Studies in the perforin-deficient and Munc13-4-deficient mouse models have shown that
when the cytotoxic function is impaired, there is a disproportional expansion of CD8+ T cells and excessive production of cytokines, including IFN-γ, and persistent macrophage activation (Badovinac et al., 2003; Badovinac et al., 2000; Crozat et al., 2007). This leads to tissue infiltration by macrophages and increased production of pro-inflammatory cytokines, giving rise to clinical features resembling those of the human disease. However, the role of hyper-activated tissue infiltrating macrophages in the pathophysiology of the disease and whether they represent the cause or the result of the disease remains to be elucidated.
1.9 RBL-2H3 cells

For many years, investigation on mast cell biology was restricted to a particular class of mast cells called connective tissue mast cells (CTMC) because they were easy to obtain, particularly from the peritoneal cavity. On the other hand, mucosal mast cells (MMC) and basophils were disregarded due to the difficulties involved in isolating these two cell types (Befus et al., 1982). Here, a brief introduction to RBL-2H3 cell generation and their granular release are discussed.

1.9.1 Isolation of RBL-2H3 cells

Rat basophilic cells were originally obtained when rats treated with the potent carcinogen developed a rare form of granulocytic leukemia characterized by a considerable peripheral blood basophil cells (Ecclleston et al., 1973). The cytochemical analysis of the infiltrated granulocytes confirmed that the features of these cells were basophils; these leukemic cells showed basophilic granules containing histamine, serotonin and chemotactic factors, and contained features that were different from those typical of mast cells. Then, the basophilic leukemia cells were serially transplanted for 20 generations back to animals and subsequent cells were isolated and cultured. This cell line, called RBL-1 had approximately a similar effect to that reported for normal rat peritoneal mast cells (RPMC) (Kulczycki et al., 1974). RBL-1 cells have also been shown to share similarities in granule biochemistry with MMC, such as glycosaminoglycans and RMCP-II. Unfortunately RBL-1 failed to give a response after IgE+antigen sensitization (Siraganian et al., 1975); therefore further search for a new mast cell lines began. A cell line called RBL-HR showed a percentage of total histamine release in respond to IgE+antigen stimulation. Subsequently, cells from RBL-HR were cloned by the limiting dilution method in an attempt to reduce cell heterogeneity and the RBL-2H3 cell line was then isolated (Buell et al., 1976).
1.9.2 RBL-2H3 release

RBL-2H3 cells, like primary mast cells, respond with degranulation following crosslinking of their IgE-bound FcεRI by multivalent allergens, with the release of a range of preformed and newly synthesized mediators that evoke a potent immune allergic response.

Histamine is certainly the most studied among the mast cells’ mediators. It is involved in allergy and inflammatory responses, including a role in mediating interactions between other cells involved in inflammation (Hasala et al., 2008). Histamine increases anti-CD3 induced IL-5 production of TH2-type T cells via histamine H2-receptors (Schmidt et al., 1994). Histamine is certainly present in RBL-2H3 cells, but the amount of histamine in this cell line, and relative to mast cells, is unclear. There are reports in the literature describing the RBL-2H3 cell line as a serotonin-releasing cell line (Harville and Dreyfus, 1996; Tamir et al., 1982; Theoharides et al., 1982; Theoharides et al., 1985). It has been demonstrated that the release of histamine can be inhibited while the release of serotonin is unaffected, suggesting that two pools of granules containing distinct cargos are present in RBL-2H3 cells (Theoharides et al., 1982).

β-hexosaminase is a granule-associated enzyme that has been extensively used to monitor mast cell degranulation as it is released in parallel with histamine. Its role during the inflammation process is not known, but it has been hypothesized that it could act in concert with tryptase and chymase for the degradation of glycoproteins and proteoglycans of the extracellular matrix (an important event during the remodeling of the inflamed tissue) (Fukuishi et al., 2014). The enzyme β-hexosaminidase shows optimal activity at low pH, a typical condition during inflammatory processes. β-hexosamidinase is commonly used as a marker for degranulation in RBL-2H3 cells since it is released in parallel with histamine.
1.10 Rationale, Hypothesis and Specific Aims

As eluted in the Chapter 1, the SNARE proteins and other proteins that regulate the SNARE proteins/complex play essential roles in the exocytosis of not only in neurons and neuroendocrine cells but also in the immune cells. Moreover, the fact that mutations in these proteins result in devastating immune disorders without neuronal or endocrinal abnormalities necessitate the needs for better understanding of their precise structural-functional relationships implicated in the immune cell exocytosis. Therefore, the objective of this research is to elucidate the molecular mechanisms underlying the functions of two important Munc proteins in immune cells, Munc13 and Munc18 using RBL-2H3 mast cells as a model system.

The study presented in the thesis is conducted based on a hypothesis that Munc18 and Munc13 play an essential role in mast cell exocytosis by regulating distinct stages of exocytosis. Based on previous findings, the following hypotheses have been generated:

**Hypothesis 1**: Munc18 play an indispensable function in the mast cell exocytosis via interactions with cognate syntaxin(s). This interaction underlies an important role of Munc18 in regulating syntaxin by governing protein level and proper trafficking.

**Hypothesis 2**: Munc13-4 is an essential priming factor of mast cell exocytosis.

These hypotheses have been assessed through following specific aims:

**Specific Aim 1**: To examine the role of interaction between Munc18 and its cognate syntaxin in the mast cell exocytosis.

**Specific Aim 2**: To examine the role of Munc13-4 as a priming factor of the mast cell exocytosis.
Chapter 2: Materials and Methods

2.1 General Materials

Parental pLKO-puro plasmid for lentivirus-mediated knockdown was purchased from Sigma-Aldrich (Oakville, ON, Canada). pLVX-IRES-puro plasmid for lentivirus-mediated Munc18-1 and -2 expression was purchased from Clontech Laboratories (Mountain View, CA). pSPAX2 was purchased from Addgene (Cambridge, MA) and pMD.G was a kind gift from Dr. Tomoyuki Mashimo (University of Texas Southwestern Medical Center at Dallas, Dallas, TX). We obtained rabbit monoclonal anti-syntaxin-3 antibody (clone EPR8532) from Abcam (Cambridge, UK), mouse monoclonal antibodies against syntaxin-1A and -1B (clone HPC-1) (Brzezinska et al., 2008) from Sigma-Aldrich; Munc18-1 (clone 31) and syntaxin-8 from BD Biosciences (Mississauga, ON, Canada); GAPDH (clone 6C5) from Millipore (Billerica, MA), GM130 (clone 35), TGN38 (clone 2) from BD Biosciences (Mississauga, ON, Canada), HA.11 (clone 16B21) from Covance (Princeton, NJ) and GFP (clone 9F9.F9) from Novus Biologicals (Lilleton, CO) and rabbit polyclonal antibodies against syntaxin-2, -3 and -4 from Synaptic Systems (Gottingen, Germany); syntaxin-7, -11 from Proteintech (Chicago, IL). Rabbit anti-Munc18-2 antibody (Broadie et al., 1994) was a kind gift from Dr. Vesa Olkkonen (National Public Health Institute, Helsinki, Finland). Rabbit antibody against full-length Munc13-4 was a kind gift from Dr. Hisanori Horiuchi (Kyoto University, Kyoto, Japan) which was described (Shirakawa et al., 2004).
2.2 Cell Culture

PC12 cells were maintained in DMEM (Invitrogen, Carlsbad, CA) containing 5% calf serum, 5% horse serum (both from HyClone Laboratories, Logan, UT), penicillin (100 U/mL)/streptomycin (0.1 mg/mL) (Sigma-Aldrich Canada), 250 ng/mL amphotericin B (Sigma-Aldrich Canada). RBL-2H3 cells were maintained in DMEM containing 11mg/mL sodium pyruvate, 5% calf serum, penicillin (100 U/ml)/streptomycin (0.1 mg/mL), 250 ng/mL amphotericin B. Human embryonic kidney-fibroblast (HEK293FT) cells were maintained in DMEM medium with 11mg/mL sodium pyruvate (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (HyClone Laboratories, Logan, UT), penicillin (100 U/ml)/streptomycin (0.1 mg/ml) (Sigma Chemical), and 250 ng/ml amphotericin B (Sigma Chemical).

2.3 Construction of Munc18-1 and -2 Knockdown and Expression Plasmids

For Munc18-1 knockdown in RBL-2H3 cells, parental pLKO-puro plasmid was modified; U6 promoter was replaced by H1 promoter + 19 nucleotides shRNA sequences of pSuper-Munc18-1 KD creating pLKO-H1-Munc18-1-puro. The Munc18-2 knockdown plasmid was previously generated (Han et al., 2011). Expression plasmids were created by modifying the parental pLVX-IRES-puro. First, puromycin-resistance gene was replaced by blasticidin-resistance gene creating pLVX-IRES-blast. Then, Emerald GFP (EmGFP) or myc sequence was subcloned into BamHI site generating pLVX-EmGFP-IRES-blast and pLVX-myc-IRES-blast, respectively. cDNA of rat Munc18-1 and -2 (kind gifts from Dr. Thomas Südhof) were mutated to create silent nucleotide mutations (SNMs) (for Munc18-1, GTCCGTGCACAGCCTGATC, for Munc18-2, AGGAAATTACGATCGTCGAGGA underlines indicate SNM) within the shRNA targeted sequences to protect exogenously expressed gene from being degraded by anti-Munc18-1 or -2 shRNA. We found our original pCMV5-rMunc18-2 wild-type (WT) cDNA had two point
mutations (G11V, T304R). These mutations were corrected by site-directed mutagenesis. Point mutations (K46E, E59K, K46E/E59K and F115E, E132A, and F115E/E132A) were also made using site-directed mutagenesis. Munc18-1 insert (WT and mutants) was digested from pCMV5-rMunc18-EmGFP with EcoRI/XbaI and was subcloned into the same sites of pLVX-EmGFP-ires-blast, pLVX-myc-ires-blast or pLVX-ires-blast. Munc18-2 insert (WT and mutants) digested from pCMV5-rMunc18-2 (without stop codon) with EcoRI/XbaI was subcloned into the same site of pLVX-EmGFP-ires-blast. Sequences of all created plasmids were verified by DNA sequencing. Knockdown and expression plasmids were co-transfected with psPAX2 and pMD.G into HEK-293FT cells for generating recombinant lentivirus.

2.4 Isolation of Stable Munc18-1/2 Knockdown and Rescued PC12 and RBL-2H3 Cells

Munc18-1/2 double-knockdown (DKD) PC12 cells were generated (Dabrazhynetskaya et al., 2012) and were maintained in DMEM (Invitrogen, Carlsbad, CA) containing 5% calf serum, 5% horse serum (both from HyClone Laboratories, Logan, UT), penicillin (100 U/ml)/streptomycin (0.1 mg/ml) (Sigma-Aldrich), 250 ng/ml amphotericin B (Sigma-Aldrich), puromycin (2.5 μg/ml) (Bishop, Toronto, Canada), and G418 (700 μg/ml) (Bioshop, Toronto, Canada). Lentivirus expressing Munc18-1 and -2 (WT or mutants) with various tags were infected to the DKD PC12 cells and were isolated by applying blasticidin (5 μg/ml) (Invivogen, San Diego CA). Wild-type RBL-2H3 cells were purchased from ATCC (Manassas, VA), and maintained in DMEM containing 10% calf serum (HyClone Laboratories, Logan, UT), penicillin (100 U/ml)/streptomycin (0.1 mg/ml) and 250 ng/ml amphotericin B. Lentivirus expressing shRNA against Munc18-1 and -2 were applied to RBL-2H3 cells with polybrene (8 μg/ml) (Sigma Aldrich) and infected cells were isolated by puromycin (10 μg/ml) for Munc18-1 and G418 (700 μg/ml) for Munc18-2. After successful down-regulation was confirmed, lentivirus expressing
EmGFP tagged rMunc18-1 or -2 wild-type and various mutants (K46E, E59K, K46/E59K, F115E, E132A, F115/E132A) were infected to the Munc18-1/2 DKD RBL-2H3 cells and selected with blasticidin (20 μg/ml).

2.5 Construction of Syntaxin-11 Knockdown Plasmids and Isolation of Stable Syntaxin-11 Knockdown RBL-2H3 cells

To knockdown rat syntaxin-11 gene, 21-nucleotides sequence of CGGTGCAGTACAAGAAGAAGA was targeted in rat syntaxin-11. CTCGAG was used as a linker sequence. These oligonucleotides containing sense and antisense of the target sequences were annealed, phosphorylated and ligated to AgeI/EcoRI sites of pLKO-puro (Sigma-Aldrich, Oakville, ON, Canada) generating the syntaxin-11 knockdown plasmid (pLKO-syntaxin-11-4). Inserted sequences were verified by DNA sequencing. This knockdown plasmid was co-transfected with psPAX2 and pMD.G into HEK-293FT cells for generating recombinant lentivirus. Lentivirus expressing shRNA against rat syntaxin-11 were applied to RBL-2H3 cells with polybrene (8 μg/ml) (Sigma Aldrich) and infected cells were isolated by puromycin (10 μg/ml).

2.6 Construction of syntaxin-3 knockdown plasmids and isolation of stable syntaxin-3 knockdown RBL-2H3 cells

To knock down the rat syntaxin-3 gene, two different constructs targeting (1) the 21-nucleotide sequence 5′-GCTCGAAAGAAATTGATAATT-3′ (named syntaxin-3–8 KD) and, (2) the 21-nucleotide sequence 5′-GTTTGTGGAGGTGATGACAAA-3′ (named syntaxin-3–5 KD) in rat syntaxin-3 were generated. The hexameric sequence CTCGAG was used as a linker sequence in both constructs. These oligonucleotides, containing sense and antisense of the target sequences, were annealed, phosphorylated and ligated into AgeI/EcoRI sites of pLKO-puro
(Sigma-Aldrich) for syntaxin-3–8 KD and pLKO-neo (Sigma-Aldrich) for syntaxin-3–5 KD, generating the syntaxin-3 knockdown plasmids. These knockdown plasmids were co-transfected with psPAX2 and pMD.G into HEK-293FT cells to generate recombinant lentivirus. Then the lentivirus expressing shRNA against rat syntaxin-3 was applied to RBL-2H3 wild-type cells (ATCC, Manassas, VA) in the presence of polybrene (8 µg/ml) (SigmaAldrich) for 2 days, and syntaxin-3–8 infected cells were isolated by using puromycin (10 µg/ml) (Bioshop, Burlington, ON, Canada) and syntaxin-3–5 infected cells were isolated by using G418 (700 µg/mL).

2.7 Generation of stable Munc13-4 KD RBL-2H3 cells

21 nucleotides shRNA sequences targeting rat Munc13-4 (GCACAGTTGAATGGTTTCACC) was annealed and ligated to pLKO-neo vector digested with EcoRI/AgeI. The hexameric sequence of CTCGAG was used as a linker sequence. After DNA sequencing, the construct was transfected into HEK-293FT cells along with psPAX2 and pMD.G for generation of lentivirus. Lentivirus was harvested 3 days later, which was applied to wild-type RBL-2H3 cells for 2 days in the presence of polybrene (8 µg/ml) (SigmaAldrich). Successfully infected cells were selected with G418 (700 µg/mL).

2.8 Generation of stable Munc13-4 rescued RBL-2H3 cells

Expression plasmids were created by modifying the parental pLVX-IRES-puro (Clontech Laboratories). First, the puromycin resistance gene was replaced by a blasticidin-resistance gene, creating pLVX-IRES-blast. Then, Emerald GFP (EmGFP; Clontech Laboratories) was subcloned into BamHI site, generating pLVX-EmGFP-IRES-blast. CMV promoter was replaced with Ubiquitin ligase C promoter from pUB-GFP (Han et al., 2009) using the Clal/EcoRI sites. cDNA of Human Munc13-4 was mutated during site-directed mutagenesis to yield single (D127N,
D133N, D941N, D947N) and double (D127N/D133N, D941N/D947N) and quadruple (D127N/D133N/D941N/D947N) mutants. Oligo sequences for generating each mutation were D127N: 5’-GAAACAGGCCAAGGGCTTGGGCAAAATGTCAGTGGGTTTCAGC-3’, D133N: 5’-GTCAGTGCTTCAGCATCCCTACTGCTCTGCTGGGCATTGAG-3’, D941N: 5’-CTCCAATGGGACTCCATTTTGCTCCAGCTTGACCCCTTTTGAGCC-3’ where underlined nucleotides indicate point mutations. After DNA sequencing, wild-type and mutants were ligated to pLVX-UBC-IB-EmGFP with EcoRI/XbaI. These constructs were transfected into HEK-293FT cells along with psPAX2 and pMD.G2 for lentivirus production which were applied to stable Munc13-4 knockdown RBL-2H3 cells. Blasidicidin (20 μg/mL) were applied to select successfully infected cells.

2.9 Construction of syntain-2–HA and syntain-3–HA expression plasmids

pLVX–HA–IRES-blast was generated by subcloning the hemagglutinin (HA) sequence in tandem into pLVX-IRES-blast using BamHI/XbaI sites. Rat syntaxin-2 and syntaxin-3 without stop codon inserts were digested from pCMV5 vector with EcoRI/XbaI for subcloning into the same sites of pLVX–HA–IRES-blast. Sequences of all created plasmids were verified by DNA sequencing. Expression plasmids were co-transfected with psPAX2 and pMD.G into HEK-293FT cells in order to generate recombinant lentivirus.

2.10 Isolation of stable syntaxin-2–HA- and syntaxin-3–HA-overexpressing RBL-2H3 cells

Munc18-1/2 DKD RBL-2H3 mast cells were maintained in DMEM containing 10% calf serum (HyClone Laboratories, Logan, UT), penicillin (100 U/ml)/streptomycin (0.1 mg/ml)
(Sigma-Aldrich) and 250 ng/ml amphotericin B (Sigma-Aldrich). For overexpression of syntaxin-2–HA and syntaxin-3–HA, lentivirus expressing HA-tagged syntaxin-2 or syntaxin-3 were applied to control and Munc18-1/2 DKD RBL-2H3 cells in the presence of polybrene (8 µg/ml) (Sigma-Aldrich) for 2 days; blasticidin (20 µg/ml) (Invivogen) was used for selection.

2.11 β-Hexosaminidase Release Assays from RBL-2H3 cells

RBL-2H3 cells were plated in 24-well plates; 2-3 d after plating, the cells that are to be stimulated IgE-dependent method were sensitized with 0.01 µg/mL DNP-IgE Spe7 antibody (Sigma Aldrich, Oakville, ON, Canada) for overnight. Cells that are to be stimulated ionomycin-dependent method were changed medium. Next day, cells were washed twice with physiological saline solution (PSS) containing 145 mM NaCl, 5.6 mM KCl, 2.2 mM CaCl₂, 0.5 mM MgCl₂, 5.6 mM glucose, 15 mM HEPES, and 0.1% BSA, pH 7.4. Degranulation was stimulated by adding 500 µl of PSS plus the following to the respective cells; 50 ng/ml DNP-HSA (Sigma-Aldrich, Oakville, ON, Canada), 0.5 µM ionomycin (Sigma-Aldrich, Oakville, ON, Canada) or 2.5 µM ionomycin with or without 0.5 µM phorbol-12-myristate-13-acetate (PMA) (Sigma-Aldrich). After 1 h of incubation at 37°C, samples was collected and centrifuged at 4°C for 3 min. Supernatant was transferred to new set of tubes for counting released β-hexosaminidase. Cells were solubilized in 500 µl of 0.5% Triton X-100 and samples was collected for counting the remaining β-hexosaminidase. 200 µl of 1mM p-nitrophenyl N-acetyl-β-D-glucosaminide (Sigma-Aldrich, Oakville, ON, Canada) were added and incubated at 37°C for 1 h. Reaction was quenched by adding 500µl of 0.05µM sodium carbonate buffer and absorbance at 405nm was measured by spectrophotometer. (OD₄₀₅ of supernatant / OD₄₀₅ of total) x 100% was calculated to be % β-hexosaminidase release. For Ca²⁺ titration assay, amounts of CaCl₂ in PSS buffer was altered to
achieve desired extracellular Ca^{2+} concentrations. For 0 mM Ca^{2+} condition, 0.1 mM EGTA was added to chelate any possible Ca^{2+} presence.

### 2.12 \[^{3}H\]-Noradrenaline Release Assays from PC12 cells

PC12 cells were plated in 24-well plates; 3–4 d after plating, the cells were labeled with 0.5 μCi \[^{3}H\]-Noradrenaline (NA) in the presence of 0.5mM of ascorbic acid for 12–16 h. The labeled PC12 cells were incubated with the fresh complete DMEM for 1–5 h to remove unincorporated \[^{3}H\] NA. The cells were washed once with PSS, and NA secretion was stimulated with 200 μl of PSS or high K^{+}-PSS (containing 81 mM NaCl and 70 mM KCl). Secretion was terminated after a 15-min (high K^{+}) incubation at 37°C by chilling to 0°C, and samples were centrifuged at 4°C for 3 min. Supernatants were removed, and the pellets were solubilized in 0.1% Triton X-100 for liquid scintillation counting.

### 2.13 Protein interaction experiments between recombinant syntaxin-11 and Munc18-2

Mouse syntaxin-11 cDNA was subcloned into pGex-KG using EcoRI/XbaI sites. pGex-KG-syntaxin-11 along with empty vector were transformed into BL21 (Codon+) cells and the bacteria were grown at 37°C until confluent. Recombinant protein expression was induced by adding 50 μM isopropyl-β-d-thiogalactopyranoside at 30°C for 3 h. Cells were lysed using 0.1% Triton X-100 and sonications in PBS containing 1 mM EDTA and protease inhibitors (10 μg/ml leupeptin and 10 μg/ml aprotinin). Supernatant portion were then mixed with glutathione agarose beads (Pierce Biotechnology, Rockford, IL). Next day, mixtures were washed extensively with PBS containing 20% sucrose, PBS containing 0.1% Triton X-100, and PBS in order. Samples were saved for binding with Munc18-2. EmGFP, wild-type Munc18-2-EmGFP, and hydrophobic pocket mutants were transfected into HEK-293FT cells. After 3 days, cells were lysed with KGlu
buffer containing 20 mM HEPES, pH 7.2, 120 mM KGl, 20 mM potassium acetate, 2 mM EGTA, and 0.1% Triton X-100 as a detergent. After centrifugation, KGl buffer plus detergent containing solubilized lysates were mixed with either ten micrograms of GST or GST-syntaxin-11. Next day, mixtures were washed extensively with KGl buffer containing 0.1% Triton X-100. Samples were then dried, and 2X SDS-PAGE sample buffer were added and boiled. The supernatant was subjected to SDS-PAGE followed by Coomassie Blue staining and immunoblots with mouse monoclonal anti-EmGFP antibody.

2.14 Yeast two-hybrid assays

Rat full-length wild-type Munc18-1 or Munc18-2 and one mutant (K46E, E59K or K46E/E59K) with SNM were subcloned into the EcoRI/PstI sites of a bait vector, pLexN. The cytoplasmic part of rat syntaxin-2 and syntaxin-3 was subcloned into the EcoRI/BglII sites of prey vector pVP16-3 (Okamoto and Südhof, 1997). The syntaxin-3 open mutant (L165E/E166A) (Dulubova et al., 1999) was generated by site-directed mutagenesis on pVP16-3-rat syntaxin-3. Yeast strain L40 (Vojtek and Cooper, 1993) was transfected with bait and prey vectors by using the lithium-acetate method (Schiestl and Gietz, 1989). Transformants were plated on selection plates lacking uracil, tryptophan and leucine. After 2 days of incubation at 30°C, colonies were inoculated into supplemented minimal medium lacking uracil, tryptophan and leucine, and placed in a shaking incubator at 30°C for 2 days.

β-Galactosidase assays were performed as follows. Yeast cells were chilled on ice and harvested by centrifugation (2000 rpm for 5 min). The collected yeast cells were resuspended in 250 μl of breaking buffer (100 mM Tris-Cl, pH 8.0, 1 mM dithiothreitol, and 20% glycerol). Then, glass beads (0.45-0.5mm; Sigma Chemical) were added to the yeast suspension to a level just below the meniscus of the liquid, followed by 12.5 μl of phenylmethylsulfonyl fluoride stock
solution (40 mM in 100% isopropanol stored at 4°C). The mixture was then vortexed six times at top speed in 15-s bursts. After that, another 250 μl of breaking buffer was added, mixed well, and centrifuged for 1 min. The liquid extract was withdrawn and transferred to new tubes. The extracted liquid was further clarified by centrifuging for 15 min in a micro-centrifuge. To perform the assay, 80 μl of the extract was added to 720 μl of Z buffer (60 mM Na₂HPO₄, 40 mM NaH₂PO₄, 10 mM KCl, 1 mM MgSO₄, and 2.7 ml/l β-mercaptoethanol, pH 7.0). The mixture was then incubated in a water bath at room temperature for 5 min. The reaction was initiated by adding 0.16 ml of stock solution (4 mg/ml o-nitrophenyl-β-D-galactoside in Z buffer; 4°C), and the reaction mixture was incubated at room temperature. The reaction was precisely terminated at the end of a 7-min incubation by addition of 0.4 ml of 1 M Na₂CO₃ stock solution in distilled water, and the optical density of the reaction mixture was measured at 420 nm by using a spectrophotometer. At the same time, the protein concentration in the extract was measured using Bradford dye-binding assay. A standard curve was prepared using serial dilutions of BSA dissolved in breaking buffer. 10 μl of the extract was added to 1 ml of the Bradford reagent (Bio-Rad Laboratories, Hercules, CA), and the change in colour was measured at 595 nm by using a spectrophotometer. The specific activity of β-galactosidase in the extract was calculated according to the following formula: (OD₄₂₀ x 1.36)/[0.0045 x protein concentration [mg/ml] x extract volume [0.08ml] x 7 min], where OD₄₂₀ is the optical density of the product o-nitrophenol at 420 nm. The factor 1.36 corrects for the reaction volume, and the factor 0.0045 is the optical density of 1 nmol/ml solution of o-nitrophenol. The unit of β-galactosidase–specific activity is therefore expressed as nano-moles per minute per milligram of protein.
2.15 Cell preparation for confocal immunofluorescence microscopy

Sterilized circular glass coverslips (0.25 mm in width, 1.8 cm in diameter) were placed in 2.2-cm wells within 12-well cell culture plates. The coverslips were then coated for 1 h with poly-d-lysine (0.1 mg/ml) at room temperature. RBL-2H3 cells were allowed to adhere to the coverslips for 3 days. Then the cells were washed with phosphate-buffered saline (PBS) and fixed for 15 min with PBS containing 4% paraformaldehyde (PFA). PFA was then removed from each well, and cells were rinsed three times (10 min each time) with 1 ml of PBS per well. The fixed cells were then permeabilized with PBS containing 0.2% Triton X-100 and 0.3% bovine serum albumin (BSA) for 5 min, followed by three washes with PBS. Nonspecific sites were blocked for 1 h at room temperature in PBS containing 0.3% BSA. For the primary antibody against syntaxin-11, we used either GST or GST-syntaxin-11 absorbed antibody to ensure specificity. Briefly, rabbit polyclonal anti-syntaxin-11 antibody was absorbed overnight with twenty micrograms of GST or GST-syntaxin-11 in PBS + 0.3% BSA such that antibody concentration is 1:500. The supernatant was then applied to the permeabilized cells as the primary antibody (1:1000) for 1 h. For the primary antibody against Munc13-4-EmGFP, anti-GFP antibody (1:1000) was used. For syntaxin-HA overexpression, the mouse monoclonal HA antibody was applied as the primary antibody (1:1000) for 1 hour. The primary antibodies were applied at 1:50 for rabbit monoclonal syntaxin-3 antibody, 1:250 for mouse monoclonal GFP antibody, 1:1000 for mouse monoclonal TGN38 and GM130, and 1:1000 for rabbit polyclonal calnexin antibody overnight at room temperature. After three washes in blocking buffer, either Alexa-Fluor-488 or Rhodamine-x-conjugated goat anti-mouse or anti-rabbit secondary antibodies were applied as 1:1000 for 1 hour at room temperature. Samples were washed again three times in blocking buffer and 300 nM DAPI was applied for 30 minutes. The slides were mounted in Fluoromount-G reagent (Southern Biotechnology, Birmingham, AL). Immunofluorescence staining was recorded with a laser
confocal scanning microscope (LSM510 or LSM710; Carl Zeiss, Jena, Germany) with an oil immersion objective lens (63×).

2.16 Quantification of confocal immunofluorescence microscopy images

The quantification of syntaxin-3 protein level was performed using ImageJ (NIH, Bethesda, MD). First, all raw files of confocal images were exported to TIFF then each image was opened on ImageJ. For syntaxin-3 overall intensity, an entire RBL-2H3 cell was manually selected using Freehand Selection, then mean intensity was calculated using the ‘Analyze→Measure’ option. For soma versus tip analysis of syntaxin-3 localization, either soma or tip of the RBL-2H3 cell was selected using Freehand Selection. Then, the intensity was calculated using the ‘Analyze→Measure’ option. The total intensity of syntaxin-3 level was calculated as above. Then, the value of intensity at the soma or tip was normalized to the total intensity of syntaxin-3 and given in percent. At least 20 cells were analyzed for each knockdown or rescue experiment and the mean values were presented.

2.17 Homology modeling of Munc18-2 and syntaxin-3

The homology model of rat Munc18-2 (GenBank accession no. U20283.1) and rat syntaxin-3 (GenBank accession no. L20820.1) was constructed based on a template crystal structure of neuronal Sec1–syntaxin-1A complex (PDB 3C98) (Misura et al., 2000). The amino acid sequences of Munc18-2 and syntaxin-3 were inputted into Zdock Server software and the predicted structure of the protein complex was visualized using Jmol software.
2.18 Homology modeling of Munc13-4 C2A and C2B domains

The homology model of human Munc13-4 C2A and C2B (NCBI Reference Sequence Accession no. NM_199242.2) was constructed based on a template crystal structure of rat Munc13-1 C2B (PDB: 3KWU) (Shin et al., 2010) and rat syt-1 C2B (PDB: 1TJX) (Cheng et al., 2004). Briefly, sequence of human Munc13-4 C2A (AA: 84-263) or C2B (AA: 904-1047) was inputted into SWISS-MODEL server along with template crystal structures (Arnold et al., 2006; Biasini et al., 2014; Guex et al., 2009; Kiefer et al., 2009). The predicted crystal structures of C2 domains of Munc13-4 were generated based on sequence homology and were visualized with Jmol software.

2.19 Total Internal Reflection Fluorescence Microscopy

RBL-2H3 cells were transfected with NPY-mCherry using electroporation (250 V, 975 μF, GenePulser, BioRad, Hercules, CA) 4 days prior to TIRFM. 2 days later, fluorescence signals were checked under epifluorescence microscope to make sure different cell-lines exhibit similar fluorescent intensity. Then the cells were plated on glass coverslip (25 mm diameter, Fisher Scientific, Waltham, MA) in 6-well plates. 2 days after plating, TIRFM was performed. TIRF microscope setup was constructed based on the prismless and through-the-lens configuration. Briefly, a condenser coupling multiple lasers (440 nm, 488 nm and 543 nm) was attached to the back port of Nikon TE2000U inverted microscope (Nikon, Mississauga, ON, Canada), equipped with a 60X oil immersion objective lens (NA =1.49). We used a 543-nm beam to excite mCherry, and a 488RDC longpass dichroic filter and a 525/50-nm band-pass emission filter (Chroma Technology Corp, Bellows Falls, VT). Images were collected by using the cooled 16-bit EM-CCD camera (Cascade 512, Roper Scientific, Ottobrunn, Germany) at the left port of the microscope. The penetration depth of the evanescent field (~100 nm) was aligned
by measuring the incidence angle of the 543-nm laser beam with a prism (n = 1.5163). A chamber containing coverslip was kept at 37°C with a microprocessor embedded temperature heater. A field of view containing 5-10 cells was chosen. Images were acquired at 5-Hz with a 100-ms exposure time by a Nikon NIS-Elements software. 30 seconds after start of image acquisition, 2.5 μM ionomycin was gently added onto the cells to minimize shifting of field of view. 2.2 mM and 10 mM Ca^{2+} containing PSS + 0.1% BSA buffer was used as bath. Total of 7 minutes were obtained per recording.

2.20 TIRFM analysis

TIRFM movies were analyzed using ImageJ (NIH, Bethesda, MD). Briefly, images were loaded on ImageJ as AVI file. Using a MultiDot function of ImageJ, every visually docked vesicles before the stimulation were marked manually (on average, 50~60 vesicles/cell). Then each dot was transformed into 2 pixel radius circle. Manual adjustment of circle location was made to make sure that the vesicles are fully surrounded by 2 pixel radius circle. Mean intensity of the circle along each success frames was quantified using MultiMeasure function of ImageJ. Vesicles that were visible before stimulation but did not undergo abrupt brightening followed by decay were considered as docked but not fusing vesicles. Number of non-fusing vesicles / total vesicles per cell * 100% was expressed as non-fusion granule %. Among the fused vesicles per cell, fluorescent intensity increase was calculated from (peak intensity after stimulation – baseline intensity) / baseline intensity * 100%. They were then expressed as a population histogram. For kinetic, the average time taken from the application of stimulation to the peak of fluorescent intensity was calculated.
2.21 Statistical Analysis

Data are presented as the means ± standard error of the mean (SEM) determined from indicated number of independent experiments. Statistical analysis was performed using independent t-test as indicated in the text or the figure legend. Statistical significance was determined at the level of p<0.05.
Chapter 3: Hydrophobic pocket of Munc18 participates in mast cell degranulation via regulation of syntaxin-11

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3.1 Rationale of the study

The SNARE protein complex orchestrates the central role in membrane fusion (Söllner, 2003) and this process is further modulated by indispensable proteins, such as Munc18 (Hata et al., 1993; Verhage et al., 2000), through interactions with one or more SNARE proteins, particularly cognate syntaxins. Recent studies have revealed the critical roles of the SNARE proteins and their regulators in exocytosis from immune cells and platelets. Particularly, genetic analysis of familial hemophagocytic lymphohistiocytosis (FHL) type-5 patients identified mutations in Munc18-2 (Côte et al., 2009; zur Stadt et al., 2009). Nonetheless, the underlying mechanisms, particularly structural determinants of the functions of Munc18 in immune cell exocytosis have not been investigated in a detailed manner.

The Munc18 family of proteins have been revealed to play multiple functions, which include (i) governing protein levels and trafficking of cognate syntaxin (Arunachalam et al.,
2008; Han et al., 2011; Han et al., 2009; Medine et al., 2007; Rowe et al., 2001; Rowe et al., 1999) and (ii) priming/stimulating membrane fusion through the interaction with the SNARE complex (Shen et al., 2010; Shen et al., 2007; Südhof and Rothman, 2009; Tareste et al., 2008). The regulation of Munc18 toward syntaxin contributes to stabilizing the protein level of cognate syntaxin and in some cases, trafficking them to appropriate intracellular compartments. In some FHL5 patients whose functional Munc18-2 protein is absent, a strong reduction of syntaxin-11 level was found in their immune cells (Côte et al., 2009; zur Stadt et al., 2009). The priming function of Munc18 is believed to be mediated in part through the interaction between Munc18 hydrophobic pocket region and syntaxin N-peptide. The functional significance of this interaction was shown that through this binding mode, Munc18-1 interacts with the SNARE complex that results in the stimulation of the SNARE-dependent liposome fusion (Shen et al., 2007). Consequently, several studies including a recent rescue experiment using syntaxin KD neurons, have demonstrated the importance of the syntaxin-1 N-peptide in synaptic vesicle exocytosis (Khvotchev et al., 2007; McEwen and Kaplan, 2008; Rathore et al., 2010; Zhou et al., 2013). On the other hand, the Munc18-1 hydrophobic pocket has been suggested to play limited or dispensable roles in exocytosis. For example, in the rescue experiments of Munc18-1 single and Munc18-1/2 double KD PC12 cells as well as Munc18-1 KO neurons, various mutants (F115E, E132A, F115E/E132A, L130K) in the hydrophobic pocket region of Munc18-1 have exhibited a very mild or no impairment in their ability to rescue exocytosis in comparison to the WT (Han et al., 2009; Malintan et al., 2009; Meijer et al., 2012), leaving the function of the hydrophobic pocket region of Munc18 to remain unclear.

Analysis of Munc18-2 in FHL5 patients has identified E132A mutation within the hydrophobic pocket region of Munc18-2 (Cetica et al., 2010), which indicates the likely importance of this residue in the function of Munc18-2 mediating degranulation. Using RBL-
2H3 cells as a model of mast cells, we evaluated the functional significance of Munc18-2 in mast cell degranulation, as well as potential molecular mechanism of its hydrophobic pocket region.
3.2 Results

3.2.1 Expression of various Munc18 and syntaxin proteins in RBL-2H3 cells

We first examined the protein expressions of Munc18 and syntaxin isoforms in RBL-2H3 cells by comparing with neurosecretory PC12 cells as a reference (Figure 3.1). Since both PC12 cells and RBL-2H3 cells are derived from rat, direct comparison is possible between the two cell lines. Level of Munc18-1 expression in RBL-2H3 cells was substantially less than in PC12 cells. In contrast, the level of Munc18-2 was more abundant in RBL-2H3 cells than in PC12 cells. Neuronal syntaxin-1A/1B isoform was strongly expressed in PC12 cells, but was completely absent in RBL-2H3 cells. Conversely, syntaxin-11 was expressed in RBL-2H3 cells, but not in PC12 cells. The levels of plasmalemmal syntaxin-2, -3, -4 as well as endosomal syntaxin-7 and -8 were similar between these two cell-lines. Selective expression of syntaxin-11 in RBL-2H3 cells is consistent with previous findings that this protein is enriched in tissues of the immune system including the thymus, spleen and lymph nodes while it is largely absent in the brain (Prekeris et al., 2000; Valdez et al., 1999).
Figure 3.1 Expression profiles of Munc18-1 and -2 as well as various syntaxin proteins present in wild-type RBL-2H3 and PC12 cells. Twenty micrograms of homogenates from wild-type RBL-2H3 and PC12 cells were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) and immunoblotting using indicated antibodies on the right. * indicates a non-specific band observed with rabbit polyclonal anti-Munc18-2 antibody.
### 3.2.2 shRNA mediated stable KD of Munc18-1, Munc18-2 single KDs and Munc18-1/2 DKD

To elucidate the function of Munc18 in degranulation of RBL-2H3 cells, we generated stable Munc18-1 and Munc18-2 single KD as well as Munc18-1/2 DKD cells using lentivirus mediated shRNA. Then the Munc18-1/2 DKD cells were examined to see whether the protein levels of various syntaxin isoforms are altered by the KD of Munc18 through immunoblot analysis (Figure 3.2, quantification in Figure 3.3). In Munc18-2 null FHL5 patients, the level of syntaxin-11 in NK cells, CTLs and platelets was found to be decreased dramatically (Al Hawas et al., 2012; Côte et al., 2009; zur Stadt et al., 2009). The immunoblotting showed that there were ~20% reductions in syntaxin-11 level in Munc18-2 KD and more dramatic decrease in Munc18-1/2 DKD cells at around ~60%. However, no changes in the protein level of plasma membrane localized syntaxin isoforms such as -2, -3 and -4 were observed. Since syntaxin-11 has been shown to be localized on endosomal membranes including late endosomes and lysosomes in macrophages and to regulates trafficking steps between late endosomes, lysosomes and the cell surface (Offenhäuser et al., 2011), other endosomal syntaxins such as syntaxin-7 and -8 were tested to see whether the reduction in protein level is limited to syntaxin-11. A very mild (~15%) reduction of syntaxin-7 level was found in Munc18-1/2 DKD cells but not in Munc18-2 single KD cells, while syntaxin-8 level was unchanged in all KD cells. Thus, Munc18-1/2 plays a critical role in maintaining the level of syntaxin-11 and, to a much lesser degree, of syntaxin-7 in mast cells.
Figure 3.2 Knockdown of both Munc18-1 and Munc18-2 results in dramatic reductions of syntaxin-11 protein level. Stable Munc18-1, -2 single KD and Munc18-1/2 DKD RBL-2H3 cells were generated by lentivirus mediated shRNA. Twenty micrograms of cell homogenates were analyzed by SDS-PAGE and immunoblotting using indicated antibodies on the right. * indicates a non-specific band observed with rabbit polyclonal anti-Munc18-2 and anti-syntaxin-8 antibodies.
Figure 3.3 Quantification of various syntaxin isoforms reveals a strong down-regulation of syntaxin-11 and a mild decrease in syntaxin-7 level in Munc18-1/2 DKD RBL-2H3 cells. Images of these proteins on the film were quantified for densitometry and normalized to the control using ImageJ. Error bars indicate SEM (n = 3-8). The statistical significance of the differences in syntaxin-11 level between control, Munc18-2 KD, and Munc18-1/2 DKD is indicated. *, p < 0.05 (Student’s t-test).
3.2.3 Munc18 is essential for mast cell degranulation

To measure secretion capability of the control and the Munc18-1/2 DKD mast cells, we examined degranulation by measuring the release of β-hexosaminidase (Figure 3.4) (Tadokoro et al., 2007). The secretion of β-hexosaminidase from RBL-2H3 cells was induced by IgE antibody and HSA antigen as well as ionomycin. Ionomycin is a Ca\(^{2+}\) ionophore that directly induces an increase in intracellular Ca\(^{2+}\) concentration therefore triggering degranulation. We found severe reductions in β-hexosaminidase release in Munc18-2 single KD cells and an almost complete abolishment in Munc18-1/2 DKD cells. In addition, a significant, yet less reduction was observed in Munc18-1 single KD cells. These reductions in β-hexosaminidase release were observed in both IgE-dependent and ionomycin-induced secretion, suggesting that the impairment lies in secretory machinery itself. The strong reduction in β-hexosaminidase release in Munc18-2 KD cells is consistent with a previous report that antisense-mediated down-regulation of Munc18-2 caused ~50% reduction in β-hexosaminidase release (Tadokoro et al., 2007).
Figure 3.4 Knockdown of both Munc18-1 and Munc18-2 results in striking degranulation defects in RBL-2H3 cells. β-hexosaminidase release was stimulated by applying 0.01 μg/ml DNP-IgE and 50 ng/ml DNP-HSA and 0.5 μM, 2.5 μM ionomycin for 1 h from control, Munc18-1 KD, Munc18-2 KD, and Munc18-1/2 DKD cells. Error bars indicate SEM (n = 5). The statistical significance of the differences in β-hexosaminidase release between control and Munc18-1 KD, Munc18-2 KD and Munc18-1/2 DKD is indicated. *, p < 0.05 (Student’s t-test).
3.2.4 Munc18-1 and -2 can effectively support mast cell degranulation

The greater impairment of degranulation by Munc18-2 KD than Munc18-1 KD could be due to either the difference in their expression levels (i.e., more abundant expression of Munc18-2 than Munc18-1) in RBL-2H3 cells or the functional specificity between these two isoforms. To examine whether there is any functional specificity exists between Munc18-1 and -2, a rescue experiment using both isoforms were performed. A previous study has shown that Munc18-1 is more effective in rescuing neurosecretion from Munc18-1-deficient chromaffin cells than Munc18-2 (Gulyás-Kovács et al., 2007). At first, we examined whether stable expression of Munc18-1 rescues neurosecretion of Munc18-1/2 DKD PC12 cells better than that of Munc18-2 (Figure 3.5). In these experiments, Munc18-1 and -2 were expressed in three different configurations: without any tag, with myc, or Emerald GFP (EmGFP) tag (Figure 3.5 A). In all cases, Munc18-1 rescued defective secretion of Munc8-1/2 DKD cells significantly better than Munc18-2 (Figure 3.5 B). Interestingly, the recovery of syntaxin-1 level was also higher in Munc18-1 rescue than that of Munc18-2 (Figure 3.5 A). Thus, our results confirmed that there is the functional specificity exists between the two isoforms in neuronal secretion and this difference seems to be related with their ability to restore syntaxin-1 protein level. This experiment also ensures that an addition of EmGFP tag to Munc18 does not affect the folding or function of the protein.

Then the rescue of Munc18-1/2 DKD RBL-2H3 cells was performed by stably expressing Munc18-1 or Munc18-2 fused with EmGFP (Figure 3.6). Upon re-expression of Munc18-1 or -2, the level of syntaxin-11 was rescued in both cases (Figure 3.6 A). In addition, the defective β-hexosaminidase release was comparably rescued by re-expression of either Munc18-1 or -2 (Figure 3.6B). The rescued cells exhibited a similar level of degranulation to that of control cells.
indicating that re-expressed Munc18-1 or -2 could fully recover the degranulation. These results suggest that there is no clear functional specificity between these two isoforms in regulating mast cell degranulation as well as stabilizing the protein level of syntaxin-11.
**Figure 3.5 Stable re-expression of Munc18-1 restores syntaxin-1 protein level and secretion defects better than Munc18-2 in PC12 cells.**

(A) Twenty micrograms of homogenates of Munc18-1/2 DKD PC12 cells rescued with EmGFP, wild-type Munc18-1, and Munc18-2 (without tag, with myc, and with EmGFP) were analyzed by SDS-PAGE and immunoblotting using indicated antibodies on the right. (B) NE release was stimulated by 70 mM KCl for 15 min in the rescued cells. Error bars indicate SEM (n = 9). The statistical significance of the differences in NE release between wild-type Munc18-1 and Munc18-2 rescued PC12 cells in each tag configuration are indicated. *, p < 0.05 (Paired t-test). * in (A) indicates non-specific band observed with rabbit polyclonal anti-Munc18-2 antibody which co-migrated with Munc18-2-EmGFP.
Figure 3.6 Stable re-expression of wild-type Munc18-1 or Munc18-2 equally restores syntaxin-11 protein levels and degranulation defects of Munc18-1/2 DKD RBL-2H3 cells. (A) Munc18-1/2 DKD RBL-2H3 cells were infected with lentiviruses that express EmGFP, wild-type Munc18-1-EmGFP, and wild-type Munc18-2-EmGFP. Twenty micrograms of cell homogenates were analyzed by SDS-PAGE and immunoblotting using indicated antibodies on the right. (B) β-hexosaminidase release was stimulated by applying 0.01 μg/ml DNP-IgE and 50 ng/ml DNP-HAS as well as 0.5 μM, and 2.5 μM ionomycin for 1 h from wild-type RBL-2H3 cells, and rescued cells. Error bars indicate SEM (n = 9). * in (A) indicates non-specific band observed with rabbit polyclonal anti-Munc18-2 antibody which co-migrated with Munc18-2-EmGFP.
3.2.5 The mutations in the hydrophobic pocket of Munc18 cause severe impairments in their rescuing activity

To elucidate the role for the hydrophobic pocket of Munc18-2 in mast cell degranulation, point mutations in this region were introduced. Namely, F115E, E132A and F115E/E132A were generated which include the mutation discovered in the FHL5 patient (E132A) (Figure 3.7 A). These mutants were expressed in DKD RBL-2H3 cells and examined their ability to restore degranulation as well as syntaxin-11 protein level in comparison with the WT (Figure 3.7 B). The protein level of all mutants was similar to that of the WT, implying that these mutations do not cause major problems in protein folding or expression. Unlike the WT Munc18-2 however, these mutants did not restore the level of syntaxin-11. Strikingly, these mutants also showed severe impairments in their ability to rescue IgE-dependent and ionomycin-induced β-hexosaminidase release of DKD RBL-2H3 cells (Figure 3.7 C). Also, any additive deteriorating effects of the double mutant (F115E/E132A) over single mutants was not observed. Our results indicate that the hydrophobic pocket of Munc18-2 plays a crucial role in mast cell degranulation at least in part via its regulation of syntaxin-11 expression.

The lack of rescue by the hydrophobic pocket mutants in Munc18-2 is surprising since it was found that the expressions of Munc18-1 with the identical mutations showed 70-80% of the rescue ability compared to the WT using Munc18-1 KD and Munc18-1/2 DKD PC12 cells (Han et al., 2009; Malintan et al., 2009). Furthermore, the same (F115E) and a similar (L130K) mutants have shown a complete rescue ability in autaptic neurotransmission of Munc18-1 deficient neurons (Meijer et al., 2012). By taking advantage of the fact that Munc18-1 can also rescue degranulation of the DKD RBL-2H3 cells, we examined whether the same hydrophobic pocket mutants of Munc18-1 can restore mast cell degranulation and syntaxin-11 level (Figure
3.8 A and B). Similar to that of Munc18-2, the mutants of Munc18-1 also showed strong impairment in restoring the degranulation and syntaxin-11 level. Therefore, the hydrophobic pocket mutations cause similar functional impairment for both Munc18 isoforms. Our results suggest that the contribution of hydrophobic pocket region of Munc18-1 are different between neuronal and immune cell exocytosis.
**Figure 3.7** Hydrophobic pocket mutants of Munc18-2 do not restore syntaxin-11 protein levels or degranulation defects of Munc18-1/2 DKD RBL-2H3 cells. (A) Sequence alignment between Munc18-1, -2, -3 indicating F115 and E132 are conserved residues in Munc18 isoforms. (B) Munc18-1/2 DKD RBL-2H3 cells were infected with lentiviruses that express EmGFP, wild-type Munc18-2-EmGFP, and hydrophobic pocket mutant Munc18-2-EmGFP (F115E, E132A, F115E/E132A). Infected cells were selected with blasticidin (20 μg/ml). Twenty micrograms of homogenates of surviving cells were analyzed by SDS-PAGE and immunoblotting using indicated antibodies on the right. * indicates non-specific band observed with rabbit polyclonal anti-Munc18-2 antibody which co-migrated with Munc18-2-EmGFP. (C) β-hexosaminidase release was stimulated by applying 0.01 μg/ml DNP-IgE and 50 ng/ml DNP-HAS as well as 0.5 μM, and 2.5 μM ionomycin for 1 h from rescued cells. Error bars indicate SEM (n = 5).
Figure 3.8 Hydrophobic pocket mutants of Munc18-1 do not restore syntaxin-11 protein level or degranulation defects of Munc18-1/2 DKD RBL-2H3 cells. (A) Munc18-1/2 DKD RBL-2H3 cells were infected with lentiviruses that express EmGFP, wild-type Munc18-1-EmGFP, and hydrophobic pocket mutant Munc18-1-EmGFP (F115E, E132A, F115E/E132A). Infected cells were selected with blasticidin (20 μg/ml). Twenty micrograms of homogenates of surviving cells were analyzed by SDS-PAGE and immunoblotting using indicated antibodies on the right. (B) β-hexosaminidase release was stimulated by applying 0.01 μg/ml DNP-IgE and 50 ng/ml DNP-HAS as well as 0.5 μM, and 2.5 μM ionomycin for 1 h from rescued cells. Error bars indicate SEM (n = 6).
3.2.6 The mutations in hydrophobic pocket of Munc18 attenuate the direct interaction with syntaxin-11

The fact that the hydrophobic pocket mutants were unable to restore syntaxin-11 in Munc18-1/2 DKD mast cells suggest that these mutants may have compromised interactions with syntaxin-11. To test whether the impairment of syntaxin-11 restoration by the hydrophobic pocket mutants are due to reduced interactions between two proteins, we performed protein interaction experiments. For this purpose, purified recombinant GST-syntaxin-11 and KGl buffer solubilized lysates of HEK-293FT cells which were transfected with WT Munc18-2 and mutants fused with EmGFP were used. Then the pull down mixtures were analyzed by immunoblotting with mouse monoclonal anti-EmGFP antibody and Coomassie Blue staining (Figure 3.9 A top panel and B). It was found that the WT Munc18-2 directly binds to syntaxin-11 as the strong Munc18-2-EmGFP band was seen in both immunoblotting and Coomassie Blue staining. On the other hand however, syntaxin-11 failed to pull down all hydrophobic pocket mutants of Munc18-2. This was not due to unequal loadings or aberrant expressions of mutants, as there were similar loadings between lanes (Figure 3.9 A bottom panel) and comparable protein levels of the mutants to that of WT were seen in lysate lanes (Figure 3.9 A top panel). Collectively, our data suggest that Munc18 works as a stabilizing regulator for syntaxin-11 and the hydrophobic pocket of Munc18 is the essential region that governs the function in mast cell degranulation.
Figure 3.9 Hydrophobic pocket mutants of Munc18-2 abolish the direct interaction with syntaxin-11. (A, Top) Solubilized lysates of HEK-293FT cells that were transfected with EmGFP, wild-type Munc18-2-EmGFP, F115E, E132A, and F115E/E132A of Munc18-2-EmGFP were incubated with ten micrograms of GST or GST-syntaxin-11 attached to glutathione agarose beads. Mixtures were washed extensively and proteins were analyzed on SDS-PAGE and immunoblotting with mouse monoclonal anti-EmGFP antibody. Solubilized lysates of HEK-293FT cells were loaded in lysate lanes. (A, Bottom) Ponceau S staining of the above membrane showing similar loadings between lanes. * indicates wild-type Munc18-2-EmGFP band that was pulled down by GST-syntaxin-11. (B) The binding mixture between solubilized lysates of HEK-293FT cells that were transfected with wild-type Munc18-2-EmGFP, F115E, E132A, F115E/E132A and ten micrograms of GST-syntaxin-11 were analyzed on SDS-PAGE followed by Coomassie Blue staining. Note only wild-type Munc18-2-EmGFP was pulled down by GST-syntaxin-11 but not any of the mutants.
3.2.7 Mislocalization of syntaxin-11 is not observed despite decreased of protein level in Munc18-1/2 DKD mast cells

Previously it was found that Munc18-1/2 DKD not only causes a strong reduction in syntaxin-1 level but also perturbation of its plasmalemmal localization in PC12 cells (Han et al., 2011; Han et al., 2009; Malintan et al., 2009). Also, in *C. elegans*, Unc-18 promotes anterograde trafficking of Unc-64, which is *C. elegans* orthologue of syntaxin-1 (McEwen and Kaplan, 2008). We therefore examined whether the sub-cellular localization of syntaxin-11 can be significantly altered by Munc18-1/2 DKD in RBL-2H3 cells (Figure 3.10 and low magnification data in Figure 3.11). Confocal immunofluorescence microscopy using rabbit polyclonal anti-syntaxin-11 antibody showed punctuate stainings. This staining was specific to syntaxin-11 since the signal was strongly reduced upon pre-absorption of the antibody with the antigen, GST-syntaxin-11, but not with GST alone. Importantly, the syntaxin-11 immunostaining was unchanged in Munc18-1/2 DKD cells (Figure 3.10 and 3.11). Thus, we conclude that although syntaxin-11 protein level is dramatically reduced, its localization is not affected by Munc18-1/2 DKD in mast cells.
Figure 3.10 Confocal immunofluorescent microscopy reveals that sub-cellular localization of syntaxin-11 is unaltered upon Munc18-1/2 DKD. Control and stable Munc18-1/2 DKD RBL-2H3 were permeabilized and stained with either GST or GST-syntaxin-11 absorbed rabbit polyclonal anti-syntaxin-11 antibody followed by Alexa488-conjugated goat anti-rabbit antibody and DAPI. Green indicates syntaxin-11, blue indicates DAPI. (A) control (B) Munc18-1/2 DKD cells. Note there is substantially diminished green intensity from right panels where GST-syntaxin-11 absorbed anti-syntaxin-11 antibody was used to stain. Bar, 10 μm.
Figure 3.11 Confocal immunofluorescent microscopy images ensuring specificity of the antibody as well as unaffected sub-cellular localization of syntaxin-11 upon Munc18-1/2 DKD. Control and stable Munc18-1/2 DKD RBL-2H3 were permeabilized and stained with either GST or GST-syntaxin-11 absorbed rabbit polyclonal anti-syntaxin-11 antibody followed by Alexa488-conjugated goat anti-rabbit antibody and DAPI. Green indicates syntaxin-11, blue indicates DAPI. Bar, 10 μm.
3.2.8 Reduction of syntaxin-11 by itself does not lead to degranulation defects in mast cells

Throughout this study, a positive correlation was observed between the syntaxin-11 level and degranulation level in the Munc18 KD RBL-2H3 cells as well as their rescued cells. However, Munc18-2 single KD cells resulted in severe degranulation defects even though its syntaxin-11 is reduced by merely 20% (Figure 3.2 and 3.3). This implies that the secretion defect of Munc18-2 single KD is unlikely due to the reduction of syntaxin-11 alone. Therefore, we investigated whether syntaxin-11 down-regulation can account for a dramatic degranulation defect in Munc18-1/2 DKD cells. To answer this, the secretion capability of stable syntaxin-11 KD RBL-2H3 cells which exhibited ~80% reduction in syntaxin-11 level was investigated (Figure 3.12). This reduction was more than the reduction (~60%) of syntaxin-11 level caused by Munc18-1/2 DKD (Figure 3.1 B). Despite stronger reduction however, a significant reduction in β-hexosaminidase release in syntaxin-11 KD cells was not observed compared to control (Figure 3.12). At first, this was unexpected because mutations in syntaxin-11 cause severe reductions in secretion from CTLs, NK cells, neutrophils and platelets in FHL4 patients as well as recently established syntaxin-11 knockout mice (Bryceson et al., 2007; D'Orlando et al., 2012; Kögl et al., 2012; Ye et al., 2012). However, our result is consistent with the result of syntaxin-11 knockout mice in which mast cell degranulation was not significantly impaired (D'Orlando et al., 2012). Thus, it is likely that other syntaxin isoforms can compensate for the reduced level of syntaxin-11 in mast cell degranulation. Taken together, the striking impairment of degranulation by Munc18 KD in mast cells is not entirely caused by the reductions in syntaxin-11 level.
Figure 3.12 Strong and stable knockdown of syntaxin-11 in RBL-2H3 cells does not show defects in β-hexosaminidase release. (A) Stable syntaxin-11 knockdown RBL-2H3 cells were generated by lentivirus mediated shRNA. Twenty micrograms of cell homogenates were analyzed by SDS-PAGE and immunoblotting using indicated antibodies on the right. (B) β-hexosaminidase release was stimulated by applying 2.5 μM ionomycin for 1 h from control and syntaxin-11 KD cells. Error bars indicate SEM (n = 6).
3.3 Conclusion

In the present chapter, we have established the method to perform stable KD and rescue of the protein of interest using RBL-2H3 mast cells. This system allowed to study the structure-vs-function relationship of Munc18 in degranulation of mast cells. Importantly, the stable KD of Munc18-1/2 in RBL-2H3 cells not only abolished degranulation but also caused a significant reduction in syntaxin-11 level without affecting its localization. Thus, the Munc18 KD RBL-2H3 cells have recapitulated the phenotype of CTLs, NK cells and platelets from some of the FHL5 patients where loss of Munc18-2 expression induces striking reductions in the level of syntaxin-11 (Al Hawas et al., 2012; Côte et al., 2009; zur Stadt et al., 2009). Furthermore, the results demonstrated that re-expression of Munc18-1 or -2 effectively restores degranulation and the protein level of syntaxin-11. These results strongly indicate that Munc18 is crucial for mast cell degranulation in part through its regulation on syntaxin-11 protein level.

Since the finding of the interaction between Munc18 hydrophobic pocket and the N-peptide of syntaxin (Burkhardt et al., 2008; Hu et al., 2007), which has been shown to support the binding of Munc18-1 to the SNARE complex and facilitates the SNARE-mediated liposome fusion (Shen et al., 2007), many efforts have been made to identify the crucial role of this interaction in physiological membrane fusion. However, the crucial roles for the hydrophobic pocket of Munc18 have not been revealed in neurotransmitter release, as the mutants in this region have been illustrated to be as equally effective in rescuing the exocytosis as the wild type (Meijer et al., 2012). But in the present Chapter, we found that the point mutants in hydrophobic pocket regions strongly impair the rescuing ability of Munc18-1 and -2 in mast cell degranulation. Therefore, the functional significance of the hydrophobic pocket of Munc18 seems to be strikingly different between neuronal and immune cell exocytosis.
Although there is a positive correlation between the syntaxin-11 level and the degranulation level in our Munc18 KD RBL-2H3 cells as well as their rescued cells, the striking impairment of degranulation by Munc18 KD in mast cells is not accounted entirely by the reductions in syntaxin-11 level. For example, syntaxin-11 KD alone does not cause severe defects in degranulation. Thus, Munc18 could potential play crucial roles other than the regulation of syntaxin-11, such as regulating other isoforms of syntaxin or the priming of the SNARE-mediated exocytosis.
Chapter 4: Regulation of closed syntaxin-3 through Lys46 and Glu59 in domain 1 of Munc18 proteins is indispensable for mast cell exocytosis

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4.1 Rationale of the study

The essential function of Munc18-1 in neuronal exocytosis (Verhage et al., 2000) is known to be mediated by its complex interactions with syntaxin-1 and/or the neuronal SNARE protein complex (Han et al., 2010). At least two important functions of Munc18-1 have been proposed: (i) priming of membrane fusion via directly interacting with the SNARE complex (Han et al., 2011; Hu et al., 2010; Ma et al., 2013; Parisotto et al., 2014; Rodkey et al., 2008; Südhof and Rothman, 2009; Tareste et al., 2008; Xu et al., 2010); (ii) molecular regulator of syntaxin-1 regulating appropriate intracellular localization and protein level of syntaxin-1 (Arunachalam et al., 2008; Han et al., 2009; Malintan et al., 2009; McEwen and Kaplan, 2008; Medine et al., 2007; Rowe et al., 2001; Rowe et al., 1999; Shen et al., 2007). A similar regulating function of Munc18-2 toward syntaxin has also been discovered; in some of FHL5 patients
whose functional Munc18-2 protein is absent, there is a strong reduction of syntaxin-11 level in the immune cells (Côte et al., 2009; zur Stadt et al., 2009). However, the underlying mechanisms governing the regulating function of Munc18-2 toward its cognate syntaxins and their significance remain unclear.

In Chapter 3, Munc18 was demonstrated to be an essential player in the mast cell degranulation partly via regulation syntaxin-11 protein level. However, the impairment of degranulation by independent syntaxin-11 KD was not observed (Figure 3.12). This indicates that the degranulation defects observed in the Munc18-1/2 DKD mast cells do not seem to be functionally attributable to the reductions in syntaxin-11 level. This is in contrast to the regulating function of Munc18-1 toward syntaxin-1 and its functional contribution to the neuronal exocytosis.

Therefore, in this Chapter, two key questions about the regulating function of Munc18-2 in mast cells were asked: 1. What are the key cognate syntaxins that play crucial roles in mast cell degranulation whose level and intracellular localization are regulated by Munc18-2? 2. Is the regulating activity of Munc18-2 dependent on the physical binding between the Munc18-2 domain-1 cleft and the close conformation of the syntaxins? In this Chapter, an attempt was made to find the key cognate syntaxin isoforms whose level and intracellular localization are governed through a direct interaction with Munc18 using the established methods of generating stable knockdown and rescued mast cells.
4.2 Results

4.2.1 Munc18 regulates syntaxin-3 protein level in addition to syntaxin-11 in mast cells

The Munc18-1/2 DKD in RBL-2H3 mast cells caused severe defects in degranulation, accompanied with specific down-regulation of syntaxin-11 among the various isoforms (syntaxin-2, 3, 4, 6, 7, 8, 11) examined (Figure 3.2). However, no significant impairment in exocytosis by the stable KD of syntaxin-11 was detected (Figure 3.12). Thus, the degranulation defects observed in the Munc18-1/2 DKD mast cells do not seem to be attributable to the reduction of syntaxin-11 level by itself. Munc18-1 and -2 are known to physically bind to syntaxin-2 and -3 in addition to syntaxin-11 and neuronal syntaxin-1 (Riento et al., 1998; Tamori et al., 1998). Recent work suggests that syntaxin-3 can potentially substitute for the function of syntaxin-11 in syntaxin-11 deficient mast cells (Hackmann et al., 2013). Therefore, we re-examined for a possible change of syntaxin-3 level in Munc18-1/2 DKD mast cells using a recently generated, rabbit monoclonal anti-syntaxin-3 antibody (EPR8543, Abcam). Immunoblot analysis revealed a clear reduction in the level of syntaxin-3 in Munc18-1/2 DKD mast cells (Figure 4.1A). The quantification revealed that more than 60% of syntaxin-3 was decreased in the DKD cells when compared with the control cells (p < 0.05, Figure 4.1B). We speculate that rabbit polyclonal antibody which was used in Chapter 3 is more cross-reactive to other proteins due to its polyclonal nature, which hampered to detect the reduction of syntaxin-3 level. Also, mild reduction in the level of syntaxin-11 in Munc18-2 single KD and more drastic decrease in Munc18-1/2 DKD mast cells were confirmed (Figure 4.1).

To examine whether the observed syntaxins-3 reduction in protein level was specifically due to the KD of Munc18 proteins and not the results of any off-target shRNA effects, shRNA-resistant WT Munc18-1 or -2 fused with Emerald GFP (EmGFP) were re-expressed back to
Munc18-1/2 DKD mast cells by lentivirus mediated expression system (Figure 4.2). The full restoration of reduced syntaxin-3 level was found, in addition to the previously reported syntaxin-11 level, in both WT Munc18-1 and Munc18-2-EmGFP rescued mast cells (Figure 4.2). Our results reveal that in addition to syntaxin-11, Munc18 regulates syntaxin-3 protein level in mast cells.
Figure 4.1 Drastic reductions of syntaxin-3 and -11 in Munc18-1/2 DKD mast cells. (A) Twenty micrograms of stable Munc18-1, -2 single KD and Munc18-1/2 DKD RBL-2H3 cell homogenates were analyzed by SDS-PAGE and immunoblotting using indicated antibodies on the right. * indicates a non-specific band observed with anti-Munc18-1 antibody. (B) Quantification of syntaxin-3 protein levels in Munc18-1, -2 single KD and Munc18-1/2 DKD RBL-2H3 cells. Blots on the film were quantified for densitometry and normalized to the respective control using ImageJ. Error bars indicate SEM (n = 6). The statistical significance of the differences in syntaxin-3 level between control and Munc18-1/2 DKD is indicated. *, p < 0.05 (Student’s t-test).
Figure 4.2 Restoration of syntaxin-3 and -11 in Munc18-1/2 DKD mast cells by wild-type Munc18-1 or -2. Stable EmGFP, wild-type Munc18-1 or -2-EmGFP rescued Munc18-1/2 DKD RBL-2H3 cells were generated by lentivirus expression system. Twenty micrograms of cell homogenates were analyzed by SDS-PAGE and immunoblotting using indicated antibodies on the right. * indicates a non-specific band observed with anti-Munc18-2 antibody which co-migrated with Munc18-2-EmGFP.
4.2.2 Munc18 controls the trafficking of syntaxin-3 intracellular localization in mast cells

Severe mislocalization of syntaxin-1 is observed in single KD of Munc18-1 and DKD of Munc18-1/2 in neuroendocrine PC12 cells (Arunachalam et al., 2008; Han et al., 2011; Han et al., 2009). Similarly, in unc-18 null C. elegans, the anterograde trafficking and synaptic localization of Unc-64, an orthologue of syntaxin-1, is significantly perturbed (McEwen and Kaplan, 2008). In contrast, localization of syntaxin-11 was not changed in the Munc18-1/2 DKD mast cells (Figure 3.10 and 3.11). Therefore, we asked whether the subcellular localization of syntaxin-3 could be perturbed in addition to its reduced protein level in Munc18-1/2 DKD mast cells. Although a change in syntaxin-2 protein level was not observed by Munc18-1/2 DKD (see Figure 3.1), a possible alteration in localization of syntaxin-2 was tested since Munc18 proteins are known to physically interact with both syntaxins-2 and -3 (Hata and Südhof, 1995). For a direct comparison of both syntaxin isoforms in a systematic manner, HA (hemagglutinin) tagged exogenous syntaxin-2 and -3 was overexpressed in the control and Munc18-1/2 DKD mast cells and examined their localizations using anti-HA monoclonal antibody. Tandem HA tags were attached to the C-terminal end of these proteins (Figure 4.3 A) and were stably expressed in control and Munc18-1/2 DKD mast cells. Immunoblotting with anti-HA antibody revealed a similar level of overexpression in both syntaxin-2 and -3-HA in control and Munc18-1/2 DKD cells (Figure 4.3 B).

Upon immunostaining, we found that syntaxin-2-HA was specifically localized at the plasma membrane (Figure 4.3 C, Top panels) while syntaxin-3-HA was shown as punctate staining patterns suggesting its localizations on secretory/lysosomal granules (Figure 4.3 D, Top panels). Interestingly, strong expressions of syntaxin-3-HA at the tips of the elongated mast cells were observed. Such plasmalemmal localization of syntaxin-2-HA was not changed upon
Munc18-1/2 DKD (Figure 4.3 C, Bottom panels). Strikingly however, severe perturbation of secretory/lysosomal localizations of syntaxin-3-HA was observed in Munc18-1/2 DKD mast cells (Figure 4.3 D, Bottom panels). Namely, there were aggregations of syntaxin-3-HA in perinuclear regions of Munc18-1/2 DKD mast cells and their expressions at the tips of the mast cells were invariably absent. Thus, our results indicate that Munc18 seems to selectively regulate the intracellular localization of syntaxin-3 but not plasmalemmal localization of syntaxin-2, although it physically binds to both isoforms.

Then the localization of the endogenous syntaxin-3 was investigated by staining control and Munc18-1/2 DKD mast cells with rabbit monoclonal syntaxin-3 antibody. In accordance with the syntaxin-3-HA overexpression study (Figure 4.3 D), the endogenous syntaxin-3 was shown as discrete punctate patterns with particularly strong expressions at the tips of elongated mast cells (Figure 4.4, Top panels). For the Munc18-1/2 DKD mast cells, some level of aggregation in syntaxin-3 expressions at the perinuclear region and their absence at the tips of the cells was recapitulated (Figure 4.4, Bottom panels). Also, dimmer overall intensity for syntaxin-3 staining in Munc18-1/2 DKD cells was observed in comparison to control, consistent with their lower protein level in those cells analyzed by immunoblotting (Figure 4.1). Quantification analysis of confocal images revealed, compared with control, an overall syntaxin-3 intensity of ~35% in Munc18-1/2 DKD (Figure 4.12 A Left panel). This reduction was similar to the reduction of syntaxin-3 protein levels in the immunoblot analysis (Figure 4.1). In addition, it was found that a large population of syntaxin-3 was expressed at the tip of the control cells (70% at the tips versus 30% in the soma). However, syntaxin-3 was strikingly aggregated in the soma with nearly 80%, whereas only 20% was found at the tips of Munc18-1/2 DKD cells (Figure 4.12 A Right panel). This result, therefore, further confirms that the localization of syntaxin-3 is perturbed by the Munc18-1/2 DKD in the mast cells.
Since the re-introduction of either WT Munc18-1 or -2 back to Munc18-1/2 DKD mast cells was able to restore the reduced protein level of syntaxin-3 (Figure 4.2), their rescue ability toward perturbed localization of syntaxin-3 was asked. For this purpose, EmGFP, Munc18-1 WT-EmGFP, and Munc18-2 WT-EmGFP rescued cells were co-stained with mouse anti-GFP monoclonal and rabbit anti-syntaxin-3 monoclonal antibodies, and syntaxin-3 localization was visualized under the confocal microscopy (Figure 4.5). In comparison to EmGFP rescued cells, both Munc18-1 and -2 WT rescued cells exhibited brighter syntaxin-3 intensity in accordance with their restored protein level in both Munc18-1 and -2 WT-EmGFP cells (Figure 4.2). In addition, the strong expression pattern at the tips was rescued in both Munc18-1 and Munc18-2 WT rescued cells but not in EmGFP rescued cells. Importantly, the perinuclearly aggregated syntaxin-3 expression pattern seen in EmGFP rescued cells were re-arranged into general spread pattern in both Munc18-1 and Munc18-2 WT rescued cells (Figure 4.5). Collectively, these results strongly indicate that Munc18-mediated syntaxin trafficking function is not only present in the neuronal cells (Arunachalam et al., 2008; Han et al., 2011; Han et al., 2009; McEwen and Kaplan, 2008) but also in the mast cells.
Figure 4.3 Systematic analysis of the intracellular localization of syntaxin-2 and -3 in control and Munc18-1/2 DKD mast cells. (A) A schematic representation of recombinant rat syntaxin-2-HA (1-290) and syntaxin-3-HA (1-289) proteins. Lys289 (shown as K) are part of C-terminal end of rat syntaxin-3 downstream of TMR domain. VDSRDL sequences constitute a linker between syntaxins and tandem HA tags. (B) Stable control and Munc18-1/2 DKD RBL-2H3 mast cells overexpressing rat syntaxin-2-HA or -3-HA were generated by lentivirus expression system. Twenty micrograms of cell homogenates were analyzed by SDS-PAGE and immunoblotting using mouse monoclonal anti-HA antibody. (C and D) Syntaxin-2-HA or -3-HA overexpressing control and Munc18-1/2 DKD RBL-2H3 mast cells were permeabilized and stained with mouse monoclonal anti-HA antibody followed by Alexa488-conjugated goat antimouse secondary antibody and DAPI. Green signal indicates syntaxin-2-HA (C), syntaxin-3-HA (D), and blue signal indicates DAPI. Control (Top panels), Munc18-1/2 DKD (Bottom panels). Left panels show wide views of the cells while right panels show magnified views focusing on individual cells. Bar, 10 µm.
Confocal microscopy of endogenous syntaxin-3 reveals mislocalization in Munc18-1/2 DKD mast cells. Control and Munc18-1/2 DKD RBL-2H3 mast cells were permeabilized and stained with rabbit monoclonal anti-syntaxin-3 antibody followed by Alexa488-conjugated goat anti-rabbit secondary antibody and DAPI. Green signal indicates endogenous syntaxin-3 and blue signal indicates DAPI. Control (Top panels), Munc18-1/2 DKD (Bottom panels). Left panels show wide views of the cells while right panels show magnified views focusing on an individual cell. Bar, 10 µm.
Figure 4.5 Rescue of syntaxin-3 localization upon re-introduction of either Munc18-1-EmGFP or Munc18-2-EmGFP. Confocal images of Munc18-1/2 DKD RBL-2H3 mast cells infected with lentivirus that express either EmGFP alone (Top panels), wild-type Munc18-1-EmGFP (Middle panels) or wild-type Munc18-2-EmGFP (Bottom panels). Cells were permeabilized and co-stained with mouse monoclonal anti-GFP antibody and rabbit monoclonal anti-syntaxin-3 antibody followed by Alexa488-conjugated goat anti-mouse secondary antibody, rhodamine-x-conjugated goat anti-rabbit secondary antibody and DAPI. Green signal indicates EmGFP, Munc18-1-EmGFP and Munc18-2-EmGFP (Left panels), red signal indicates syntaxin-3 (Middle panels) and merged images (Right panels). Note dimmer staining intensity of syntaxin-3 in EmGFP rescued cells compared to wild-type Munc18 rescued cells. Bar, 10 µm.
4.2.3 Syntaxin-3 aggregation seen in Munc18-1/2 DKD mast cells does not co-localize with Golgi or ER markers

The aggregated syntaxin-3 near the nucleus was found in Munc18-1/2 DKD mast cells. Since Munc18 was hypothesized as a molecular regulator mediating trafficking of syntaxin-3, a possible aggregation at Golgi or ER compartments of Munc18-1/2 DKD cells was tested. To look at the Golgi co-localization, syntaxin-3 was co-stained with two different Golgi markers; GM130 for cis-Golgi and TGN38 for trans-Golgi network in Munc18-1/2 DKD cells and analyzed their localizations under the confocal microscopy (Figure 4.6 A). The syntaxin-3 aggregation did not co-localize with either of Golgi markers. Then the possible aggregation at ER compartment was tested by staining cells with anti-calnexin antibody (Figure 4.6 B). Since this antibody was made against rabbit, co-stain with anti-syntaxin-3 antibody was not possible. However, we noticed calnexin expressions were found to be spread throughout the cells unlike aggregated syntaxin-3 pattern in Munc18-1/2 DKD cells. Therefore, we conclude that perinuclear aggregation of syntaxin-3 does not occur at ER or Golgi compartments on Munc18-1/2 DKD mast cells.
Figure 4.6 Syntaxin-3 aggregation seen in Munc18-1/2 DKD mast cells does not co-localize with Golgi or ER markers. Munc18-1/2 DKD RBL-2H3 mast cells were permeabilized and co-stained with rabbit monoclonal anti-syntaxin-3 antibody and Golgi markers (A); GM130 (Top panel) and TGN38 (Bottom panel) followed by Alexa488-conjugated goat anti-rabbit secondary antibody, rhodamine-x-conjugated goat anti-mouse secondary antibody and DAPI. For ER staining, Munc18-1/2 DKD mast cells were co-stained with rabbit polyclonal anti-calnexin antibody and Golgi markers (B); GM130 (Top panel) and TGN38 (Bottom panel) followed by Alexa488-conjugated goat anti-rabbit secondary antibody, rhodamine-x-conjugated goat anti-mouse secondary antibody and DAPI. Green signal indicates syntaxin-3 (A, Left panels) and calnexin (B, Left panels), red signals indicates GM130 and TGN38 (Middle panels) and merged images including DAPI signal as blue (Right panels). Note syntaxin-3 aggregation does not show clear co-localization with either Golgi markers or ER marker. Bar, 10 µm.
4.2.4 Lys46 and Glu59 of Munc18 play crucial roles in mediating binary interaction with syntaxin-2 and -3

The binding of Munc18-1 to monomeric syntaxin-1 is mediated by interactions of many residues revealed by X-ray crystallography (Misura et al., 2000). Among them, Lys46 and Glu59 in domain-1 of Munc18-1 were demonstrated to play a crucial role in governing the binary interaction; each single point mutation of K46E or E59K caused significant disruptions in binding to syntaxin-1 while double mutations of K46E/E59K resulted in complete abolishment of interaction using yeast two-hybrid assays and isothermal titration calorimetry (ITC) (Han et al., 2011; Han et al., 2009). Lys46 and Glu59 are conserved not only between Munc18-1 and -2 but also in Unc-18 in C. elegans and Rop in Drosophila (Figure 4.7 A). Also, a homology model of Munc18-2 in complex with monomeric syntaxin-3 based on the crystal structure of Munc18-1-syntaxin-1A suggested that these two residues are in close proximity to Asp230 and Arg114 of closed syntaxin-3, possibly mediating electrostatic interactions (Figure 4.7 B). Therefore, we next investigated whether the same mutations in Munc18-1 and -2 would affect the binary interactions (if so, how much) with the syntaxin-2 and -3, using yeast two-hybrid assays.

Significant disrupting effects of K46E and E59K mutations on the binary interactions between Munc18 and syntaxin-2 or -3 were found. However, intriguing differences existed between syntaxin-2 and -3. Even a single mutation of K46E or E59K in Munc18-1 or -2 completely abolished the binding to syntaxin-3 and the double mutations of K46E/E59K did not exhibit any additive effects (Figure 4.8 A and B). In contrast, the single binary interaction mutants of Munc18-1, and to a lesser extent those of Munc18-2 retained some ability to bind syntaxin-2. However, like those observed in syntaxin-3, the double mutants of Munc18-1 or -2 similarly abrogated the binding to syntaxin-2 (Figure 4.8 C and D). Thus, this result suggests
that binding mode of Munc18-1 and -2 toward syntaxin-2 seems similar to the binding mode
between Munc18-1 and syntaxin-1 which was previously reported (Han et al., 2011; Han et al.,
2009), while their binding mode to syntaxin-3 is significantly different.
Figure 4.7 Sequence alignment and homology modelling illustrating conserved residues of Lys46 and Glu59 and predicted interactions with syntaxin-3. (A) Sequence alignment of domain 1 residues of rat Munc18-2 and Munc18-1, C. elegans UNC-18, and Drosophila Rop indicate conservation of Lys46 and Glu59 among different Munc18 isoforms and homologs (highlighted in red). (B) Predicted structure of Munc18-2 in complex with syntaxin-3, by using homology modeling based on Munc18-1/syntaxin-1A crystal structure (Misura et al., 2000). Each domain of Munc18-2 is represented in a different color. Domains 1, 2, and 3 are shown in green, purple and pink, respectively. Syntaxin-3 is shown in cyan. An enlarged representative structure is shown on the right, illustrating the location of Lys46 and Glu59 of Munc18-2, and their potential electrostatic interaction partners Asp230 and Arg114, respectively, in syntaxin-3.
Figure 4.8 K46E and E59K mutations in domain 1 of Munc18-1 or Munc18-2 result in the abolishment of binding to closed syntaxin-3. Direct binding between Munc18-1 or Munc18-2 wild-type and the binary interaction mutants (K46E, E59K, K46E/E59K) with syntaxin-3 (A and B), and syntax-2 (C and D) was analyzed by yeast two-hybrid assays. Munc18 proteins were expressed as bait, whereas the cytoplasmic domains of wildtype syntaxins were expressed as prey. In each assay, β-galactosidase activities of the transformed yeast clones were quantified and normalized so that the activity of the yeast clones transformed with the wild-type Munc18 was set to 100%. pLexN indicates empty bait vector. Error bars indicate SEM (n=6–15). *, p < 0.05 (Student’s t-test).
4.2.5 Munc18 primarily binds to closed syntaxin-3

Based on the homology model illustrating Munc18-2 bound to closed syntaxin-3 (Figure 4.8 B), it was speculated that the WT syntaxin-3 adopts a closed conformation upon binding to Munc18. To verify whether the WT syntaxin-3 indeed adopts a closed conformation and that Munc18-2 primarily binds to this closed conformation, we examined whether binding between Munc18-2 and syntaxin-3 is significantly reduced when syntaxin-3 adopts an open conformation. In a previous NMR study of syntaxin-1A (Dulubova et al., 1999), mutations (L165A/E166A) in a linker region between its N-terminal Habc domain and its SNARE domain were found to cause this protein to open. These residues are highly conserved from nematode to human; similar mutations (L165A/E166A) in the syntaxin-1 ortholog Unc-64 also cause this protein to adopt an open conformation (Richmond et al., 2001). Therefore, a syntaxin-3 mutant that has an open conformation was generated (L165E/E166A) and its interaction with both isoforms of Munc18 was tested using yeast two-hybrid assays. We found that, compared to WT syntaxin-3, the syntaxin-3 open mutant merely exhibited ~10% of binding to either Munc18-1 or Munc18-2 isoforms (Figure 4.9). This indicates that the interaction of WT syntaxin-3 with Munc18 is mainly governed by syntaxin-3 in a closed conformation. Importantly, the fact that K46E and E59K alone completely abolished the interaction with WT syntaxin-3 indicates that these two mutants play an essential role in mediating a binary interaction with closed syntaxin-3 (Figure 4.8 A and B). In addition, although the binding mode of Munc18-1 and Munc18-2 towards closed syntaxin-2 seems similar to the binding mode of Munc18-1 and closed syntaxin-1 in neuronal exocytosis, as previously reported (Figure 4.8 C and D) (Han et al., 2011; Han et al., 2009), their interactions with the closed syntaxin-3 are substantially different, in that even a single mutation is enough to disrupt such interaction.
Figure 4.9 Wild-type syntaxin-3 adopts a closed conformation upon binding to Munc18 proteins. The syntaxin-3 LE open mutant was expressed as prey and binding to wild-type Munc18-1 or Munc18-2 was tested. Resulting β-galactosidase activity of the syntaxin-3 LE open mutant was quantified and normalized to the binding of wild-type syntaxin-3 to Munc18. pLexN indicates empty bait vector. Error bars indicate SEM (n=6–15). *, p < 0.05 (Student’s t-test).
4.2.6 Lys46 and Glu59 of Munc18 are essential for regulating syntaxin-3 protein level, localization and mast cell degranulation

To test the functional outcomes of these binary interaction mutations in regards to the regulation of syntaxin-3 protein level, localization and mast cell degranulation, we stably expressed the EmGFP fused mutants of Munc18-2 (K46E, E59K single mutants and K46E/E59K double mutant) in Munc18-1/2 DKD mast cells along with the WT and examined their ability to restore decreased syntaxins-3 protein level, subcellular localization as well as defective degranulation. Immunoblot analysis illustrated that all mutants were expressed at the similar level to the WT, implying that there were no major folding issues caused by these point mutations (Figure 4.10). Strikingly, none of the binary interaction mutants of Munc18-2 was able to restore the reduced syntaxins-3 protein level of Munc18-1/2 DKD mast cells (Figure 4.10 A). Upon examining the localization of syntaxin-3 in Munc18-1/2 DKD mast cells that are rescued by these mutants, dimmer overall staining intensities were observed in a comparison to WT rescued cells (Figure 4.10 C). Localizations of syntaxin-3 in the binary mutants rescued cells were present in an aggregated manner and their expressions at the tips of the cells was virtually absent (Figure 4.12 B and C). Finally, none of the Munc18-2 binary mutants was able to rescue the secretion to response to any stimulations (Figure 4.10 B). In accordance with yeast two-hybrid assay results (Figure 4.8 B), any additive effects of the double mutant (K46E/E59K) over the single mutants (K46E and E59K) were not observed, suggesting that even a single mutation is detrimental in abolishing the functional outcomes of the binary interactions in mast cells.

In neuronal exocytosis, the ability of Munc18-1 to rescue the phenotypes of Munc18-1/2 DKD PC12 cells was correlated to their ability of binding to syntaxin-1 (Han et al., 2011;
Taking advantage of the fact that Munc18-1 can also rescue the phenotypes of Munc18-1/2 DKD mast cells, additional experiments were performed to observe the effects of the same mutations in Munc18-1 on the mast cell degranulation and regulation of syntaxin-3 protein level and localization (Figure 4.11). Likely Munc18-2, mutants in Munc18-1 were not able to restore reduced syntaxin-3 protein level, its mislocalization and the impaired degranulation (Figure 4.11 and 4.12 D and E). Thus, in the case of mast cells, the ability of Munc18-1 and -2 to rescue degranulation was well reflected on their ability to bind to syntaxin-3 which were shown in the yeast two-hybrid assays (Figure 4.8 A)
**Figure 4.10 Binary interaction mutants of Munc18-2 do not restore syntaxin-3 levels, localization or degranulation defects of Munc18-1/2 DKD mast cells.** (A) Munc18-1/2 DKD RBL-2H3 cells were infected with lentiviruses that express EmGFP, wild-type Munc18-2–EmGFP or the binary interaction mutants of Munc18-2–EmGFP (K46E, E59K, K46E/E59K). Infected cells were selected with blasticidin (20 µg/ml). Homogenates of surviving cells (20 µg) were analyzed by SDS-PAGE and immunoblotting using the antibodies indicated on the right. *, a non-specific band observed with anti-Munc18-2 antibody, which co-migrated with Munc18-2–EmGFP. (B) β-hexosaminidase release from the rescued cells was stimulated by 1-hour incubation with 0.01 µg/ml DNP-IgE and 50 ng/ml DNP-HSA, or 0.5 µM or 2.5 µM ionomycin. Error bars indicate s.e.m. (n=6). (C) Munc18-1/2 DKD RBL-2H3 expressing the Munc18-2–EmGFP variants were permeabilized, and co-stained with mouse monoclonal anti-GFP and rabbit monoclonal anti-syntaxin-3 antibodies followed by Alexa-Fluor-488-conjugated goat anti-mouse secondary antibody, Rhodamine-x-conjugated goat anti-rabbit secondary antibody and DAPI. Green, Munc18-2–EmGFP of wild-type and mutants (Left panels); red, syntaxin-3 (Middle panels) and merged images (Right panels). Notice the absence of tip staining and the increased perinuclear aggregation of syntaxin-3 in the mutant-rescued cells compared to the wild-type Munc18-rescued cells. Scale bars: 10 µm. *, p < 0.05 (Student’s t-test).
Figure 4.11 Binary interaction mutants of Munc18-1 do not restore syntaxin-3 levels, localization or degranulation defects of Munc18-1/2 DKD mast cells. (A) Lentivirus expressing EmGFP, wild-type and binary interaction mutants (K46E, E59K, K46E/E59K) of Munc18-1–EmGFP were applied to Munc18-1/2 DKD RBL-2H3 cells. Homogenates of isolated cells (20 µg) were analyzed by SDS-PAGE and immunoblotting using antibodies indicated on the right. (B) β-hexosaminidase release from the rescued cells was stimulated by 1-hour incubation with 0.01 µg/ml DNP-IgE and 50 ng/ml DNP-HSA, or 0.5 µM or 2.5 µM ionomycin. Error bars indicate s.e.m. (n=6). (C) Munc18-1/2 DKD RBL-2H3 cells expressing Munc18-1–EmGFP variants were permeabilized and co-stained with mouse monoclonal anti-GFP antibody and rabbit monoclonal anti-syntaxin-3 antibody followed by Alexa-Fluor-488-conjugated goat anti-mouse secondary antibody, Rhodamine-x-conjugated goat anti-rabbit secondary antibody and DAPI. Green, Munc18-1–EmGFP of wildtype and binary interaction mutants (Left panels); red, syntaxin-3 (Middle panels) and merged images (Right panels). Notice the absence of tip staining and the decreased intensity of syntaxin-3 in the mutant-rescued cells compared to the wild-type Munc18-1-rescued cells. Scale bars: 10 µm. *, p < 0.05 (Student’s t-test).
Figure 4.12 Quantification of syntaxin-3 expression, and expression at the tips versus soma of control and Munc18-1/2 DKD mast cells as well as wild-type Munc18-1- or Munc18-2-rescued and binary mutant-rescued Munc18-1/2 DKD mast cells. (A) Quantification of syntaxin-3 expression in control and Munc18-1/2 DKD RBL-2H3 mast cells was performed using ImageJ. Left graph shows the fluorescence intensity; right graph indicates distribution of the fluorescence signal (tip versus soma). Analyzed were 22 cells for control and 32 cells for DKD cells, and their mean values are presented. Error bars indicate SEM. (B and C) Overall syntaxin-3 expression levels (B) and syntaxin-3 expression at the tips versus soma (C) of EmGFP, Munc18-2 WT, Munc18-2 K46E-, E59K- and K46E/E59K-rescued Munc18-1/2 DKD RBL-2H3 cells. Confocal microscopy images were quantified by ImageJ. (D and E) The same quantification was performed for Munc18-1 WT-, Munc18-1 K46E-, E59K- and K46E/E59K-rescued Munc18-1/2 DKD RBL-2H3 cells illustrating overall syntaxin-3 expression levels (D) and syntaxin-3 expression at the tips versus soma (E). Note, in both Munc18 isoforms, WT rescued both overall intensity as well as tip localization of syntaxin-3, whereas all binary mutants failed to rescue this phenotype. Error bars indicate SEM (n=23–40). *, p < 0.05 (Student’s t-test).
4.2.7 Knockdown of syntaxin-3 results in severe impairment in mast cell degranulation

If the regulation of syntaxin-3 by Munc18-1 and -2 is functionally important in mast cell degranulation, one would anticipate that this syntaxin isoform should play an independent role in exocytosis. To test this hypothesis, two independent stable KD RBL-2H3 mast cell lines of endogenous syntaxin-3 each targeting different regions of rat syntaxin-3 were generated using lentivirus mediated shRNA (named as syntaxin-3-5 KD and syntaxin-3-8 KD). Immunoblotting with rabbit monoclonal anti-syntaxin-3 antibody revealed some reductions in the level of syntaxin-3 in both cell-lines, but syntaxin-3-8 KD resulted in a greater level of decrease than syntaxin-3-5 KD (Figure 4.13). Also, there were no obvious changes in syntaxins-2 and -11 protein levels in this strong syntaxin-3 KD cells indicating there is no possible up-regulation of those isoforms (Figure 4.13 A). When performed degranulation assay using IgE- and ionomycin-dependent stimulations, both syntaxin-3 KD mast cells had significant defects in secretion (Figure 4.13 B and D). However, the degree of degranulation defects was correlated to the level of syntaxin-3 reductions seen in two different KD cell-lines. Thus, this result suggests that syntaxin-3 plays an important independent role in mast cell degranulation and that severe secretion defects seen in Munc18-1/2 DKD mast cells can be attributed to the lack of syntaxin-3 regulations.
Figure 4.13 Stable knockdown of syntaxin-3 in mast cells results in severe impairment of β-hexosaminidase release. (A and C) Stable syntaxin-3 knockdown RBL-2H3 cells were generated by two different lentivirus-mediated shRNAs targeting different sequences of rat syntaxin-3. Cell homogenates (20 µg) were analyzed by SDS-PAGE and immunoblotting using antibodies indicated on the right. The signals were detected using an enhanced chemiluminescence detection system. Numbers on the left indicate molecular mass markers. (B and D) β-hexosaminidase release from the syntaxin-3–8 KD (B) and syntaxin-3–5 KD (D) RBL-2H3 cells was stimulated by applying 0.01 µg/ml DNP-IgE and 100 ng/ml DNP-HSA, or 0.5 µM or 2.5 µM ionomycin for 1 hour. Error bars indicate s.e.m. (n=19, B; and n=8, D). *, p < 0.05 (Student’s t-test).
4.3 Conclusion

In this Chapter, an attempt to find the key cognate syntaxin protein whose level and localization are regulated by Munc18 was made. Through a re-examination of Munc18-1/2 DKD mast cells, another syntaxin isoform, syntaxin-3, was identified whose level was reduced in addition to syntaxin-11 (Figure 4.1). This decreased syntaxin-3 was completely restored upon re-expression of Munc18-1 or -2 illustrating the specificity of such phenotype (Figure 4.2). Furthermore, the finding of mislocalized syntaxin-3 in perinuclear region of Munc18-1/2 DKD mast cells which was rescued by re-introduction of WT Munc18 (Figure 4.3, 4.4 and 4.5) provides an additional strong evidence that Munc18 plays a crucial regulatory activity toward syntaxin-3.

In neuronal exocytosis, a single binary interaction mutation is not sufficient to disrupt binding between Munc18-1 and monomeric syntaxin-1 (Han et al., 2011). However, the results from present Chapter illustrate that a single binary interaction mutant (K46E or E59K) of either Munc18-1 or Munc18-2 is as detrimental as the double mutant (K46E/E59K) in both binding closed syntaxin-3 (Figure 4.8) and rescuing the phenotype of the Munc18-1/2 DKD mast cells (Figure 4.10 and 4.11). These results are strikingly different from the binary interaction between Munc18-1 and syntaxin-1, and their functional implications.

In attempt to investigate an independent role of syntaxin-3 in mast cell degranulation, two lines stable syntaxin-3 KD mast cells were generated. In response to stimulation, both syntaxin-3 KD cells exhibited strong impairments in both IgE- and ionomycin-dependent degranulation (Figure 4.13). This is unlike a previous attempt to see the role of syntaxin-11 in mast cell exocytosis, where strong KD of endogenous syntaxin-11 did not result in any defects in degranulation (Figure 3.12). Thus, the results in current Chapter suggest that although Munc18
proteins regulate both syntaxins-3 and -11 isoforms in mast cells, the former interactions provide more functional contributions to the mast cell exocytosis.
Chapter 5: Munc13-4 is an essential priming factor and a Ca\textsuperscript{2+}-sensor of mast cell exocytosis

The contents of the following chapter are unpublished.

5.1 Rationale of the study

In previous Chapters, investigations were made to examine the function of Munc18 and its functional contribution to the mast cell degranulation through regulating cognate syntaxin proteins, namely syntaxin-3 and -11. In order for the secretory granules to be released, they are required to be primed which renders a fusion-competent state. Moreover, the exocytosis of the immune cells is Ca\textsuperscript{2+} dependent, however the critical Ca\textsuperscript{2+} sensor for this type of exocytosis is yet to be investigated. This is in contrast to the extensively studied and highly established roles of Munc13 as a priming factor and syntaptotagmin-1 as a Ca\textsuperscript{2+} sensor for the neuronal exocytosis (Augustin et al., 1999; Brose et al., 2000; Fernández-Chacón et al., 2001; Geppert et al., 1994b).

The Munc13 protein family, which consists of Munc13-1, -2, -3 (Brose et al., 2000), play essential roles in priming of synaptic vesicles (Augustin et al., 1999). Munc13-4 and BAP3 (brain-specific angiogenesis inhibitor 1-associated protein 3) are distantly related with the core Munc13 family (Figure 5.1). Munc13-4 is expressed ubiquitously but exhibits higher expression in immune tissues. Structurally, Munc13-4 and BAP3 differ from the core Munc13 proteins that they lack N-terminal C2 domain, calmodulin binding domain and diacylglycerol-binding C1 domain. Yet, they share two Munc13 homology domains (MHDs) and two C2 domains. In Munc13-1, -2 and -3, four aspartic acid residues necessary to support Ca\textsuperscript{2+}-dependent
phospholipid binding are preserved only in the C2B domain while the Ca\textsuperscript{2+} binding sequence in the C-terminal C2C domain is degenerated (McEwen and Kaplan, 2008). By contrast, the key aspartic acid residues are preserved in both C2 domains (C2A and C2B) of different Munc13-4 and BAP3 orthologues across various species including \textit{C. elegans} and \textit{Drosophila} (Figure 5.2). Importantly, each of the C2 domain of Munc13-4 was found to bind Ca\textsuperscript{2+} (Boswell et al., 2012), which is also predicted by the homology model (Figure 5.3). The Ca\textsuperscript{2+} binding to both C2 domains is similar to that of syt-1, a key Ca\textsuperscript{2+} sensor in neuronal exocytosis (Fernández-Chacón et al., 2001).

The essential role of Munc13-4 was demonstrated as mutations in this protein has been implicated with a devastating immune disease, FHL type 3 (Feldmann et al., 2003; Yamamoto et al., 2004). Since then, many attempts were made to investigate the potential priming mechanisms of Munc13-4 in hematopoietic cells including CTLs, mast cells, NK cells, neutrophils and platelets (Brzezinska et al., 2008; Elstak et al., 2011; Higashio et al., 2008; Marcenaro et al., 2006; Ménager et al., 2007; Neeft et al., 2005; Pivot-Pajot et al., 2008; Ren et al., 2010; Savage et al., 2013; Shirakawa et al., 2004). However, despite the fact that both C2 domains of Munc13-4 can bind to Ca\textsuperscript{2+} similar to syt-1, how Ca\textsuperscript{2+}-binding properties of the C2 domains can contribute to the function of Munc13-4 remains unanswered. Therefore, in this final Chapter, we asked two questions regarding function of Munc13-4. 1. Is Munc13-4 a priming factor of the mast cell degranulation? 2. Is Munc13-4 a Ca\textsuperscript{2+}-sensor of the mast cell degranulation? To answer these questions, an attempt was made to investigate the priming and/or Ca\textsuperscript{2+} sensing role of Munc13-4 in the mast cell degranulation using established methods of generating stable knockdown and rescued mast cells.
Figure 5.1 Schematic diagram of Munc13 family and BAP. Asterisks indicate Ca\textsuperscript{2+} binding residues in C2 domains of each protein.
Figure 5.2 Sequence alignment C2A and C2B domains in Munc13 family orthologues, BAP3 and Syt1. (A and B) Sequence alignment of C2A (A) and C2B (B) between Munc13 isoforms across different species as well as BAP3 and Syt1. Red highlight denotes Ca\textsuperscript{2+} binding aspartic acids. M: Mus musculus, R: Rattus norvegicus, H: Homo sapiens, C: C. elegans, D: Drosophila melanogaster.
Aspartic acid residues in C2 domains of human Munc13-4 are predicted to bind to Ca$^{2+}$.

(A and B) Homology modeling of C2 domains of human Munc13-4 that are generated based on sequence homology and available crystal structure. (A) Homology modeling of human Munc13-4 C2A domain (purple) was generated using C2B domain of rat Munc13-1 (PDB: 3KWU) (yellow). (B) Homology modeling of human Munc13-4 C2B domain (purple) was generated using C2B domain of rat syt-1 (PDB: 1TJX) (yellow). In both C2 domains, four aspartic acid residues of human Munc13-4 are conserved and are predicted to interact with Ca$^{2+}$ ions (green spheres).
## 5.2 Results

### 5.2.1 Munc13-4 is an essential priming factor of Ca$^{2+}$ dependent mast cell degranulation

To test the function of Munc13-4 in mast cell degranulation, stable Munc13-4 KD mast cells were generated (Figure 5.4). Immunoblotting revealed that the levels of crucial proteins for immune cell exocytosis such as various isoforms of Munc18 and syntaxin remained unchanged, despite drastic loss of Munc13-4 in KD cells (Figure 5.4 A and B). Then the secretion capabilities of control and Munc13-4 KD cells were measured by triggering exocytosis using IgE and ionomycin with or without PMA (Figure 5.4 C). In all cases, vast reductions of exocytosis from Munc13-4 KD cells was observed confirming a crucial function of Munc13-4 in mast cell exocytosis.

Drastic loss of exocytosis from Munc13-4 KD cells suggests lack of priming of secretory granules. At the same time, absence of Munc13-4 may lead to altered Ca$^{2+}$ sensitivity of the secretion due to the fact Munc13-4 contains two C2 domains that both can bind to Ca$^{2+}$. Therefore, to see the Ca$^{2+}$-dependence of mast cell exocytosis, the exocytosis was triggered and measured while titrating the extracellular Ca$^{2+}$ from 0.05 mM to 30 mM (Figure 5.5 A and B). In both cases, the exocytosis of control cells peaked at the 2.2 mM Ca$^{2+}$, while there were dramatic reductions of the secretion beyond this optimal physiological Ca$^{2+}$ concentration. In contrast however, Munc13-4 KD strongly suppressed the exocytosis throughout the wide range of extracellular Ca$^{2+}$ concentrations. Thus, the severe loss of the Ca$^{2+}$-dependent exocytosis in the absence of Munc13-4 indicates Munc13-4 as an essential priming factor of the mast cell exocytosis.
Figure 5.4 Severe degranulation defects from Munc13-4 knockdown mast cells. (A) Immunoblotting analysis of various secretory proteins in control and Munc13-4 knockdown cells. (B) Quantification of protein levels in Munc13-4 knockdown cells normalized to control cells. Error bars indicate SEM (n=6-9). (C) β-hexosaminidase release from the control and Munc13-4 knockdown cells was triggered by 1-hour incubation with indicated stimulations at 2.2 mM extracellular Ca²⁺. Error bars indicate SEM (n=6). *, p < 0.05 (Student’s t-test).
Figure 5.5 Loss of Ca\textsuperscript{2+}-dependency in exocytosis from Munc13-4 knockdown mast cells. (A and B) Titration of extracellular Ca\textsuperscript{2+} of control and Munc13-4 knockdown cells when stimulated with IgE+DNP-HSA (A) or 2.5 \mu M ionomycin (B).
5.2.2 Mutations in C2 domains of Munc13-4 results in defects of mast cell degranulation

To investigate the functions of the C2 domains of Munc13-4 in the immune cell exocytosis in more details, the KD cells was rescued by stably expressing Emerald GFP (EmGFP) fused Munc13-4 proteins in which the conserved aspartic acid residues in the C2 domains are mutated to asparagine. As referred in the Rationale of the Study, Munc13-4 is the only isoform of Munc13 family that contains intact Ca$^{2+}$ binding aspartic acid residues in both C2A and C2B domains (Figure 5.1) and these residues are conserved throughout species as well as syt-1 (Figure 5.2). Moreover, homology modeling of the C2A and the C2B domains of Munc13-4 predict that these aspartic acid residues are in close proximity thus binding to Ca$^{2+}$ ions (Figure 5.3). The mutations in these residues were recently been reported to result in almost completely abolishment of Ca$^{2+}$ binding properties of the recombinant Munc13-4 protein (Boswell et al., 2012). Specifically, two aspartic acid residues in the C2A (D127N/D133N) or the C2B (D941N/D947N) as well as combination of the both (the quadruple mutant, D127N/D133N/D941N/D947N) were mutated and expressed in Munc13-4 KD mast cells (Figure 5.6 A). The immunoblot analysis revealed the double and quadruple mutants are expressed at the comparable level of WT (Figure 5.6 B) indicating the mutations did not impose any issues in foldings and expression of proteins. WT Munc13-4 completely restored the defective exocytosis of Munc13-4 KD cells in response to both IgE and ionomycin thereby confirming specificity of our KD and rescue approaches (Figure 5.6 C). However, the double mutants of either the C2A or the C2B domain failed to exhibit rescue ability toward IgE-dependent and ionomycin-induced secretion. Interestingly they did show some levels of rescue in secretion upon the ionomycin+PMA induced degranulation, with the C2A double mutant exhibiting better rescue ability than that of the C2B double mutant. Quadruple mutant rescued
cells exhibited very limited level of degranulation even with the strongest stimulation (Figure 5.6 C).
Figure 5.6 Point mutations in C2 domains of Munc13-4 failed to rescue mast cell degranulation. (A) Schematic diagram showing locations of point mutations in C2 domains of human Munc13-4. (B)Immunoblotting analysis of Munc13-4 knockdown cells rescued with EmGFP-fused wild-type or C2A, C2B double mutants or quadruple mutant. (C) β-hexosaminidase release from the Munc13-4 knockdown cells rescued with double and quadruple mutants in C2 domains was triggered by 1-hour incubation with indicated stimulations at 2.2 mM Ca²⁺. Error bars indicate SEM (n=6). *, p < 0.05 (Student’s t-test).
5.2.3 Specific mutations in C2 domains of Munc13-4 alter Ca$^{2+}$ sensitivity of mast cell degranulation

The partial rescue of degranulation exhibited by the C2 domain mutants is interesting as it suggests two possible scenarios; (i) the C2 domain mutants have partial defects in priming of secretory granules, (ii) the priming function of Munc13-4 is still possessed by the mutants, rather the Ca$^{2+}$-sensitivity of the exocytosis is altered suggesting Ca$^{2+}$-sensing function of the C2 domains. In addition, the fact that two C2 domain mutants resulted in distinct level of partial rescue illustrates that there could be a different degree of importance in the function of the C2 domains to mediate the exocytosis. To delineate potential mechanisms of the C2 domains of Munc13-4, the rescued cells were stimulated with ionomycin (0.5 or 2.5 µM) and the extracellular Ca$^{2+}$ concentrations were titrated with or without the presence of 0.1 µM of PMA (Figure 5.7). If the C2 domains are involved in priming step, then one would expect to see the same Ca$^{2+}$ titration curve as WT rescue albeit lower secretion levels. However, if scenario #2 is correct, then altered Ca$^{2+}$-sensitivity would be expected. From the WT Munc13-4-EmGFP rescued cells, the bell shaped response of exocytosis was observed indicating the full restoration of Ca$^{2+}$-sensitivity of mast cell exocytosis (Figure 5.7 A-D). The level of degranulation was evident even at 0.05 mM Ca$^{2+}$, while the peak of secretion occurred at 2.2 mM. Further increase in concentrations (5 and 10 mM) caused a rather dramatic reduction in exocytosis. Strikingly however, double and quadruple point mutants of C2 domains exhibited markedly altered Ca$^{2+}$-sensitivity of exocytosis. They showed some level of secretion at 2.2 mM Ca$^{2+}$ while they invariably displayed a trend of positive increase in exocytosis at concentrations beyond 2.2 mM (Figure 5.7 A-D). This pattern of shifted Ca$^{2+}$-sensitivity was obvious regardless of stimulation methods used. Importantly, although all mutants displayed altered Ca$^{2+}$-sensitivity, mutants exhibited different level of exocytosis. The double mutant in C2A showed better secretion than
the double mutant in C2B, followed by quadruple mutant in both C2 domains (Figure 5.7 A-D).

While, the Munc13-4 KD cells rescued by EmGFP not only exhibited almost complete abolishment of exocytosis even at 10 mM Ca$^{2+}$, but also did not result in any Ca$^{2+}$-dependency of the exocytosis (Figure 5.7 A-D).

Finer titration (between 0.1 mM and 2.2 mM) of extracellular Ca$^{2+}$ was performed to further examine whether there is significant difference between the C2A mutants versus C2B mutants in the alteration of the Ca$^{2+}$-sensitivity (Figure 5.8 A and B). A similarly shifted Ca$^{2+}$-sensitivity was found in these mutants, which suggests that both domains need to be functional for Munc13-4 to be a Ca$^{2+}$ sensor for exocytosis. Collectively, these results suggest that specific mutations in the Ca$^{2+}$ binding residues of the C2 domains seem to impair the Ca$^{2+}$-sensing functions of this protein in the mast cell exocytosis.
Figure 5.7 Ca\textsuperscript{2+}-sensitivity of mast cell exocytosis is altered upon mutations in C2 domains of Munc13-4. (A to D) Titration of extracellular Ca\textsuperscript{2+} from 0.05 mM to 10 mM of Munc13-4 knockdown cells rescued with double and quadruple mutants in C2 triggered by 0.5 µM ionomycin (A), 2.5 µM ionomycin (C), 0.5 µM ionomycin + 0.1 µM PMA (B), 2.5 µM ionomycin + 0.1 µM PMA (D). Error bars indicate SEM (n=6).
Figure 5.8 Finer titration at low Ca\(^{2+}\) concentrations still result in altered Ca\(^{2+}\) sensitivity exerted by the C2 domain mutants. (A and B) Titration of extracellular Ca\(^{2+}\) between 0.1 mM and 2.2 mM for Munc13-4 knockdown cells rescued with double and quadruple mutants in C2 triggered by 0.5 µM ionomycin + 0.1 µM PMA (A), 2.5 µM ionomycin + 0.1 µM PMA (B). Error bars indicate SEM (n=6).
5.2.4 Single mutation in C2 domains of Munc13-4 is enough to alter Ca$^{2+}$-sensitivity of mast cell degranulation

After testing the importance of two C2 domains, we sought to narrow down the critical aspartic acid residue in the C2 domain that are involved in Ca$^{2+}$ sensing of exocytosis. For this purpose, the KD cells were rescued with Munc13-4 that has a single mutation in the C2A or the C2B domain and tested their rescue ability toward Ca$^{2+}$-sensitivity of exocytosis (Figure 5.9). Like the double or quadruple mutants, all single mutants were expressed as well as the WT (Figure 5.9 A). Upon testing their rescue ability, we saw that the single point mutants seem to result in better secretion than their respective double mutants at 2.2 mM (Figure 5.9 B). Nevertheless, Ca$^{2+}$-sensitivities of all single mutants are still strikingly altered (Figure 5.9 C-F). All single mutants had a positive trend of secretion elevation as the increasing extracellular Ca$^{2+}$ and their exocytosis at 10 mM Ca$^{2+}$ was even better than that of control and WT rescued cells. In agreement with double mutant data, two single mutants in the C2A showed a better secretion than those in the C2B throughout the range of extracellular Ca$^{2+}$. Collectively, the results illustrate that even a single mutation in Ca$^{2+}$ binding residue of the C2A or the C2B is sufficient to alter Ca$^{2+}$-sensitivity of mast cell degranulation.
Figure 5.9 Single mutation in C2 domain of Munc13-4 is enough to alter Ca\(^{2+}\)-sensitivity of mast cell exocytosis. (A) Immunoblotting analysis of Munc13-4 knockdown cells rescued with EmGFP-fused wild-type or single mutants in C2 domains of Munc13-4. (B) β-hexosaminidase release from the Munc13-4 knockdown cells rescued with single mutants in C2 domains was triggered by 1-hour incubation with indicated stimulations at 2.2 mM Ca\(^{2+}\). (C - F) Titration of extracellular Ca\(^{2+}\) between 0.05 mM to 10 mM of Munc13-4 knockdown cells rescued with single mutants in C2 triggered by 0.5 μM ionomycin (C), 2.5 μM ionomycin (D), 0.5 μM ionomycin + 0.1 μM PMA (E), 2.5 μM ionomycin + 0.1 μM PMA (F). Error bars indicate SEM (n=6). *, p < 0.05 (Student’s t-test).
5.2.5 Mutations in C2 domains do not alter subcellular localization of Munc13-4

As a potential mechanism to explain the effects of the C2 domain mutants, subcellular localization and translocation of Munc13-4 proteins before and after stimulation with ionomycin were examined (Figure 5.10 and 5.11). Munc13-4 is known to be associated with lysosomal granules by interacting with Rab27A on granules (Elstak et al., 2011; Shirakawa et al., 2004). Indeed, EmGFP-fused Munc13-4 WT and mutants stained by anti-GFP antibody exhibit similar punctate signals, which agree that they are associated with secretory lysosomes (Elstak et al., 2011; Shirakawa et al., 2004). After the stimulation with ionomycin in the presence of 2.2 mM or 10 mM of extracellular Ca$^{2+}$ concentration, Munc13-4 WT or mutants were found to be translocated to the plasma membrane. This is in agreement with a finding that Munc13-4 are recruited to the plasma membrane following stimulation in human neutrophils (Pivot-Pajot et al., 2008). The translocation to the plasma membrane was consistently observed regardless of the presence of mutations or regardless of the different Ca$^{2+}$ concentrations. Thus, the defective Ca$^{2+}$-sensitivity of immune cell exocytosis seen from the mutants does not seem to be explained by their localization.
**Figure 5.10 Translocation of Munc13-4 to plasma membrane upon stimulation does not exhibit Ca\(^{2+}\)-dependency.** Munc13-4 knockdown cells expressing EmGFP alone, EmGFP-fused wild-type, double and quadruple mutants in C2 domain were stimulated with 2.5 μM ionomycin at 2.2 mM or 10 mM extracellular Ca\(^{2+}\). Then, the cells were permeabilized and stained with mouse monoclonal anti-GFP antibody followed by Alexa-Fluor 488-conjugated anti-mouse secondary antibody and DAPI. Images were acquired by confocal microscope. Scale bars: 10 μm.
Figure 5.11 Translocation of single mutants in C2 domains of Munc13-4 upon stimulation does not exhibit Ca\(^{2+}\)-dependency. Munc13-4 knockdown cells expressing EmGFP-fused single mutants in C2 domain were stimulated with 2.5 μM ionomycin at 2.2 mM or 10 mM extracellular Ca\(^{2+}\). Then, the cells were permeabilized and stained with mouse monoclonal anti-GFP antibody followed by Alexa-Fluor 488-conjugated anti-mouse secondary antibody and DAPI. Images were acquired by confocal microscope. Scale bars: 10 μm.
5.2.6 TIRFM analysis reveals crucial role of Munc13-4 in single granule fusion dynamics in a Ca\(^{2+}\) dependent manner

Next, we investigated to see if the secretion phenotypes exhibited by the C2 domain mutants can be attributed to the alterations in the dynamics of single vesicle fusions in response to Ca\(^{2+}\). Specifically, we asked whether the altered Ca\(^{2+}\)-sensitivity is due to the changes of the frequency in the vesicular fusion events and/or whether it is due to different sizes of fusion pore formation of single vesicle fusion at different Ca\(^{2+}\) concentrations. For this purpose, we transfected the mast cells with cDNA of a cargo molecule (Neuropeptide Y fused with mCherry, NPY-mCherry) and examined their release using total internal reflection fluorescence microscopy (TIRFM). Under evanescent field, we observed a comparable number of mCherry-containing granules in the control and Munc13-4 KD cells suggesting that the NPY-mCherry is similarly expressed in these cells, stored in secretory lysosomal granules, and that these vesicles are attached or very close to the plasma membrane in the absence of Munc13-4 (Figure 5.12 A).

To reveal the Ca\(^{2+}\)-dependence on single vesicle fusion, we used two different extracellular Ca\(^{2+}\) concentrations (2.2 and 10 mM) and stimulated the exocytosis by applying 2.5 \(\mu\)M ionomycin and monitored release of NPY-mCherry from fusion of granules under TIRFM. A granule fusion event was evident as a rapid increase of fluorescent intensity after the stimulation followed by a steady decay (Figure 5.12 C). Interestingly, we saw that single granule fusion events almost always exhibited two successive peaks in the fluorescent intensity (Figure 5.12 C). This could be due to compound exocytosis which allows sequential fusions of granules onto previously fused granules that are known to occur prevalently in the mast cells but further work needs to be done to address this possibility (Blank, 2011; Lorentz et al., 2012). In addition, there were granules that appear in the evanescent field but failed to exhibit a rapid increase of
fluorescent signal and a steady decay afterward. Rather, these granules were present for the entire recording period even after stimulation (Figure 5.12 B). These granules were classified as non-fusion granules. This observation is consistent with the analysis of non-fusing vesicles from neuroendocrine cells under TIRFM (Yamaga et al., 2015). In the WT control cells at 2.2 mM Ca\textsuperscript{2+}, the release of NPY-mCherry in response to ionomycin caused a rapid and large increase in the fluorescence signal, which was then decayed (Figure 5.12 C). Most of granules seemingly underwent fusion events from the control cells, as the non-fusion granules only consisted of 9.7% of total granules population per cell (Figure 5.12 D). However, upon triggering exocytosis at 10 mM, the control cells exhibited defective single granule fusion events. The non-fusion events of granules from the control cells increased to nearly 31.4% indicating frequency of fusion events was reduced at this high Ca\textsuperscript{2+} (Figure 5.12 D). Unexpectedly, the kinetics and amplitudes of NPY-mCherry release from fused granules were also severely compromised (Figure 5.12 C, E and F). Namely, time to reach to the peak of the fluorescent intensity was substantially delayed at 10 mM compared to 2.2 mM Ca\textsuperscript{2+} (85 sec vs. 22 sec) (Figure 5.12 E). In addition, increase of fluorescent intensity amplitude from a single granule fusion event was dramatically lower at 10 mM Ca\textsuperscript{2+} (Figure 5.12 F). In contrast, Munc13-4 KD cells had exhibited substantially augmented population of non-fusion granules at 2.2 mM Ca\textsuperscript{2+} as they constituted 35% of total granules (Figure 5.12 D). Moreover, among the fused granules, the increase of fluorescent intensity amplitude was substantially smaller than control cells (Figure 5.12 C and F). These phenotypes of single granule fusion events from the KD cells remained similar at 10 mM Ca\textsuperscript{2+} (Figure 5.12 D and F), and time to reach to the peak of the fluorescent intensity was invariable (Figure 5.12 E). These results suggest important functions of Munc13-4 in single granule fusion events of mast cells; not only it regulates frequency of fusion events, but also the
kinetics and magnitudes of cargo releases presumably by regulating opening and size of fusion pore in an Ca\textsuperscript{2+}-dependent manner.
Figure 5.12 Single granule fusion analysis using TIRFM reveals role of Munc13-4 in regulating fusion events in a Ca$^{2+}$-dependent manner. (A) Example of NPY-mCherry expressing lysosomal granules in control and Munc13-4 knockdown cells before stimulation (left) or at the peak of exocytosis (right) under TIRFM. Yellow circles were drawn to indicate single granules from the cells. Scale bars: 10 µm. (B) Typical trace of granules that were docked but failed to exhibit a rapid increase in fluorescent intensity followed by a decay in response to stimulation. Granules with this behaviour were classified as non-fusing granules. (C) Representative trace of release of NPY-mCherry from single granule fusions from the control and Munc13-4 knockdown cells at 2.2 mM (black) and 10 mM (red) extracellular Ca$^{2+}$. (D) Non-fusion events were calculated by normalizing number of granules which exhibited non-fusing behaviour to the total number of granules of control and Munc13-4 knockdown cells at 2.2 mM and 10 mM extracellular Ca$^{2+}$. Error bars indicate SEM (n=25-50 per cell). * P < 0.05, student t-test. (E) Kinetic of NPY-mCherry release from fused single granules was calculated from the time of adding stimulation (2.5 µM ionomycin) to reach the peak of fluorescent intensity; control and Munc13-4 knockdown cells at 2.2 mM and 10 mM extracellular Ca$^{2+}$. Error bars indicate SEM (n=25-50 per cell). * P < 0.05, student t-test. (F) Normalized histogram and Gaussian fits illustrating fluorescent intensity increase of single granule fusion events from control and Munc13-4 knockdown cells at 2.2 mM and 10 mM extracellular Ca$^{2+}$. % increase was calculated by normalizing peak fluorescent intensity as a result of fusion event to the baseline before stimulation of each granule. Error bars indicate SEM (n=25-50 per cell).
5.2.7 Point mutants in C2 domains of Munc13-4 alters Ca\textsuperscript{2+}-sensitivity of single granule fusion dynamics

We next tested the effects of Ca\textsuperscript{2+}-binding mutations in the C2 domains of Munc13-4 on single granule fusion events. First, we observed that the re-expression of WT Munc13-4, but not EmGFP, was able to restore the decreased frequency as well as altered kinetics and amplitudes of fusion events of KD cells at 2.2 mM (Figure 5.13 A and 5.14 A-C). More importantly, we saw the restoration of Ca\textsuperscript{2+}-dependency of the granule fusions as the population of non-fusion granules increased accompanied with slower and lowered amplitudes of fused granules at 10 mM. Upon testing double mutants and quadruple mutant of the C2 domains, we found that the Ca\textsuperscript{2+}-sensitivity of their frequency and opening of fusion pore of single granule fusion events were significantly shifted (Figure 5.13 B and 5.14). At 2.2 mM, all of the mutants in the C2 domains illustrated impaired single granule fusions such that there were substantially increased non-fusion events and the granules that were fused exhibited reduced amplitudes compared to WT rescued cells (Figure 5.13 B and 5.14 A and D). Moreover, average time taken to reach the peak of fluorescent intensity was significantly longer than that of WT (Figure 5.13 B and 5.14 B). Among the mutants, the double mutant in the C2A had lower population of non-fusion granules than double mutant of the C2B domain followed by quadruple mutant at 2.2 mM Ca\textsuperscript{2+} (Figure 5.14 A). In addition, the fluorescent intensity increase following a single fusion event was the highest for the C2A, followed by the C2B and the quadruple mutant having substantial population with only marginal increase (Figure 5.13 B and 5.14 D). But these compromised fusion dynamics were invariably alleviated when it was triggered at 10 mM Ca\textsuperscript{2+}. Percentage of non-fusion granules was significantly decreased compared to 2.2 mM. Also, the amplitude and kinetics of the fusion events were both greater and faster at this elevated Ca\textsuperscript{2+} (Figure 5.13 B and 5.14). Then we performed TIRFM to observe whether single mutations in the C2 domains
can also give rise to alterations in Ca\(^{2+}\)-sensitivity of single granule fusion dynamics (Figure 5.15 and 5.16). Upon trigger exocytosis at two different Ca\(^{2+}\) concentrations, we observed single mutations in the C2 domains are enough to disrupt the Ca\(^{2+}\)-dependency of single granule fusion events. The percentage of non-fusing granules was significantly reduced at 10 mM Ca\(^{2+}\) (Figure 5.16 A). Among the fused granules, their kinetics and amplitude of the release were faster and greater at this elevated Ca\(^{2+}\) (Figure 5.15 and 5.16 B and C). In conclusion, mutations in the C2 domains of Munc13-4 alters two independent parameters of vesicle fusion in a Ca\(^{2+}\)-dependent manner: the size and kinetic of fusion pore opening in single vesicles and the frequency of the fusion events.
Figure 5.13 Mutations in C2 domains of Munc13-4 strikingly alters the Ca\(^{2+}\)-sensitivity of single granule fusion. (A and B) Representative traces of release of NPY-mCherry from Munc13-4 knockdown cells rescued with EmGFP, wild-type (A) and double, quadruple mutants (B) in C2 domains upon exocytosis triggered at 2.2 mM Ca\(^{2+}\) (black) and 10 mM Ca\(^{2+}\) (red).
Figure 5.14 Mutations in C2 domains of Munc13-4 strikingly alters the Ca\(^{2+}\)-sensitivity of single granule fusion. (A) Non-fusion events were calculated by normalizing number of granules which exhibited non-fusing behaviour to the total number of granules of Munc13-4 knockdown cells rescued with EmGFP, wild-type Munc13-4, double mutants as well as quadruple mutant in C2 domains at 2.2 mM and 10 mM extracellular Ca\(^{2+}\). Error bars indicate SEM (n=25-50 per cell). * P < 0.05, student t-test. (B) Kinetic of NPY-mCherry release from fused single granules was calculated from the time of adding stimulation (2.5 \(\mu\)M ionomycin) to reach the peak of fluorescent intensity; Munc13-4 knockdown cells rescued with EmGFP, wild-type Munc13-4, double mutants as well as quadruple mutant in C2 domains at 2.2 mM and 10 mM extracellular Ca\(^{2+}\). Error bars indicate SEM (n=25-50 per cell). * P < 0.05, student t-test. (C and D) Normalized histogram and Gaussian fits illustrating fluorescent intensity increase of single granule fusion events from Munc13-4 knockdown cells rescued with EmGFP, wild-type Munc13-4 (C), double mutants as well as quadruple mutant in C2 domains (D) at 2.2 mM and 10 mM extracellular Ca\(^{2+}\). % increase was calculated by normalizing peak fluorescent intensity as a result of fusion event to the baseline before stimulation of each granule. Error bars indicate SEM (n=25-50 per cell).
Figure 5.15 Single mutation in either C2A or C2B domain of Munc13-4 is enough to alter the Ca\(^{2+}\)-sensitivity of single granule fusion. Representative traces of release of NPY-mCherry from Munc13-4 knockdown cells rescued with single mutants in C2 domains upon exocytosis triggered at 2.2 mM Ca\(^{2+}\) (black) and 10 mM Ca\(^{2+}\) (red).
Figure 5.16 Single mutation in either C2A or C2B domain of Munc13-4 is enough to alter the Ca\textsuperscript{2+}-sensitivity of single granule fusion. (A) Non-fusion events were calculated by normalizing number of granules which exhibited non-fusing behaviour to the total number of granules of Munc13-4 knockdown cells rescued with single mutants in C2 domains at 2.2 mM and 10 mM extracellular Ca\textsuperscript{2+}. Error bars indicate SEM (n=25-50 per cell). * P < 0.05, student t-test. (B) Kinetic of NPY-mCherry release from fused single granules was calculated from the time of adding stimulation (2.5 µM ionomycin) to reach the peak of fluorescent intensity; Munc13-4 knockdown cells rescued with single mutant in C2 domains at 2.2 mM and 10 mM extracellular Ca\textsuperscript{2+}. Error bars indicate SEM (n=25-50 per cell). * P < 0.05, student t-test. (C) Normalized histogram and Gaussian fits illustrating fluorescent intensity increase of single granule fusion events from Munc13-4 knockdown cells rescued with single mutant in C2 domains at 2.2 mM and 10 mM extracellular Ca\textsuperscript{2+}. % increase was calculated by normalizing peak fluorescent intensity as a result of fusion event to the baseline before stimulation of each granule. Error bars indicate SEM (n=25-50 per cell).
5.3 Conclusion

In this Chapter, an attempt to investigate the molecular mechanism of Munc13-4 in the mast cell degranulation was made. Munc13-4 has been known to be a crucial player in the immune cell exocytosis, as the KO of Munc13-4 in mice led to defective degranulation from platelets and CTLs and the mutations in Munc13-4 has been shown to result a devastating immune disorder, FHL3 in humans (Feldmann et al., 2003).

Our results suggest that Munc13-4 mediate the mast cell exocytosis by performing a dual function; (i) as a whole protein, it is an essential priming factor, (ii) aspartic acid residues in the C2 domains are important to regulate the Ca^{2+}-sensitivity of exocytosis. The priming role of Munc13-4 was demonstrated by the complete abolishment of exocytosis throughout a wide range of Ca^{2+} concentrations by the Munc13-4 KD cells and EmGFP rescued cells. On the other hand, mutations on Ca^{2+} binding residues of the C2 domains exhibited markedly shifted Ca^{2+}-sensitivity (Figure 5.4 to 5.9).

In addition, we found Ca^{2+}-dependent variations in the kinetics and sizes of fusion pore opening as a consequence of Munc13-4 C2 domain mutations (Figure 5.12 to 5.16). In neuronal exocytosis, the kinetics of each single vesicle fusion is believed to be highly uniform. Although null mutations of the proteins critical for exocytosis, such as syt-1, VAMP-2, SNAP-25, and Munc13-1, strikingly reduces the frequency of the fusion events, the kinetics of the remaining fusion events are similar, suggesting that the fusion event is unitary therefore leaving the entity that mediates the fusion pore opening remains unclear. However, this unitary nature may be partly because the size of synaptic vesicles is small, the concentration of transmitters is high and release of the fusion event is too rapid. These make it technically difficult to measure the variations in fusion pore opening in neuronal exocytosis. For example, although two types of
synaptic vesicle fusions (kiss-and-run versus full membrane fusion) have been suggested, the measurement of the kinetics of miniature post-synaptic current does not normally distinguish these two types. Unlike the ultrafast neurotransmitter exocytosis, the fusion of the large lysosomal vesicles occurs in the order of seconds thereby studying fusion pore opening in this system much approachable (Fernández-Chacón and Alvarez de Toledo, 1995; Oberhauser and Fernandez, 1996; Spruce et al., 1990). In this aspect, the future study of the lysosomal vesicle fusion can uniquely contribute to unravel mechanisms which regulate the fusion pore opening and closure.
Chapter 6: Discussion

6.1 Essential role of Munc18 in mast cell degranulation

Previous studies which demonstrated the identification of important players on mast cell degranulation have relied on siRNA mediated acute KD or use of a particular gene deficient-mast cells. Through these, the crucial function of Munc18-2 and other SNARE proteins have been demonstrated (Brochetta et al., 2014; Frank et al., 2011; Kim et al., 2012; Lippert et al., 2007; Martin-Verdeaux et al., 2003; Sander et al., 2008; Tadokoro et al., 2007; Tiwari et al., 2008; Vaidyanathan et al., 2001). However, without a rescue approach, specificities of the phenotypes are of a concern in KD studies, due to nonspecific off-target effect of siRNA. Moreover, without a way to re-introduce a gene, mutational studies cannot be performed thus hampering detailed structural-functional analysis of a protein. In present thesis, lentivirus-mediated stable KD and rescue of protein of interest using RBL-2H3 mast cells was established and this system allowed to study the structure-vs-function relationship. Also, these cells were stimulated by IgE-dependent as well as ionomycin-induced methods to trigger the degranulation to observe the secretory phenotypes mediated by a specific protein of interest.

In Chapters 3 and 4, the role of Munc18 and the functional significance of the interactions with cognate syntaxins in mast cell exocytosis has been investigated. The structure-vs-function relationship of the SNARE proteins and their regulators has been intensively studied in neuronal secretion using genetically engineered neurons of mouse, Drosophila, C. elegans, adrenal chromaffin cells and PC12 cells. However, there have been a significantly fewer studies
conducted based on immune cell exocytosis despite similar SNARE-mediated exocytosis are known to occur (Al Hawas et al., 2012; Elstak et al., 2011; Ren et al., 2010; Ye et al., 2012).

In Chapter 3, through the stable KD of Munc18-1/2 in RBL-2H3 cells, we have demonstrated that there is not only abolished degranulation but also a significant reduction in syntaxin-11 protein level. Thus the phenotypes of Munc18-1/2 DKD RBL-2H3 cells have recapitulated the phenotype of CTLs, NK cells and platelets from FHL5 patients where loss of Munc18-2 expression induces striking reductions in the level of syntaxin-11 (Al Hawas et al., 2012; Côte et al., 2009; zur Stadt et al., 2009). Moreover, re-expression of Munc18 effectively rescued the defective degranulation and restored the protein level of syntaxin-11. These results strongly indicate that Munc18 is crucial for mast cell degranulation in part through its regulating function toward syntaxin-11. Furthermore, in Chapter 4, we have identified another syntaxin isoform, syntaxin-3, whose level was strikingly reduced in Munc18-1/2 DKD mast cells. Like syntaxin-11, this decreased syntaxin-3 was completely restored upon re-expression of Munc18-1 or -2 illustrating the specificity of such phenotype. In addition, observation of mislocalized syntaxin-3 in a perinuclear region of Munc18-1/2 DKD mast cells which was rescued by re-introduction of WT Munc18 provides an additional strong evidence that Munc18 plays additional regulatory activity toward syntaxin-3 as well.

In neuronal exocytosis, absence of Munc18-1 lead to complete abolishment of exocytosis and this phenotype can partly be explained by the lack of regulatory activity toward its binding partner, syntaxin-1. Syntaxin-1 null or KD has a profound defect in neuronal secretion as it is one of the essential SNARE apparatus in neuronal exocytosis. In an attempt to investigate the functional contribution of regulation toward cognate syntaxin partners by Munc18 in mast cells, stable syntaxin-3 and -11 KD mast cells were generated and tested for their degranulation.
Surprisingly, the syntaxin-11 KD mast cells did not exhibit any significant defect in degranulation despite stronger down-regulation of its protein level than the observed in Munc18-1/2 DKD mast cells. Syntaxin-11 has been implicated to be an important player in the degranulation of many granulocytes as genetic mutation in syntaxin-11 has led to a devastating immune disorder, FHL4 where the isolated CTLs and NK cells from human patients exhibit defective degranulation (Bryceson et al., 2007; D'Orlando et al., 2012; Dabrazhynetskaya et al., 2012; Hellewell et al., 2014; Kögl et al., 2012; Offenhäuser et al., 2011; Prekeris et al., 2000). Moreover, syntaxin-11-deficient human platelets exhibited a robust defect in agonist-induced secretion (Ye et al., 2012). Also, in the same study, syntaxin-11 was shown to be able to form SNARE complexes with VAMP-8 and SNAP-23, indicating a functional candidate of t-SNARE in hematopoietic cell exocytosis. However, our syntaxin-11 KD mast cells did not exhibit any degranulation defects. One possibility that can explain differences in phenotypes might be that the functional importance of syntaxin-11 is variable in distinct types of blood-residing cells. Although many granulocytes share SNARE-mediated exocytosis to be a common mechanism, different expression and therefore different functional contributions of specific players are observed. For example, antibody mediated blocking of syntaxin-4 was shown to block Ca\(^{2+}\) and GTP\(\gamma\)S-induced exocytosis in human neutrophils (Mollinedo et al., 2003). However, platelets isolated from syntaxin-4 single or syntaxin-2, 4 double KO mice did not exhibit any secretion defects (Ye et al., 2012). Moreover, while VAMP-7 is highly expressed in all neutrophils and low concentration of VAMP-7 antibody prevented the release from neutrophils (Logan et al., 2006), VAMP-8 seems to be the importance player in platelet and mast cells (Ren et al., 2007; Tiwari et al., 2008). Furthermore, in CTLs, VAMP-2 seems to be the only v-SNARE localized to the lytic granules and ablation of VAMP-2 resulted in robust reduction in secretion (Matti et al., 2013), while it is dispensable in mast cells and platelets (Ren et al., 2007; Sander et al., 2008).
Thus, although syntaxin-11 has profound effects on exocytosis of CTLs, NK cells and platelets, its role may be minor in mast cells. Therefore, unlike neuronal exocytosis where the functional relationship between Munc18-1 and syntaxin-1 is well reflected based on their binding properties, actions of Munc18 toward its cognate syntaxins and their functional contributions might be variable due to heterogeneous expression profiles in different hematopoietic cells. Another possible explanation as to why we did not see the degranulation defects from the syntaxin-11 KD mast cells could be perhaps different types of granules in mast cells require distinct SNARE proteins for the degranulation. Thus, depending on the types of assay methods measuring different cargo molecules, the interpretation of the role of proteins could be different. In addition to β-hexosaminidase-containing secretory granules, mast cells are known to have different types of granules containing different cargos as well as conventional lysosomes that can fuse with the plasma membrane *Puri and Roche, 2008*, demonstrated that VAMP-8-deficient mast cells resulted in defective degranulation of β-hexosaminidase, serotonin and cathepsin D (Puri and Roche, 2008). However, this VAMP-8 KO mast cells had normal histamine and TNF-α releases indicating VAMP-8 in mast cells are specifically important for the release of a subset of granules. This notion is also observed in other hematopoietic cells such as platelets, where they contain α granules, dense granules and lysosome that all exhibit fusion with plasma membrane. Although Munc18-2-, syntaxin-11- and VAMP-8-deficient platelets exhibited defects of all three different types of granules fusion (Al Hawas et al., 2012; Ren et al., 2007; Ye et al., 2012), SNAP-23 and syntaxin-2 seem to be required for release of dense granules only (Chen et al., 2000). Therefore, it is possible that syntaxin-11 might be playing an important role in mediating fusion of other type of secretory granules (lysosome or histamine containing granules) and this could be also regulated by Munc18. Further studies will decipher precise function of Munc18/syntaxin-11 interaction toward mast cell exocytosis.
Unlike syntaxin-11, syntaxin-3 KD resulted in a significant reduction in mast cell degranulation. Syntaxin-3 was demonstrated to be an important t-SNARE in mast cell (Brochetta et al., 2014; Frank et al., 2011; Tadokoro et al., 2007; Tadokoro et al., 2010) as well as other hematopoietic cells such as neutrophil and NK cells (Martín-Martín et al., 1999; Naegelen et al., 2015; Pattu et al., 2012). These studies revealed that syntaxin-3 is localized to the secretory granules in these granulocytes and translocation to the plasma membrane occurs when stimulated. Also recently, it was demonstrated that expression of syntaxin-3 increased in dendritic cells upon activation by lipopolysaccharide, and KD of syntaxin-3 resulted in profound reduction in the secretion of IL-6 (Collins et al., 2014). Therefore, syntaxin-3 exhibits conserved important function in fusion events of distinct populations of hematopoietic cells. In addition to drastically decreased protein level of syntaxin-3 in our Munc18-1/2 DKD mast cells, there was a profound mislocalization of syntaxin-3. This granule localized syntaxin-3 was invariantly aggregated at the perinuclear region in the Munc18-1/2 DKD mast cells. Moreover, the fact that these phenotypes were all rescued by the re-introduction of WT Munc18 provide a strong evidence that the Munc18 participates in the process of mast cell exocytosis via regulating both protein level and trafficking of syntaxin-3. Thus, although Munc18 proteins regulate both syntaxins-3 and -11 isoforms of mast cells, the former interactions could provide more functional contributions to the mast cell exocytosis.
6.2 Regulatory function of Munc18 toward syntaxin

The results from Chapters 3 and 4 indicate that Munc18 performs an essential role in the mast cell degranulation partly via regulating its cognate syntaxin proteins. In Munc18-1/2 DKD RBL-2H3 cells, not only the protein levels of syntaxin-3 and -11 was reduced but the localization of syntaxin-3 was perturbed. Moreover, these phenotypes were successfully rescued by the re-introduction of WT but not by the mutants which failed to interact with syntaxins. Thus, this provides a strong evidence that regulatory function of Munc18 toward cognate syntaxin is dependent on direct interaction. Such regulating phenotype was also observed in CTLs and NK cells from FHL5 patients where level of syntaxin-11 was strongly reduced (Côte et al., 2009). Moreover, this is in line with previous results indicating the importance of interaction between Munc18-1 and syntaxin-1 and their implications in neuronal exocytosis. In particular, syntaxin-1 level was dramatically decreased in Munc18-1 KO neurons, Munc18-1 deficient chromaffin cells as well as Munc18-1/2 DKD PC12 cells (Han et al., 2009; Toonen et al., 2005; Verhage et al., 2000; Voets et al., 2001). This phenotype seems to be conserved in other species as it has been reported that deletion of Vps45p, a SM protein in yeast, results in reduction of cognate syntaxin, Tlg2p, as a result of proteasome-mediated degradation. Furthermore, another yeast homologue of syntaxin, Ufe1, has been shown to be degraded through ER associated degradation pathway in the mutant lacking Sly1p (Braun and Jentsch, 2007). Although many studies have shown that in the absence of Munc18, protein level of syntaxin is severely affected, no clear mechanism has been established as to how. One possible scenario would be that Munc18 may affect the synthesis of syntaxin perhaps through interacting with transcription factors. However, it has not been demonstrated that Munc18 can interact with any transcription factors and whether such factors can have impact on syntaxin gene regulation. Therefore, a more plausible case might be that Munc18 regulates the level of syntaxin protein by ensuring the stability of syntaxin through
protein-protein interaction. Munc18 was originally discovered as it was tightly bound to syntaxin (Hata et al., 1993). Indeed, the protein level of other SNARE proteins such as SNAP-25 or VAMP which does not exhibit as strong interactions with Munc18 as syntaxin, was found to be unchanged in Munc18-1 KO brain (Toonen et al., 2005). This provides a strong evidence that high affinity binding is necessary for Munc18 to protect its cognate protein from being degraded. Previously, it has been hypothesized that the decreased levels of secretory activity may lead to down regulation of proteins important for secretion. However, this possibility could be excluded by the observation that the level of another synaptic protein that is critical for secretion such as SNAP-25 is not affected even when the secretion is severely impaired in the absence of Munc18-1 (Arunachalam et al., 2008; Han et al., 2009). Moreover, syntaxin-1 level was not changed in Munc13-1/2 DKO mice despite the arrested neurotransmitter release (Varoqueaux et al., 2002). Therefore, it is highly likely that increased syntaxin-3 and -11 degradation is caused by the direct effect of Munc18 on syntaxin stability. Perhaps, it could be postulated that the syntaxin-3 or -11 without Munc18 become highly unstable and thus highly susceptible to be degraded by the cell via the ubiquitin-proteasome degradation pathway (Glickman and Ciechanover, 2002). Likewise, it has been reported that in the absence of CSPα, which is known as a chaperone of SNAP-25, protein level of SNAP-25 decreases post-transcriptionally due to impaired stability of this protein (Sharma et al., 2011). Therefore, like CSPα and SNAP-25 in neurons, Munc18 and syntaxin-3 and -11 may behave in a similar chaperone-substrate relationship.

In addition, results shown in Chapter 4 clearly demonstrated the importance of Munc18 on proper secretory granule localization of syntaxin-3. This notion is in line with the previous study that showed that when syntaxin-1 is ectopically expressed in intracellular compartments, it correctly localizes to the plasma membrane only when co-transfected with Munc18-1 in normal rat kidney (NRK) fibroblast cells and other non-neuronal cells (Medine et al., 2007; Rickman et
al., 2007; Rowe et al., 2001). Likewise, Munc18-1/2 DKD PC12 cells exhibited vastly mislocalized syntaxin-1, which was rescued when WT Munc18-1 was re-expressed (Han et al., 2011; Han et al., 2009). It has been suggested that this proper trafficking of syntaxin relies on binding of syntaxin to Munc18 as this binding mode is thought to prevent the formation of ectopic SNARE complexes within cells. It has become clear that complex formation between SNARE proteins is promiscuous than previously recognized. The SNARE proteins have been shown to be able to form SNARE complex via unspecific interactions with non-cognate SNARE partners (Shen et al., 2007), possibly due to the high resemblance in their core sequence within SNARE motifs which participates in complex formation (Fasshauer et al., 1999). Therefore, interaction of Munc18 and syntaxin-3 might begin only shortly after syntaxin-3 protein is made to prevent any ectopic SNARE complex formation that could trap syntaxin-3 in an inappropriate compartment. From the co-localization studies, the results demonstrated that perinuclearly mislocalized syntaxin-3 in the absence of Munc18-1/2 does not co-localize with the Golgi or ER markers. This is in contrast to a study which shown that ectopically expressed syntaxin-1 is trapped at the Golgi or the ER compartments (Rickman et al., 2007). In mast cells, fusion-competent secretory granules are generated through extensive maturations of initial progranules which were formed from budding from Trans-Golgi network. This maturation processes include acidification of granules, sorting of granule contents, and importantly, by fusing with other granules and/or lysosomes and recycling endosomes which would require SNARE machinery (Wernersson and Pejler, 2014). Therefore, it is possible that syntaxin-3 may get engaged in an ectopic SNARE complex formation at the progranules and gets stuck in the absence of proper regulation provided by Munc18. In this scenario, Munc18 would protect syntaxin-3 from any ectopic SNARE complex until the secretory granules are fully maturated (Figure 6.1). Then, in the presence of appropriate SNARE partners and other regulators, binary interaction of Munc18
and closed syntaxin-3 halts, and syntaxin-3 would go on to form functional SNARE complex for efficient membrane fusion. Therefore, the binary interaction between Munc18 and syntaxin-3 serves an important role in proper syntaxin-3 trafficking by preventing inappropriate interaction with non-cognate SNARE partners.
Figure 6.1 Model illustrating regulatory role of Munc18 on subcellular localization of syntaxin-3. Binding of Munc18/syntaxin at immature progranules prevents syntaxin from engaging ectopic SNARE complex formation thus aiding trafficking to mature granules that are important for degranulation. Such regulatory role of Munc18 is not observed in the case where Munc18 is absent or in the presence of mutant Munc18 that does not bind to syntaxin-3.
6.3 Contrasting mechanism of binary interaction between Munc18 and syntaxin from neuronal exocytosis

Since the finding of the interaction between Munc18 hydrophobic pocket and the N-peptide of syntaxin (Burkhardt et al., 2008; Hu et al., 2007) which has been shown to support the binding of Munc18-1 to the SNARE complex and facilitates the SNARE-mediated liposome fusion (Shen et al., 2007), many efforts have been made to identify the crucial role of this interaction in physiological membrane fusion. From the side of syntaxin, several studies suggest the important role of syntaxin-1 N-peptide toward Munc18-1 for Munc18-1’s fusion promoting role. It was demonstrated that a synthetic N-terminal peptide of syntaxin-1 inhibits neuronal secretion from neurons and neuroendocrine PC12 cells, by competitively binding to Munc18-1 (Khvotchev et al., 2007). In addition, phosphorylation of syntaxin-1 N-peptide at Ser14, which disrupts binding to Munc18-1 in neuroblastoma cells, dramatically decreased the neurosecretory response (Rickman et al., 2010). Moreover, deletion of N-peptide in syntaxin-1A failed to rescue the defective secretion phenotype of syntaxin-1 null neurons (Zhou et al., 2013). However, in alternative studies, the mutations in the Munc18-1 hydrophobic pocket (F115E, E132A, F115E/E132A) that abolish N-terminal binding were shown to have limited impact on DCV exocytosis in Munc18-1 single (Malintan et al., 2009) and Munc18-1/2 double KD (Han et al., 2009) PC12 cells. Moreover, a recent study has revealed that the F115E mutation in Munc18-1 that loses binding to the SNARE complex due to impaired N-terminal interaction can still support normal synaptic transmission in Munc18-1 null neurons (Meijer et al., 2012).

In contrast, the results presented in Chapter 3 demonstrated the importance of hydrophobic pocket in mediating interaction with N-terminal syntaxin-11 in mast cell exocytosis. An introduction of point mutations in hydrophobic pocket of Munc18 resulted in lack of
restoration of decreased syntaxin-11 protein level as well as defective degranulation of Munc18-1/2 DKD mast cells. Moreover, the fact that even a single point mutation of either F115E or E132A was able to abrogate the rescue ability of Munc18 indicates that this binding mode seems to play much important role in mast cells. The reason why the hydrophobic pocket mutations in Munc18 cause substantial different outcomes in exocytosis between neuronal cells and mast cells awaits further studies. We found that all hydrophobic pocket mutants of Munc18-1 or -2 do not restore syntaxin-11 level in the DKD RBL-2H3 cells. On the other hand, previous studies showed that the level of syntaxin-1 is significantly restored by the same Munc18-1 mutants in the DKD PC12 cells, albeit slightly lower than by the WT Munc18-1 (Han et al., 2011; Han et al., 2009; Meijer et al., 2012). Thus, the functional significance of Munc18 hydrophobic pocket in different types of exocytosis seems to be explained at least in part by the differential degree of involvement of this region in regulation of the cognate syntaxins. To explain the difference in the functional implication of hydrophobic pocket binding to exocytosis, the following is hypothesized. Perhaps, the relative importance of the binding between Munc18 hydrophobic pocket and syntaxin N-peptide is inversely related to the degree of tightness of the binding between the cleft formed by domain-1 and 3a of Munc18 and closed syntaxin. The binding of Munc18 to closed syntaxin is a major binding mode for the binary interaction between Munc18-1 and syntaxin-1A (Burkhardt et al., 2008; Han et al., 2009; Misura et al., 2000), while the N-peptide binding alone cannot support the binary interaction but seem to further secure the binary interaction (Burkhardt et al., 2008; Shen et al., 2007). For example, Munc18-1 and syntaxin-1A still bind each other at low nanomolar concentrations even when the hydrophobic pocket of Munc18-1 is mutated or N-peptide of syntaxin-1A is removed (Burkhardt et al., 2008; Malintan et al., 2009). In line with this, it was recently demonstrated that closed syntaxin-1 with N-peptide bearing mutations was still able to rescue the defective secretion phenotypes of syntaxin-1 KD
PC12 cells and locomotion defects of *Unc-64* null *C. elegans* (Park et al., 2016). This indicates that in neuronal exocytosis, the binary interaction between Munc18-1 and syntaxin-1 is mostly mediated through closed syntaxin binding and N-peptide binding seems to be further securing an already existing interaction. In contrast, we found that even a single mutation (F115E or E132A) in hydrophobic pocket region of Munc18-2 failed to interact with syntaxin-11. Therefore, a plausible explanation can be that the binding of Munc18-2 to closed syntaxin-11 is much weaker than that of between Munc18 and syntaxin-1A. Indeed, a recent study has demonstrated that binding of Munc18-2 to N-peptide of syntaxin-11 was fully abrogated when E132A mutation was introduced and importantly, E132A mutant Munc18-2 did not exhibit any binding to full-length syntaxin-11 (Hackmann et al., 2013). This study also revealed a crystal structure of human Munc18-2 which demonstrated that compared to Munc18-1, Munc18-2 has an enlarged hydrophobic pocket which would provide physically permissive environment for binding toward syntaxin-11 N-peptide (Hackmann et al., 2013). Thus, it seems that N-peptide binding is a key determinant (if not all) for mediating interaction between Munc18-2 and syntaxin-11. Moreover, another structural study demonstrated that the crucial role of syntaxin N-peptide in monomeric interaction with Munc18 is isoform-dependent; deletion of N-peptide in syntaxin-1 has little effect on its binding to Munc18-1 whereas similar deletion in syntaxin-4 dramatically reduces its binding with Munc18-3 (Christie et al., 2012).

Not only N-peptide binding to hydrophobic pocket, but also a binding of closed, monomeric syntaxin to Munc18 seems to involve a different mechanism and therefore functional contribution to mast cell exocytosis from neuronal exocytosis. In neuronal exocytosis, a single binary interaction mutation of either K46E or E59K was not sufficient to disrupt the binding between Munc18-1 and monomeric syntaxin-1 (Han et al., 2011; Han et al., 2009; Malintan et al., 2009; Meijer et al., 2012). Compared to WT, single binary interaction mutants exhibit only
marginally reduced ability to rescue the defective secretion phenotype of Munc18-1/2 DKD PC12 cells and the synaptic transmission of hippocampal neurons that lack Munc18-1 (Han et al., 2011; Han et al., 2009; Meijer et al., 2012). However, the results in Chapter 4 clearly illustrate that even a single binary interaction mutant (K46E or E59K) of Munc18 is as detrimental as the double mutant (K46E/E59K) in both binding closed, monomeric syntaxin-3 and rescuing the phenotypes of Munc18-1/2 DKD mast cells. These results are strikingly different from the binary interaction between Munc18-1 and syntaxin-1, and their functional implications. Therefore, we speculate although the importance of the binary interaction between Munc18 and cognate syntaxin partners is well conserved in both neuronal and mast cell exocytosis, the binding affinity and, thus, its functional outcome are different between neuronal vs mast cell exocytosis. Since even a single mutant was able to fully abrogate the binary interaction between Munc18 and closed syntaxin-3, it is possible that the binary interaction between Munc18 and cognate syntaxin in immune cells is weaker than that in neuronal cells of between Munc18-1 and syntaxin-1. Therefore, if even one single residue is altered, the whole binary interaction would be abolished because binding by the other residue is not strong enough to sustain the interaction on its own. This hypothesis seems to be in line with our finding that the interaction between the N-peptide of syntaxin and the hydrophobic pocket of Munc18 is also functionally important in mast cell degranulation. Because the interaction between Munc18 and closed syntaxin itself is weak, binding between the Munc18 hydrophobic pocket and the syntaxin N-peptide may work in concert to maintain interaction of Munc18 with its syntaxin partners in mast cells (Figure 6.2). Thus, unlike neuronal exocytosis, where the latter interaction mode is regarded to be of limited function (Arunachalam et al., 2008; Han et al., 2009; Malintan et al., 2009; Meijer et al., 2012), both modes of interactions are equally valuable molecular actions between Munc18 and cognate syntaxin that are essential for mast cell exocytosis.
Figure 6.2 Model illustrating differences of Munc18-syntaxin interaction in neuronal vs immune cell exocytosis. The binary interaction between binding cavity of Munc18 formed between domains 1 and 3 to closed cognate syntaxin(s) in immune cell exocytosis is weaker than Munc18-1 and syntaxin-1 of neuronal exocytosis. Therefore, N-terminal peptide binding to the hydrophobic pocket of Munc18 provides an equally important binding affinity to regulate the protein level and trafficking of cognate syntaxin(s) in immune cell exocytosis.
6.4 Role of Munc13-4 in mast cell exocytosis

Since the identification of Munc13-4 as a gene causing devastating immune disease, FHL3 (Feldmann et al., 2003), different laboratories have attempted to understand the molecular basis on the function of this protein in immune cell exocytosis. Munc13-4 is highly expressed in immune cells, where it participates in regulated granule exocytosis in CTLs, mast cells, neutrophils and platelets (Higashio et al., 2008; Marcenaro et al., 2006; Neeft et al., 2005; Pivot-Pajot et al., 2008; Shirakawa et al., 2004; Yamamoto et al., 2004). Several studies have converged on the function of Munc13-4 has a regulator of immune cell exocytosis through interaction with Rab27A, a small GTPase found in lytic and secretory granules of CTLs, neutrophils and platelets (Elstak et al., 2011; Neeft et al., 2005; Shirakawa et al., 2004). Moreover, these studies revealed that granules from Munc13-4-deficient CTLs failed fuse despite normal docking. Therefore, these results suggest a post-docking role for Munc13-4 in granule priming, consistent with a general role of Munc13 proteins in neuronal exocytosis.

The results in Chapter 5 demonstrate the Munc13-4 is an essential protein mast cell exocytosis. We demonstrated that strong KD of Munc13-4 results in complete abolishment mast cell degranulation regardless of extracellular Ca\textsuperscript{2+} concentrations despite having a similar number of visibly docked granules. This observation is in line with a proposed function of Munc13-4 as a putative priming factor of immune cell exocytosis (Feldmann et al., 2003). This priming action could be mediated through a conserved MHD domain found in Munc13-4. Studies on Munc13-1 illustrates that the MHD is primarily responsible for the priming function of Munc13s, although the adjacent C2C domain may also assist in this function (Basu et al., 2005; Stevens et al., 2005). Early studies suggested that Munc13-1 fragments corresponding to the MHD sequences and the C2C domain bind to the N terminus of syntaxin-1 (Betz et al.,
1997). These data, together with the finding that the open syntaxin-1 LE mutant partially rescues the strong phenotype of unc13-null mutants in *C. elegans* (Richmond et al., 2001), have led to a model in which the MHD helps to open syntaxin-1 by binding to SNARE motif from the closed conformation and providing a template to assemble the SNARE complex, a process which could be aided by interactions of the MHD with Munc18-1 and the membranes. Indeed, further biophysical experiments demonstrated that the MHD dramatically accelerates the transition from the Munc18-1/closed syntaxin-1 complex to the Munc18-1/SNARE-complex assembly (Ma et al., 2011). Therefore, given that Munc13-4 also contains a conserved MHD region, it is possible that the MHD of Munc13-4 controls the activity of cognate syntaxin for proper SNARE complex assembly. Indeed, a study has demonstrated that Munc13-4 interacts with syntaxins-1, -4 and -11 (Boswell et al., 2012). Moreover, the same study has shown that Munc13-4 binds to SNARE domain of the syntaxins as the binding did not occur when fully cytoplasmic domain of syntaxins was used; presumably due to closed conformation. Therefore, Munc13-4 may possess a conserved mechanism of its priming function, via interacting with syntaxins.

Unlike other Munc13 isoforms implicated in priming of synaptic vesicles, Munc13-4 contains two C2 domains (C2A and C2B) where both domains are predicted to bind Ca^{2+}. Moreover, these Ca^{2+}-binding residues are not only conserved throughout Munc13-4 and BAP3 orthologues in different species but also with syt-1, a Ca^{2+}-sensor for neuronal exocytosis. The potential importance of the C2 domains in the function of Munc13-4 was also suggested by the identification of missense mutations in the C2A and C2B domains of Munc13-4 from FHL3 individuals (Sieni et al., 2011). A Munc13-4 protein lacking both C2 domains seemed to localize to recycling endosomes in CTLs but failed to promote endosome fusion (Ménager et al., 2007). Similarly, a Munc13-4 protein lacking its C2A domain localized to secretory lysosomes in RBL-2H3 mast cells but failed to support granule exocytosis (Neeft et al., 2005). Despite the fact C2
domains seem to play an important function in immune cell exocytosis, no attempt was made to understand its mechanism in a relation to the Ca$^{2+}$ binding properties of these domains. By performing Ca$^{2+}$-titration assay, our results indicate that Ca$^{2+}$-binding residues in both C2 domains mediate Ca$^{2+}$-sensing of the mast cell exocytosis. From the control and WT Munc13-4 rescued cells, the secretion exhibited a bell-shaped curve, where increasing extracellular Ca$^{2+}$ concentrations seems to induce more secretion until an optimal Ca$^{2+}$ concentration of 2.2 mM. Then, elevated Ca$^{2+}$ concentrations beyond 2.2 mM rather caused decrease in secretion. However, Munc13-4 mutants bearing mutations in C2 domains had exhibited markedly shifted Ca$^{2+}$-sensitivity on the mast cell degranulation where they showed a trend of positive increase in the secretion at elevating Ca$^{2+}$ concentrations. This altered Ca$^{2+}$-sensitivity is similarly observed in syt-1 null Drosophila and mouse hippocampal neurons (DiAntonio and Schwarz, 1994; Fernández-Chacón et al., 2001; Littleton et al., 1994; Shin et al., 2009). These studies revealed point mutations in syt-1 that altered the Ca$^{2+}$ requirements for exocytosis. For example, AD3 mutant allele harboring a point mutation at Y311N which disrupts the Ca$^{2+}$-sensing ability of C2B domain, shifted the Ca$^{2+}$ requirements for exocytosis to right (DiAntonio and Schwarz, 1994). Moreover, the same mutant impaired the exocytosis at a step after synaptic vesicle docking, indicating that C2B domain of syt-1 must sense Ca$^{2+}$ in order for docked synaptic vesicles to fuse in response to Ca$^{2+}$. In addition, R233Q, which plays a role in both syt-membrane and syt-SNARE interactions, altered the Ca$^{2+}$ requirements for synaptic vesicle exocytosis in cultured hippocampal neurons (Fernández-Chacón et al., 2001). Therefore, it seems a different family of proteins performs Ca$^{2+}$-sensing function in exocytosis between neuronal and immune cells: syt-1 for neuronal exocytosis and Munc13-4 for immune cell exocytosis (Figure 6.3). Both proteins contain intact two C2 domains which mediate the Ca$^{2+}$ binding. In addition, unlike Munc13-1 where it is tethered to the active zone via RIM-binding C2A domain (Betz et
Munc13-4 has been shown to localize at the secretory granules, in part, by interacting with Rab27A (Shirakawa et al., 2004). Syt-1 is also known to exhibit this vesicular localization, thus vesicular localization with two C2 domains seem to a common mechanism that mediates Ca^{2+}-sensitivity of membrane fusion events. Also, it is surprising that one protein Munc13-4, seems to mediate two different functions in mast cell exocytosis: priming and Ca^{2+} sensor. Although precise mechanism awaits further studies, one speculation can be that perhaps the requirement of very fast exocytosis has made it necessary for two proteins (Munc13-1 and syt-1) to mediate the two separate functions in neurons. In the process of specialization, the C2C domain of Munc13-1 were degenerated and lost the function of Ca^{2+} sensor (Lu et al., 2006; Shin et al., 2010). The remaining C2B domain of Munc13-1 then performs a role in synaptic plasticity by its Ca^{2+} binding property (Shin et al., 2010). On the other hand, in slow immune cell exocytosis, a single molecule Munc13-4 may be sufficient to mediate the dual functions.

Finally, we have found Ca^{2+} dependent variations in the kinetics and sizes of fusion pore opening as a consequence of Munc13-4 C2 domain mutants. Although initially fusion pore was regarded as pure lipid due to large diameter and smoothness of the membrane, it is now accepted that fusion pore are made of proteins due to similar conductance as ion channels and gap function (Breckenridge and Almers, 1987). Present models of fusion pore envision the C-terminal ends of VAMP and syntaxin to be located close to each other right in the centre of the pore. The C-terminal end of SNAP-25 is located near the putative location of transmembrane domains of VAMP and syntaxin. Deletion of a few amino acids at C-terminus of SNAP-25 reduce exocytosis in chromaffin cells (Criado et al., 1999; Wei et al., 2000). In addition to SNARE complex, free V0 part of the proton ATPase pump has been implicated in fusion pore formation of yeast vacuoles (Peters et al., 2001). V0 trans-complexes may form a proteolipid-lined channel at the fusion site and radial expansion of such a protein pore may be a mechanism
for membrane fusion. Through observation of single granule release in NPY-mCherry transfected RBL-2H3 cells, the results in Chapter 5 demonstrated that Munc13-4 can regulate the kinetics and size of fusion pore opening. Interestingly, this fusion pore regulation by Munc13-4 was Ca\(^{2+}\)-dependent, as the C2 domain mutants resulted in markedly altered Ca\(^{2+}\)-dependent regulation of fusion pore. Unlike the ultrafast neurotransmitter exocytosis, the fusion of the large lysosomal vesicles occurs in the order of seconds thereby studying fusion pore opening in this system much approachable (Fernández-Chacón and Alvarez de Toledo, 1995; Oberhauser and Fernandez, 1996; Spruce et al., 1990). Therefore, the intrinsic nature of slow release of large granules perhaps has made it possible to identify the novel role of Munc13-4 as a Ca\(^{2+}\)-dependent fusion pore regulator. The precise molecular mechanism as to how Munc13-4 performs such function await future studies. Syt-1 also interacts with lipid in a Ca\(^{2+}\)-dependent manner and Ca\(^{2+}\) triggers partial penetration of syt-1 C2 domains into the lipid bilayer, induced positive curvature (Chapman, 2008; Martens et al., 2007). Therefore, based on Ca\(^{2+}\)-dependent SNARE protein and phospholipid binding properties of Munc13-4, perhaps it may regulate the formation of fusion pore both directly (through lipid binding) or indirectly (through other SNARE proteins).
Figure 6.3 Model illustrating independent roles of Munc13-1 and synaptotagmin-1 in neuronal exocytosis while Munc13-4 performing a dual role in immune cell exocytosis.
6.5 Possible mechanisms of Ca$^{2+}$-sensing by Munc13-4

In our study, we have seen that mutations in Ca$^{2+}$-binding residues in either C2 domain can lead to disruption in Ca$^{2+}$-sensitivity. This is unlike the studies on syt-1 where the primarily function of Ca$^{2+}$-sensing is mediated by the C2B domain. For example, mutations of all five Ca$^{2+}$ binding residues of the C2A had no apparent defect in synaptic transmission (Stevens and Sullivan, 2003). In contrast, Ca$^{2+}$ binding to the C2B domain is essential for syt-1 function as a Ca$^{2+}$-sensor as mutations of D309N or D363N completely disrupted the function (Pang et al., 2006). Precise mechanism as to why both C2 domains of Munc13-4 are critical for Ca$^{2+}$-sensing would require detailed quantitative analysis. Both C2A and C2B domains of syt-1 exhibits low Ca$^{2+}$-affinity (Fernandez et al., 2001; Radhakrishnan et al., 2009; Ubach et al., 1998). Based on Munc13-1 C2B domain, it was found that Ca$^{2+}$ affinity of the C2B domain was indistinguishable from that of syt-1 C2A and C2B fragment (Shin et al., 2010), therefore Ca$^{2+}$ affinity of the C2 domains of Munc13-4 are likely to be low. For syt-1, low Ca$^{2+}$ affinity can be overcome by the high Ca$^{2+}$ concentration around Ca$^{2+}$-channels where syt-1 are localized through the presence of PIP$_2$ and interaction between vesicle localized Rab3 with active zone protein RIM (Wang et al., 1997). However, whether such granule tethering molecules can juxtapose Munc13-4 close to Ca$^{2+}$-microdomain in mast cell is unknown. Therefore, it is possible that due to this intrinsic low Ca$^{2+}$-affinity, binding of Ca$^{2+}$ to both C2A and C2B are needed to co-operate the Ca$^{2+}$-sensing function of Munc13-4. For syt-1, both C2A and C2B domains seem to mediate similar levels of phospholipid and t-SNARE protein binding (Chapman, 2008). Nonetheless, greater functional implications assessed from the C2B domain could mainly be due to its interaction with PIP$_2$ (Bai et al., 2004; Schiavo et al., 1996). Although isolated C2B domain exhibits slightly weaker binding to lipid membrane than the C2A, it preferentially interacts with PIP$_2$, which are highly concentrated in microdomains where the release occur (Fernandez et al., 2001). Therefore, this
has led to a model in which binding to PIP$_2$ may navigate syt-1 to the membrane-penetration activity to the functionally meaningful location where then it can assist in exocytosis. However, it seems that the mechanisms of action mediated from two C2 domains of Munc13-4 seem different from that of syt-1. A study has shown that Ca$^{2+}$-dependent phospholipid binding property of Munc13-4 are mainly mediated through the C2B domain, while Ca$^{2+}$-dependent syntaxin binding are mediated by the C2A domain (Boswell et al., 2012). This suggests that two C2 domains of Munc13-4 exhibit independent roles, which need to work in concert for Munc13-4 to promote Ca$^{2+}$-dependent SNARE-mediated mast cell exocytosis.

The results from Ca$^{2+}$-titration demonstrate that there seems an optimal Ca$^{2+}$ concentration to trigger mast cell exocytosis in such too high concentrations of Ca$^{2+}$ rather inhibit the exocytosis. This is not simply due to the cytotoxicity induced by elevated Ca$^{2+}$ because the C2 domain mutants Munc13-4 exhibited better secretion at this higher Ca$^{2+}$. This is markedly different from observations from syt-1 and Munc13-1 studies, where although the EPSC or IPSC amplitude gets saturated, higher Ca$^{2+}$ amount (i.e. >10mM) does not result in decrease in secretion (Shin et al., 2010; Shin et al., 2009). One possible scenario could be that Munc13-4 exhibits different binding affinities toward cognate syntaxin according to the Ca$^{2+}$ amounts such that binding affinity is stronger at higher Ca$^{2+}$. It was demonstrated that Munc13-4 interacts with syntaxin proteins in a Ca$^{2+}$-dependent manner, and like syt-1, the C2 domains of Munc13-4 interacts with the SNARE domain of syntaxin. (Boswell et al., 2012). Therefore, at normal 2.2 mM Ca$^{2+}$, Munc13-4 engages in binding to syntaxin and it gets displaced easily allowing syntaxin to form SNARE complex to drive fusion. However, at 10 mM, the SNARE complex assembly and therefore fusion could be hampered by the tight binding of Munc13-4 to syntaxin which juxtaposes bulky Munc13-4 in the region where the SNARE assembly has to occur. For C2 domain mutants, higher Ca$^{2+}$ is rather beneficial due to loss of Ca$^{2+}$-affinity. This
speculates that Ca\(^{2+}\)-sensing mechanism of the C2 domains of Munc13-4 could perhaps be providing the optimal binding affinity toward SNARE proteins and/or phospholipid in Ca\(^{2+}\)-dependent manner.

From the results of Munc13-4 KD and the C2 domain mutants rescue experiments, it seems clear that absence of Munc13-4 results in complete abolishment but altered Ca\(^{2+}\)-sensitivity when the C2 domains are mutated. Therefore, when whole Munc13-4 is present, it is an essential priming factor while the C2 domains affect Ca\(^{2+}\)-sensitivity of mast cell exocytosis. However, an important question has to be asked. If Munc13-4 protein is solely functioning as a Ca\(^{2+}\) sensor, then the absence of Munc13-4 should still lead to some level of secretion at higher Ca\(^{2+}\) concentrations despite altered Ca\(^{2+}\) sensitivity, similar what it is observed from syt-1 KO in neuronal exocytosis (Shin et al., 2009). On the other hand, if Munc13-4 is solely functioning as a pure priming factor, then selective mutations in the C2 domains would still lead to similar Ca\(^{2+}\)-sensitivity as WT, albeit lower level of secretion throughout Ca\(^{2+}\) concentrations. Although the interpretations regarding the function of Munc13-4 in Chapter 5 may have given an impression that priming and Ca\(^{2+}\)-sensing roles are completely unrelated, the reality is that Munc13-4 may mediate its priming role in a Ca\(^{2+}\)-dependent manner. From the single C2 domain mutant experiments, it was found that even a single mutation in the C2 domains of Munc13-4 is enough to alter the Ca\(^{2+}\)-sensitivity. However, compared to double and quadruple mutants, these single mutants exhibited better secretion throughout wide range of Ca\(^{2+}\) concentrations despite similarly altered Ca\(^{2+}\)-sensitivity. Moreover, it was observed that the C2A mutants had more rescue ability toward the secretion than the mutants of the C2B domain. In addition, the quadruple mutants exhibited a markedly reduced secretion throughout Ca\(^{2+}\)-concentrations. Thus, another possible interpretation of the results seems that the C2 domains of Munc13-4 are involved in both priming and Ca\(^{2+}\)-sensing that are functionally related. Moreover, it appears that both C2 domains of
Munc13-4 are involved in Ca\textsuperscript{2+}-dependent priming functions, but the C2B domain of Munc13-4 is functionally more important than the C2A domain. At present, it remains unknown how these C2 domains of Munc13-4 are involved in Ca\textsuperscript{2+}-dependent priming function. Mechanistically, two potential possibilities can be postulated in regards to this function (Figure 6.4). 1) Based on the speculative role of MHD domain in priming function, the C2 domains could induce changes in the conformation of the Munc13-4 upon Ca\textsuperscript{2+}. Indeed Ca\textsuperscript{2+}-induced conformational switch often occurs in other proteins containing Ca\textsuperscript{2+}-binding domains such as EF-hand containing calmodulin, and C2 domains containing in Doc2β and even C2B of syt-1 (Groffen et al., 2004; Nelson and Chazin, 1998; Wu and Schulten, 2014). Thus, perhaps Ca\textsuperscript{2+}-binding to the C2 domains of Munc13-4 induces changes in a conformation which would then allow the MHD domain to exert augmented priming function presumably by providing increased affinity toward syntaxin. 2) Also, it could be postulated that the priming step is composed of two separate stages where later stage is Ca\textsuperscript{2+}-dependent. This idea was demonstrated in adrenal chromaffin cells where CAPS1/2 DKO phenotypes cannot be rescued by the expression of Munc13-1 (Liu et al., 2010). Interestingly, Munc13-1 was able to rescue the secretion defects of single CAPS2 KO chromaffin cells, suggesting a functional interaction between two proteins. Furthermore, open syntaxin-1 LE mutant was able to rescue CAPS1/2 DKO phenotypes. Therefore, it seems both Munc13-1 and CAPS are functioning at the same priming function but Ca\textsuperscript{2+}-dependent priming by CAPS is a downstream pathway. For mast cell exocytosis, Munc13-4 could be the player that mediates both stages of priming yet by different domains. In this scheme, MHD domain of Munc13-4 are involved in the first stage of priming that is Ca\textsuperscript{2+}-independent, while the C2 domains mediate second priming stage which is downstream and Ca\textsuperscript{2+}-dependent. This model will predict that in the absence of the MHD (i.e. Munc13-4 KD), there is no Ca\textsuperscript{2+}-dependency of the fusion as the first priming stage of granules cannot occur. But when the C2 domains are
mutated, second priming stage of granules will exhibit Ca\textsuperscript{2+}-dependency, thus altered Ca\textsuperscript{2+}-
sensitivity in exocytosis. Delineating precise mechanisms of the MHD and the C2 domains
would need future studies. Collectively, our data suggest that Munc13-4 is an essential player of
the mast cell exocytosis by performing a dual function: 1) priming factor and 2) Ca\textsuperscript{2+}-sensor.
Two possible scenarios underlying Ca²⁺-dependent priming of Munc13-4 in immune cell exocytosis. Scenario 1 suggests that binding of Ca²⁺ to the C2 domains changes conformation of Munc13-4, leading to efficient priming function through MHD-syntaxin interactions. Scenario 2 suggests that priming stage is composed of two steps; MHD partially opens syntaxin in a Ca²⁺-independent manner, followed by fully opening of syntaxin by the C2 domains in a Ca²⁺-dependent manner to complete priming stage.
6.6 **Strength/Limitations of the study**

The experimental models and approaches used in this thesis have both advantages and limitations that must not be overlooked in order to avoid overstatement of conclusions that are drawn from the findings.

6.6.1 **Strength/Limitations in the model used in the study**

Currently available experimental models range widely from *in vitro* reconstituted system, cell lines to *in vivo* animals. It is expected that the experimental models should be selected based on the aim of the experiment and careful consideration of the limitations of each model. In this thesis, the mechanisms underlying the functions of Munc18 and Munc13 were studied using RBL-2H3 cell line. The use of this cell line over primary mast cell line is due to the advantages that they can be cultured indefinitely while being extremely versatile for genetic manipulation. Therefore, they are easier to study the molecular mechanism of secretory pathway compared to the mast cells. Moreover, using immortalized cell line is more economically efficient and the results provided from this model are highly reproducible. Another great advantage is that the cell line is derived from single colony that was isolated after confirming the phenotype. Therefore, it eliminates the possibility of contamination by other confounding cell types. Furthermore, RBL-2H3 cells exhibits a robust secretion in response to Ca$^{2+}$ influx, providing a set of important experimental advantages for cell biological and biochemical studies of the secretory pathway. In addition, they are responsive to IgE/antigen dependent stimulation. In an attempt isolate mast cell lines, researchers obtained RBL-1 cell lines, which exhibited mast cells like features but failed to response to IgE antibody which hampered the use of this cell lines further (Kulczycki et al., 1974). In contrast, RBL-2H3 cells possess binding of IgE antibody to its FceRI receptor and subsequent downstream events. Therefore, use of IgE/antigen and ionomycin in this current
study allows to distinguish phenotypes that are caused by defects in IgE dependent signaling pathway or actual degranulation steps. From the results of Munc13 and Munc18, it appears these proteins are involved in the exocytosis stage, as both IgE/antigen and ionomycin induced degranulation yielded similar level of secretion. Furthermore, other aspects of mast cell physiology are also seen in RBL-2H3 cells such as expression of rat mast cell protease II (Seldin et al., 1985) and similar expression of c-kit receptor tyrosine kinase to that in human and murine mast cells (Tsujimura et al., 1995). However, at the same time, it is clear that RBL-2H3 cells do exhibit features of basophils. Basophils and mast cells are two functionally similar but distinct cell types. The original rat basophilic cells that were obtained from rats treated with a potent carcinogen, were the predecessors of RBL-2H3 cells, and these cells rather exhibited basophil features; basophilic granules containing histamine, serotonin and chemotactic factors that were different from mast cells (Eccleston et al., 1973). Although the SNARE-mediated granule exocytosis is similarly occurring in different hematopoietic cells, it seems distinct players and precise molecular mechanisms are involved in each specific cell type. Thus, whether current findings from RBL-2H3 cells can also be observed in pure primary mast cells still remains to be questionable.

In current thesis, shRNA-mediated Munc18, syntaxin and Munc13-4 cells were used to study the functions of these proteins. These KD cells exhibit very severe phenotypes and the defective phenotypes could be rescued upon re-expression of WT proteins. This is a great advantage for assessing the functional deficits of the mutations introduced in a particular protein to study the structure-vs-function. However, protein KD approach using shRNA or antisense treatment is usually thought to result in reduction of gene expression rather than complete ablation from the gene as for KO method. Therefore, it is often considered that the effect of KD is transient and does not result in 100% deletion of the protein, raising a concern regarding the
possible functional contribution of residual WT protein when assessing the phenotype of the mutant. However, our KD cells were engineered in such way that shRNA designed to target a specific protein are stably expressed through continuous drug selection using puromycin and G418. Initial selection of shRNA expressing cells using these drugs followed by continuous culturing in presence of drugs ensures the stable expression of shRNA for the target mRNAs in surviving cells. Although it is arguable that there may be residual WT proteins in the KD cells, we always compare the phenotypes of the mutants to that of the negative control, which is KD cell rescued with empty EmGFP. Moreover, it is important to acknowledge that our KD mast cells exhibited similar severe phenotypes as the immune cells without functional protein. For example, in our Munc18-1/2 DKD mast cells, we have seen not only defective degranulation but also drastic downregulation of syntaxin-11. This was similar observed in CTLs and NK cells isolated from FHL5 patients where functional Munc18-2 is absent (Côte et al., 2009). This suggests that such a minimum trace of endogenous Munc18 is insufficient to fully perform the functional role of Munc18 in our RBL-2H3 cells. Another limitation associated with this system involves inability to control the level of exogenously expressed protein. For my study, the KD RBL-2H3 mast cells were stably rescued with various mutants through lentiviral infection system with IRES system. An IRES (internal ribosomal entry site) is a nucleotide sequence that allows for translation in the middle of mRNA sequence during protein synthesis (Pelletier and Sonenberg, 1988). The presence of an IRES sequence before the drug resistance gene in this vector facilitates translation of drug resistance gene and ensures simultaneous expression of the gene of interest and gene for drug resistance from bicistronic mRNA transcript (Jang et al., 1988). This allows drug resistance to be used as an indicator of transduction efficiency and a marker for selection, thereby reducing the probability of survival of uninfected cells and consequently increasing the infection rate in drug resistant cells. This is great for ensuring
expression of the gene of interest in the cells. However, exogenously expressed Munc18 and Munc13 variants seem to be often over-expressed compared to the endogenous WT proteins. Although comparable level of expression of exogenously introduced WT and the mutant proteins have allowed reasonable comparison of the phenotypes, whether there is any artifactual effect exerted by the overexpression on the general secretory phenotypes of the cells need to be carefully considered during the interpretation of the results before generating conclusions.

6.6.2 Strengths/Limitations in the experimental approaches

6.6.2.1 Analysis of mast cell degranulation

Throughout the thesis, the degranulation from RBL-2H3 cells was assessed by measuring the activity of released β-hexosaminidase. Although this is an established method to measure the degree of degranulation, other methods of measuring different cargos need to be employed. Unlike synaptic vesicles, mast cells and other hematopoietic cell contain different types of granules which contain different contents and markers. For example, EM studies of mast cell revealed granular heterogeneity of three different types of granules from I, II and III, that are differentiated on the basis of a specific marker and access to endocytotic tracers (Blank, 2011). Type I granules are multi-vesicular lysosomes that release their content only under non-physiological situations. Type II and III represent the compartment that are regulated by a physiological stimulus. Reports have shown that the release of histamine can be inhibited while the release of serotonin is unaffected (Theoharides et al., 1982). Also, it was shown that absence of histamine led to an increase in serotonin levels, suggesting that the cargo molecules can interact for amine homeostasis (Theoharides et al., 1985). Moreover, studies on mast cells have shown that there are different SNARE molecules expressed in various types of granules (Puri and Roche, 2008). Therefore, although β-hexosaminidase is known to be co-released with
histamine from mast cells, it is equally important to measure the release of contents from various types of granules to draw a holistic mechanism of mast cell exocytosis.

Moreover, our method of measuring β-hexosaminidase release after 1 hour of stimulation does not reveal any kinetic aspects involved in the process of mast cell exocytosis. From earlier studies, researchers have successfully analyzed the kinetics of mast cell exocytosis using electrophysiology (Fernández-Chacón and Alvarez de Toledo, 1995). Due to relatively large diameter of mast cell granules, the mast cells exhibited stepwise changes in the capacitance upon stimulation where each step represents fusion of a granule. Moreover, these studies have revealed mast cells can exhibit compound exocytosis by observing a larger increase in capacitance change than a step. In addition to capacitance measurement, mast cell exocytosis can also be measured by amperometry, due to release of monoamine serotonin. Also, these patch clamp techniques can offer much sensitive detection of granule exocytosis, especially for compound fusion of numerous granules such as mast cell degranulation. Together, assessing the role of Munc13 and Munc18 in the mast cell exocytosis using additional assays would help to further address the importance of these proteins not only for the fusion of different types of granules but also to delineate precise mechanisms.

6.6.2.2 Studying protein-protein interaction

In this thesis, the yeast two-hybrid assay (Fields and Song, 1989) has been employed to detect the binary interaction between Munc18 variants and monomeric syntaxin-2 and -3. Studying protein interaction using yeast two-hybrid method has a great advantage as it provides a more in vivo-like environment that greatly resembles that of higher eukaryotic systems for protein interaction to occur compared to a system based on a bacterial host (Chien et al., 1991; Koegl and Uetz, 2007). Moreover, in contrast to the classic biochemical approaches that require
high quantities of purified proteins, only the cDNA of the gene of interest is required for yeast two-hybrid assay. This system is ideal for analyzing the known interactions, which then can be further used to test the effect of modification of important residues of the protein on its interactive property. This can lead to discoveries of important domains of the protein for known interactions. Nonetheless, it is important to be aware of some of the drawbacks of this approach (Chien et al., 1991; Koegl and Uetz, 2007). Firstly, the expression level of protein cannot be regulated thus may lead to over- or under-expression of the protein of interest. Secondly, there is a possibility of auto-activation in which protein of interest activates transcription of reporter gene on its own or through a third protein that bridges two proteins which in all cases may lead to detection of false positives. Therefore, it is very important to use proper controls to exclude these possibilities when interpreting the results. Another factor to be aware of when interpreting the data is that in yeast two-hybrid assay, weak and transient interactions are more readily detected as the reporter gene response often leads to significant amplification of signal. Thus the degree of interaction can be overemphasized than it actually may be. Therefore, it is essential to consider these limitation of the technique when interpreting the data and drawing the conclusion from the data.

Although pull-down assay using GST-syntaxin-11 was employed to detect the interaction with Munc18-2, other biochemical assay that are more quantitative are necessary. For example, isothermal titration calorimetric (ITC) measurements have been frequently employed to detect and measure the interaction between SNARE proteins and its regulators such as syt-1, Munc13 and Munc18 (Burkhardt et al., 2008; Lu et al., 2006; Radhakrishnan et al., 2009). This sensitive biophysical assay can reveal not only whether an interaction occurs, but how tight or weak the binding affinity is. Thus knowing the precise binding affinity will be beneficial to conclude the binding interactions in a more quantitative way. Moreover, ITC measurement will also
differentiate the inter vs intra-molecular interactions of protein of interest, thus providing more hints to detailed binding modes that could exist among these proteins.
6.7 Future directions

Chapters 3 and 4 of current thesis have been focused on identifying critical cognate syntaxin partner that are involved in direct binding to Munc18. Chapter 5 has been focused on delineating the role of Munc13-4 in mast cell exocytosis as a potential Ca\(^{2+}\) sensor. The results from the thesis clearly deliver two important messages regarding the mechanisms underlying Munc18 and Munc13 functions in exocytosis. Firstly, Munc18 regulates both syntaxin-3 and -11 through a binding cavity between domains-1 and -3 and hydrophobic pocket, respectively. These interactions are critical for protein levels of both syntaxin isoforms as well as subcellular localization of syntaxin-3 that are important for mast cell exocytosis. Second, Munc13-4 plays a dual role in mast cell exocytosis; as a whole protein, it is an essential priming factor whereas C2 domains are critically involved in Ca\(^{2+}\)-sensing of mast cell exocytosis. Moreover, Munc13-4 regulates the mast cell exocytosis by regulating fusion pore opening in a Ca\(^{2+}\)-dependent manner. Despite the messages delivered in the study, much more work needs to be done in order to completely understand the functions of these proteins in mast cell exocytosis. The first step towards this relies on filling in the gaps of the current studies. In the thesis, it has been suggested that syntaxin-3 and -11 protein levels depend on the interaction between Munc18 and these syntaxins as mutations in Munc18 which disrupt this binding result in a reduction in level of syntaxin in mast cells. However, the mechanism of this decreased syntaxin level is unclear although it is highly likely caused by instability of syntaxin that leads to rapid degradation. Therefore, this hypothesis needs to be verified. Firstly, any change in mRNA level of syntaxin the absence of Munc18 should be verified, perhaps by using RT-PCR. Also, whether syntaxin is more prone to degradation in the absence of Munc18 can be assessed by investigating for any upregulation in ubiquitylated syntaxin. This could be done by immunoprecipitating syntaxin and further probing with antibody against ubiquitin. Alternatively, proteasome-specific inhibitors
which interfere with the ubiquitin/proteasome pathway could be used to see if this alleviates the
degradation of syntaxin in the absence of Munc18 (Corey and Li, 1999; Fenteany et al., 1994;
Meng et al., 1999; Sin et al., 1999). Furthermore, protein-synthesis inhibitor, cycloheximide, can
be used to measure the half-life of protein without confounding contributions from
transcription/translation of that protein of interest (Sharma et al., 2011). This will allow
comparing the stability of syntaxin in presence or absence of functional Munc18. Clarifying the
mechanism underlying Munc18-dependent syntaxin expression/degradation would provide
important information regarding whether Munc18 affects syntaxin synthesis or stability. In
addition, it has been demonstrated that lack of Munc18-1 results in severe defects in docking of
DCV in chromaffin cells and PC12 cells (Han et al., 2011; Han et al., 2009; Voets et al., 2001)
and synaptic vesicles in *C. elegans* (Weimer et al., 2003). It has been suggested that the ability of
Munc18-1 to restore defective docking relies on its ability to regulate syntaxin-1 (Han et al.,
2011). Therefore, it would be very interesting to investigate whether lack of Munc18-1/2 in
RBL-2H3 cells can also results in similar defects in docking of its granules. This will provide an
additional mechanism on how Munc18 are involved in mast cell exocytosis. Moreover, Munc18
has been speculated to be important for its priming role through interacting with the whole
SNARE complex. Indeed, although our syntaxin-3 KD caused strong impairments in the mast
cell degranulation (Chapter 4), this cannot fully attribute the almost complete abolishment of
degranulation found in Munc18-1/2 DKD mast cells (Chapter 3). Moreover, although even
Munc18-2 single KD mast cells exhibit severe degranulation defects, the level of syntaxin-3 was
unchanged. Thus, we speculate that there are other possibilities through which Munc18
participates in the mast cell degranulation in addition to the regulation toward syntaxin-3. For
example, Munc18 might directly interact with the SNARE complex, thereby controlling the
priming step of the exocytosis. Further analyses to test the veracity of these possibilities will provide a clearer view on how Munc18 is involved in mast cell exocytosis.

Ca^{2+}-sensing and priming role of Munc13-4 needs to be uncoupled. In Chapter 5, we have demonstrated that mast cell degranulation is severe defective when Munc13-4 is absent while Ca^{2+}-sensitivity is shifted when C2 domains are mutated. However, from double and quadruple mutant experiments, it is clear that although one single mutation of C2 domain is enough to alter the Ca^{2+}-sensitivity, additional mutations seem to impair the function of this protein as a priming factor. Since it is believed that priming function of Munc13 is to open syntaxin to allow formation of SNARE complex, a LE mutant syntaxin-3, which is constitutively open can be expressed in the mast cells. If C2 domain mutants can invariably alter the Ca^{2+}-sensitivity of exocytosis in this priming “bypassed” condition, then we can perhaps claim C2 domain of Munc13-4 as a Ca^{2+}-sensor. Moreover, in addition to Munc13-4, there are expression of other C2 domain containing proteins in mast cells. There are different isoforms of synaptotagmins such as -1, -2, -3 and -9 (Baram et al., 1999; Baram et al., 2001; Grimberg et al., 2003; Haberman et al., 2003). Although syt-1 has been shown to be expressed in mast cells, whether it possesses similar function and mechanism as neuronal syt-1 is unknown (Kimura et al., 2001). Rather, BMMC grown from syt-2-deficient mice showed a severe defect in both β-hexosaminidase and histamine release (Tuvim et al., 2009). Syt-3 and -9 have been demonstrated to be important for regulator of endocytic recycling compartment. Also, mast cell express Doc2α, although its function and mechanism in exocytosis is still unclear (Blank, 2011). Therefore, a comparative study of observing phenotypes of Munc13-4 and different syt KD and rescue is necessary to appreciate the function of Munc13-4 as a potential Ca^{2+} sensor of mast cell exocytosis.
The current thesis has focused on integrating how the structural specificity and interactive property of Munc18 and Munc13 contribute to its essential roles in mast cell exocytosis. With the knowledge obtained from the studies discussed in this thesis, the next big step would be to understand how insufficiency in functional Munc18 and Munc13 is related to diseases in humans. Munc13-4, syntaxin-11 and Munc18-2 are genes implicated to cause devastating immune disease, FHL-3, -4 and -5, respectively (Bryceson et al., 2007; Santoro et al., 2006; zur Stadt et al., 2009). FHL is characterized by proliferation and infiltration of hyperactivated macrophages, and T-lymphocytes manifesting as acute illness with prolonged fever, cytopenias and hepatosplenomegaly (Zhang et al., 2014). In addition, some neurologic abnormalities such as increased intracranial pressure, neck stiffness, convulsions, blindness and coma can be present initially or may develop later. Without treatment, children with FHL can only survive up to 2 months, where progression of hemophagocytic lymphohistiocytosis and infection account for the majority of deaths. Current treatments of FHL is to use antibiotic or antiviral agents to manage symptoms but it eventually requires hematopoietic stem cell transplantation. From sequencing of genes from FHL patients, it was found that are many deletion, missense and point mutations that caused abnormal function of the proteins. However, detailed analysis on how these mutants affect exocytosis in the hematopoietic cells is lacking. Therefore, it would be very interesting to characterize the nature of these mutations by investigating the mechanisms underlying the effect of these FHL-associated mutants in exocytosis. In Chapter 3 of the thesis, we have demonstrated that one of the point mutants implicated with FHL5, E132A, failed to rescue the defective degranulation phenotype of Munc18-1/2 DKD mast cells. Moreover, this mutant did not interact with syntaxin-11, therefore unable to mediate the regulatory function. This result was re-confirmed by a recent crystal structure of Munc18-2 where it elucidated the essential role of E132 in mediating interaction with syntaxin-11 (Hackmann et al., 2013). For FHL5 mutants,
their ability to mediate regulatory function toward syntaxin-3 or -11 and whether they can rescue the defective secretion of Munc18 KD mast cells can be thoroughly assessed. The mutants are expected to have one of the following outcomes: 1) the mutation lead to structural instability leading to inefficient protein levels; 2) the mutation impairs binding to syntaxin-3 or -11 and consequently its regulatory function; 3) the mutant exhibit normal regulatory activity toward syntaxins, but failed to rescue the defective secretion, suggesting impairment of priming function of this protein. For mutations associated with FHL3, those mutants of Munc13-4 can be tested for whether they exhibit loss of priming function or altered Ca\textsuperscript{2+}-sensitivity. Information obtained from these FHL3-associated mutants will clarify the mechanism of this protein in immune cell exocytosis. Therefore, the results from this mutational studies will elucidate a possible convergence in molecular mechanisms exhibited by different mutants of these proteins. Understanding the molecular mechanisms underlying disease phenotypes will provide new insight into potential therapeutic interventions.
Concluding Remarks

In summary, the results from the study presented in the thesis demonstrated that the Munc18 and Munc13-4 are playing essential roles in the mast cell exocytosis where Munc18 participates partly through regulating its cognate syntaxin whereas Munc13-4 functions as a priming factor and a Ca\textsuperscript{2+}-sensor. Using Munc18-1/2 DKD KD RBL-2H3 cells as a model system, it was found that interaction between Munc18 and syntaxin-3 and -11 is critical for Munc18’s regulatory activity toward cognate syntaxin, which stabilizes and properly localizes syntaxin (Chapters 3 and 4). From these results, we demonstrated that similar binding modes work together for regulating the cognate syntaxins in both neuronal and mast cell exocytosis, however the relative contributions of these two binding modes vary depending on cell types. Furthermore, using Munc13-4 KD RBL-2H3 cells as a model system, it was illustrated that Munc13-4 functions as an important Ca\textsuperscript{2+}-sensor of mast cells in addition to its essential role as a priming factor. Moreover, we demonstrated Ca\textsuperscript{2+}-dependent fusion pore regulation exerted by Munc13-4 (Chapter 5). Overall, the findings from this study have provided thorough mechanistic insights into the structural/functional relationships of Munc18 and Munc13-4 in regulations of mast cell exocytosis. Mast cell exocytosis is critical for release of diverse molecules such as histamine, heparine, proteases, chemokines and cytokines which serve a wide range of physiological functions that is essential for maintaining immunological homeostasis of an organism. Exocytosis is a fundamental cellular process that is crucial for many cells and organs: from communication between neurons to hormonal regulation to immune responses. Many similarities exist between the secretory mechanisms in the variety of cells. Therefore, the findings from this study will have broader implications for our knowledge in other secretory organs that utilize similar mechanisms for secretion.
Chapter 7: References


by an evolutionarily conserved motif and is sensitive to inositol hexakisphosphate. J Biol Chem 279, 12574-12579.


