Molecular insights into the mechanisms underlying the pleiotropic functions of Munc18-1 in dense-core vesicle exocytosis

by

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

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Abstract

Exocytosis refers to a cellular process in which an intracellular vesicle fuses its membrane with the plasma membrane to release its contents to the extracellular space. This process underlies diverse physiological processes such as communication between neurons, hormonal regulation, and immune responses. Munc18-1 is an indispensable protein that regulates exocytosis during neuronal communication and hormonal signaling. It is known to specifically interact with one or more SNARE proteins (syntaxin-1, SNAP-25, and synaptobrevin-2) through diverse binding modes. Multiple lines of evidence suggest the pleiotropic functions of Munc18-1 although they are sometimes contradictory: as a molecular chaperone of syntaxin-1, in priming of dense-core vesicles (DCVs) to fusion competent state, and in docking of DCVs to the plasma membrane. Munc18-1 consists of multiple domains: domain-1, -2, -3a, and -3b. However, the molecular mechanisms underlying the functions of Munc18-1 in exocytosis remain unclear. It was hypothesized that the multiple roles of Munc18-1 occur through its distinctive binding modes with SNARE proteins, which in turn are mediated by its specific domains. The structure and interactive properties underlying its essential roles were investigated through detailed phenotypic analyses of
various domain-specific Munc18-1 mutants upon re-expression in Munc18-1/-2 double knockdown PC12 cells through lentivirus mediated infection. The results demonstrate that domain-1 and domain-3a of Munc18-1 are critical for a high affinity binary interaction with the closed form of syntaxin-1, which is crucial for syntaxin-1 trafficking and consequently in DCV docking and secretion. Furthermore, domain-3a of Munc18-1 additionally plays a crucial role in priming exocytosis by regulating SNARE complex interaction, which occurs independently from its role in syntaxin-1 stabilization and trafficking. Taken together, we postulate that Munc18-1 sequentially adopts distinctive binding modes at different stage of exocytosis that enable it to regulate each step through highly specialized mechanisms. The findings from this study provide novel insights into the obscure mechanisms underlying the functions of Munc18-1 in regulated exocytosis.
Acknowledgments

First and foremost, I would like to express my great appreciation to my supervisor, Dr. Shuzo Sugita, who has guided me throughout my graduate studies. His constant encouragements, support and guidance have allowed me to go forward for the past five years in the lab. I am very grateful to him for providing me with exceptional learning experience that has been profoundly rewarding.

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<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>(H(^3))NE</td>
<td>Tritium Labeled Norepinephrine</td>
</tr>
<tr>
<td>Å</td>
<td>Ångström (unit of length equal to 10(^{-10}) m)</td>
</tr>
<tr>
<td>Ach</td>
<td>Acetylcholine</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>C</td>
<td>Control</td>
</tr>
<tr>
<td>CAPS1</td>
<td>Calcium-dependent activator protein for secretion 1</td>
</tr>
<tr>
<td>CHO</td>
<td>Chinese hamster ovary</td>
</tr>
<tr>
<td>CMV</td>
<td>Cytomegalovirus</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>Doc2</td>
<td>Double C2 like domain containing protein</td>
</tr>
<tr>
<td>dsRNA</td>
<td>double stranded Ribonucleic acid</td>
</tr>
<tr>
<td>E1EE</td>
<td>Early infantile epileptic encephalopathy</td>
</tr>
<tr>
<td>EM</td>
<td>Electron microscopy</td>
</tr>
<tr>
<td>EmGFP</td>
<td>Emerald green fluorescent protein</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>GABA</td>
<td>γ-aminobutyric acid</td>
</tr>
<tr>
<td>GLUT4</td>
<td>glucose transporter 4</td>
</tr>
<tr>
<td>GST</td>
<td>Glutathione S-transferase</td>
</tr>
<tr>
<td>hPLAP</td>
<td>Human placental alkaline phosphate</td>
</tr>
<tr>
<td>IRES</td>
<td>Internal ribosome entry site</td>
</tr>
<tr>
<td>ITC</td>
<td>Isothermal titration calorimetry</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>KCl</td>
<td>Potassium chloride</td>
</tr>
<tr>
<td>KD</td>
<td>Knockdown</td>
</tr>
<tr>
<td>KO</td>
<td>Knockout</td>
</tr>
<tr>
<td>Lat A</td>
<td>Latrunculin A</td>
</tr>
<tr>
<td>LDCV</td>
<td>Large dense core vesicle</td>
</tr>
<tr>
<td>M int</td>
<td>Munc18 interacting protein</td>
</tr>
<tr>
<td>M HD</td>
<td>Munc13 homology domain</td>
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<tr>
<td>NAT</td>
<td>Noradrenaline transporter</td>
</tr>
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<td>NE</td>
<td>Norepinephrine</td>
</tr>
<tr>
<td>NGF</td>
<td>Nerve growth factor</td>
</tr>
<tr>
<td>NRK</td>
<td>Normal rat kidney</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
</tr>
<tr>
<td>NPY</td>
<td>Neuropeptide Y</td>
</tr>
<tr>
<td>NSF</td>
<td>N-ethylmaleimide sensitive factor</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>PH</td>
<td>Pleckstrin homology</td>
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<tr>
<td>PIP₂</td>
<td>Phosphatidylinositol 4,5-bisphosphate</td>
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<td>PIP5KI</td>
<td>Type I phosphatidylinositol 5-kinase</td>
</tr>
<tr>
<td>PITP</td>
<td>Phosphatidylinositol transfer protein</td>
</tr>
<tr>
<td>PM</td>
<td>Plasma membrane</td>
</tr>
<tr>
<td>PSS</td>
<td>Physiological Saline Solution</td>
</tr>
<tr>
<td>PTB</td>
<td>Phosphotyrosine-binding</td>
</tr>
<tr>
<td>RNAi</td>
<td>Ribonucleic acid interference</td>
</tr>
<tr>
<td>RRP</td>
<td>Readily releasable pool</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>shRNA</td>
<td>Short hairpin ribonucleic acid</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>siRNA</td>
<td>Short interfering ribonucleic acid</td>
</tr>
<tr>
<td>Slp</td>
<td>Synaptotagmin-like protein</td>
</tr>
<tr>
<td>SM</td>
<td>Sec1/Munc18</td>
</tr>
<tr>
<td>SMLV</td>
<td>Synaptic-like microvesicles</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>SV</td>
<td>Synaptic Vesicle</td>
</tr>
<tr>
<td>Syx1</td>
<td>Syntaxin-1</td>
</tr>
<tr>
<td>VAMP2</td>
<td>Vesicle associated membrane protein 2</td>
</tr>
<tr>
<td>VMAT</td>
<td>Vesicular monoamine transporter</td>
</tr>
<tr>
<td>Vps</td>
<td>Vacuolar protein sorting</td>
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Chapter 1: Introduction

1.1 Exocytosis

Membrane fusion underlies every vital process in all multicellular organisms, ranging from organelle biogenesis and compartmentalization to synaptic transmission in the brain (Bacaj et al., 2010; Südhof and Rothman, 2009). Membrane fusion is mediated through the physical merge between two lipid bilayers of previously separate compartments allowing for the content exchange (Söllner, 2003). Exocytosis refers to a cellular process in which an intracellular vesicle fuses its membrane with the plasma membrane, followed by the release of its contents from the intravesicular space to the cell exterior (Lin and Scheller, 2000). Exocytosis may occur through two pathways; constitutive and regulated. Constitutive exocytosis is a basic feature of all eukaryotic cells and occurs in the absence of external signals. This allows for the maintenance of plasma membrane composition and the extracellular environment (Lin and Scheller, 2000). Regulated exocytosis is a more specialized process in which the vesicles undergo exocytosis only in response to physiological signal, such as an increase in intracellular calcium ion concentration (Lin and Scheller, 2000). This process is both temporally and spatially tightly regulated which ensures the efficient release of diverse cargo molecules such as neurotransmitters, hormones, enzymes, and cytokines (Söllner, 2003). The most extensively studied form of regulated exocytosis is chemical transmission of neurotransmitters and hormones from neurons and/or neuroendocrine cells to target cells. Overall, this ensures the maintenance of proper physiological functions in all organisms.
1.1.1 Secretory vesicles that undergo regulated exocytosis

Intercellular signaling by neurons and neuroendocrine cells depends on the release of distinctive cargo molecules from different types of secretory vesicles. Synaptic vesicles (SVs) (~50 nm diameter) are the most prominent type of secretory organelle in neurons. They have a translucent core when visualized with conventional electron microscopy (Hannah et al., 1999; Thomas-Reetz and De Camilli, 1994). Synaptic vesicles store and secrete classical non-peptide neurotransmitters such as γ-aminobutyric acid (GABA), glutamate, glycine, and acetylcholine, which account for the fast signaling characteristic of neurons (Sugita, 2008; Thomas-Reetz and De Camilli, 1994). Membrane composition analyses on highly purified preparations of synaptic vesicles revealed a wide diversity of integral membrane proteins. Vacuolar proton pump (V-ATPase), ion channels, and neurotransmitter carriers of vesicular membrane mediate the uptake and storage of neurotransmitters while other numerous membrane proteins are involved in vesicle trafficking (Jahn and Südhof, 1994; Llona, 1995; Südhof and Jahn, 1991; Takamori et al., 2006).

Synaptic vesicles tend to cluster in the presynaptic nerve terminals (active zone). When an action potential reaches the nerve terminal and depolarizes the presynaptic plasma membrane, voltage-gated Ca\(^{2+}\) channels at the active zone open. This is followed by the transient increase in the cytosolic Ca\(^{2+}\) concentration which triggers synaptic vesicle exocytosis (Katz and Miledi, 1967).

In endocrine and neuroendocrine cells, a population of microvesicles that exhibit high resemblance to neuronal synaptic vesicles in morphology and membrane composition have been identified and thus were named synaptic-like microvesicles (SLMVs) (Bacaj et al., 2010; Linstedt and Kelly, 1991; Thomas-Reetz and De Camilli, 1994). It has been found that SLMVs in β-cells of the pancreas contain GABA whereas those in PC12 cells contain acetylcholine (Cameron et al., 1991; Linstedt and Kelly, 1991; Reetz et al., 1991). Furthermore, a depolarization-induced, calcium-dependent release of the neurotransmitters GABA and acetylcholine from SLMVs in the
amphicrine pancreatic cell line, AR42J, and neuroendocrine cell line, PC12, respectively, has been reported (Ahnert-Hilger and Wiedenmann, 1992; Bauerfeind et al., 1995). However, the SLMVs do not accumulate at the plasma membrane of neuroendocrine cells, which is a requirement for vesicles that undergo calcium-dependent exocytosis (Thomas-Reetz and De Camilli, 1994). Therefore, it remains unclear whether SLMVs are able to release their content in a regulated manner homologous to synaptic vesicles.

Dense-core vesicles (DCVs) (~70-200 nm diameter) are key organelles required for the secretion of hormones, neuropeptides and biogenic amines from endocrine cells, neuroendocrine cells and neurons. DCV release from these cells mediates modulatory changes in target cells (Kim et al., 2006; Sugita, 2008). The eponymous dense core results from the presence of highly condensed granule matrix that can be visualized by conventional electron microscopy (Burgoyne and Morgan, 2003; Thomas-Reetz and De Camilli, 1994). Although some neurons possess DCVs that can undergo exocytosis independently of synaptic vesicles, the most studied neuroendocrine cells for DCV exocytosis are the adrenal chromaffin cell and its tumor counterpart, the PC12 cell line (Burgoyne and Morgan, 1998; Burgoyne and Morgan, 2003; Gillis and Chow, 1997; Morgan and Burgoyne, 1997). The mechanism of DCV exocytosis appears to be similar to that of synaptic vesicle exocytosis, which involves sequential stages such as docking of vesicles to the plasma membrane, priming of vesicles to fusion competent state, and finally the Ca$^{2+}$-dependent fusion of vesicles (Burgoyne and Morgan, 2003; Sugita, 2008). However, how each stage of vesicle exocytosis is regulated still remains to be resolved.

The study presented in this thesis focuses on elucidating the mechanism underlying dense-core vesicle exocytosis in PC12 cells.
1.1.2 Physiological roles of dense-core vesicle exocytosis

A wide range of neuronal and non-neuronal cell types contain dense-core vesicles (also referred to as secretory granules in some cell types) in which their specific vesicular contents such as hormones, growth factors, neuropeptides, processing enzymes, and catecholamines serve a diverse range of physiological functions upon release (Burgoyne and Morgan, 2003).

The ventral hypothalamus of the central nervous system regulates the endocrine secretion of the anterior lobe of the pituitary gland which serves an important role in regulating homeostasis (Guillemin, 1978; Hökfelt, 1991). This hypothalamic control of pituitary hormones involves secretion of releasing and inhibiting hypothalamic hormones such as dopamine, thyrotropin-releasing hormone, corticotropin-releasing hormone, gonadotropin-releasing hormone, and growth-hormone-releasing hormone from hypothalamic neurons. Likewise, hormonal secretion from dense-core vesicles of the anterior and posterior pituitary gland plays important roles in regulating target endocrine glands such as thyroid, gonads, liver, breast gland, adrenal cortex, and kidney in addition to overall somatic growth and maintenance of homeostasis (Bean et al., 1994; Guillemin, 1978).

Chromaffin cells of the adrenal medulla secrete catecholamines such as epinephrine and norepinephrine stored in dense-core vesicles (also referred to as chromaffin granules) into systemic circulation to send signals to multiple organs as part of the fight-or-flight response. Chromaffin cells also secrete a number of neuroactive peptides, which are involved in paracrine signaling (Aunis and Langley, 1999). Moreover, insulin secretion from β-cells of the endocrine pancreas constitutes another important physiological event as this is critical for the regulation of the energy balance in body. The β-cell is devoted to the biosynthesis and processing of hormone pre-cursors to produce mature insulin which is then stored in dense-core secretory vesicles (also called β-granules) followed by timely release of mature insulin in response to glucose-uptake
Aside from neuroendocrine and endocrine cells within tissues that are specialized for secretory function such as the pituitary gland, adrenal medulla, and pancreas, dense-core vesicle exocytosis also occurs in non-secretory tissues such as the heart and kidney, although less abundantly. In heart, atrial muscle cells were shown to release atrial natriuretic peptide, a potent vasodilator. This contributes to the homeostatic control of body water, sodium, potassium and adipose tissue (Newman and Severs, 1990; Potter et al., 2009). Moreover, juxtaglomerular cells of the kidney have been shown to secrete renin, which plays a major role in regulating blood pressure and volume (Hackenthal et al., 1990).

The release of diverse signaling molecules through dense-core vesicle exocytosis from a variety of cells serves a crucial role in maintaining normal physiology in organisms. Therefore, it is critical to understand the molecular mechanisms underlying this process.

In the study presented in this thesis, the molecular mechanisms underlying dense-core vesicle exocytosis have been investigated using PC12 cell line, established from a tumor of adrenal chromaffin cells (Greene and Tischler, 1976), due to a variety of experimental advantages provided by this cell line.

1.2 The SNAREs: the conserved core machinery

Exocytosis of secretory vesicles relies on the fusion between the vesicular membrane and plasma membrane. The neuronal SNARE (Soluble N-ethylmaleimide-sensitive factor attachment protein receptor) proteins which include synaptobrevin-2 (also known as VAMP2, vesicle-associated membrane protein 2), syntaxin-1, and SNAP-25 (synaptosomal-associated protein of 25 kDa) are the central components of membrane fusion.
1.2.1 Discovery of SNAREs

In 1988, the cytosolic protein, NSF (N-ethylmaleimide sensitive factor) was isolated from cytosol of Chinese hamster ovary (CHO) cells. NSF was found to be an essential component for catalyzing the fusion between transport vesicles and cisternae of the Golgi stack based on its ability to restore intercisternal Golgi transport in mammalian cell-free system (Malhotra et al., 1988). A subsequent study revealed that the NSFs work together with the cytoplasmic factors, α, β, γ-SNAPs (α, β, γ-soluble NSF-attachment proteins). These two proteins were since then identified as essential components of the intracellular membrane fusion apparatus (Clary et al., 1990). Moreover, the sequence of this purified NSF was found to exhibit a striking homology to the Sec18p in yeast which is known to be essential for the vesicle transport from the endoplasmic reticulum (ER) to Golgi in vivo (Wilson et al., 1989). The Sec18p was also shown to be able to replace NSF in a mammalian system for cell-free Golgi transport (Wilson et al., 1989). This suggested that the mechanism of vesicular fusion is highly conserved across different species and throughout a variety of intracellular fusion processes. The first clue to which molecules mediate synaptic membrane fusion was obtained when syntaxin-1, SNAP-25, and synaptobrevin-2 were identified as targets of clostridial botulinum and tetanus toxins (Blasi et al., 1993a; Blasi et al., 1993b; Link et al., 1994; Schiavo et al., 1992). In 1993, three synapse-associated membrane proteins – synaptobrevin-2 (VAMP-2), syntaxin-1, and SNAP-25 – were isolated via binding to NSF/α- and γ-SNAP fusion proteins from bovine brain. These proteins were identified as receptors for the α- and γ-SNAPs, hence were named SNAREs (Soluble N-ethylmaleimide-sensitive factor attachment protein (SNAP) receptor) (Söllner et al., 1993a; Söllner et al., 1993b).
1.2.2 Structure of SNAREs

VAMP-2 (residues 1-116), also referred to as synaptobrevin-2, consists of a short N-terminal sequence, a SNARE motif, and a C-terminal transmembrane region (Figure 1.1). The cytoplasmic domains of synaptobrevin-2 are unfolded in solution as observed by circular dichroism (CD) and solution NMR experiment (Fasshauer et al., 1998; Hazzard et al., 1999). The C-terminal two thirds of the cytoplasmic domain of synaptobrevin-2 (25-96) participate in SNARE complex formation (Fasshauer et al., 1998; Hazzard et al., 1999). Synaptobrevin-2 is known to be anchored to the membrane through the carboxyl-terminal transmembrane domain.

SNAP-25 (synaptosomal-associated protein 25) is a hydrophilic protein that consists of 206 residues (Figure 1.1). It is anchored on the plasma membrane through palmitoyl side chains attached to four closely spaced cysteine residues in the middle of the sequence (Hodel, 1998). SNAP-25 contains two highly conserved SNARE motifs (residues 1-80 and residues 142-206) that are thought to form intermolecular coiled coils motifs (Hodel, 1998; Wang et al., 2008).

Syntaxin-1 consists of residue 1-288 and is largely helical. Syntaxin-1 is characterized by several domains: N-terminal peptide (1-26), H$_{abc}$ domain (27-156), linker region (157-187), H3 domain (187-248), and transmembrane region (266-288) (Misura et al., 2000) (Figure 1.1). The H$_{abc}$ domains are independently folded and form a three-helical bundle. H3 domain serves as a SNARE motif that participates in SNARE complex formation (Sutton et al., 1998). These two domains are connected by a highly flexible linker region (Margittai et al., 2003). Syntaxin-1 is anchored to the membrane by the carboxyl-terminal transmembrane domain. Syntaxin-1 adopts two conformations: closed and opened (Dulubova et al., 1999). Syntaxin-1 is defined as “closed” when its N-terminal H$_{abc}$ domain folds back onto the H3 domain. Syntaxin-1 in this conformation has been reported to be compatible with binding to Munc18-1, while incompatible with SNARE complex formation which requires syntaxin-1 to be opened. Syntaxin-1 can also adopt an
“opened” conformation in which $H_{abc}$ domain-1 of syntaxin-1 extends away from H3 domain. Syntaxin-1 in this “opened” conformation is known to be able to participate in SNARE complex assembly as the H3 domain is exposed to interact with other SNAREs (Dulubova et al., 1999).

Figure 1.1 Structure of neuronal SNAREs (A) Domain diagrams of the neuronal SNAREs. The number of residues of each protein is indicated above each diagram on the right. TM, transmembrane; NTS, N-terminal sequence. (B) Ribbon diagram of the structures of the SNARE complex and the syntaxin-1 $H_{abc}$ domain (shown in orange). Dashed curve line represents the syntaxin-1 sequence linking $H_{abc}$ domain to H3 domain. Synaptobrevin-2 contributes residues 25-96, SNAP-25 contributes residues 1-80 and 142-206, and syntaxin-1 contributes residues 187-248 for SNARE complex formation. (C) The amino acid sequence of the partial SNARE domains of proteins contributing to the ternary SNARE complex. The 15 hydrophobic layers (-7 to -1 and +1 to +8) are labeled in bold and the 0 ionic layer is italicized. Boxed hydrophobic layers follow packing observed in tetrameric leucin-zipper proteins. Adapted and modified from (Rizo and Rosenmund, 2008) and (Parpura and Mohideen, 2008).
1.2.3 SNAREs form a stable four-helical bundle to mediate membrane fusion

The SNARE proteins mediate fusion by forming a trans-complex that bridges the vesicle and target membranes (Rothman and Warren, 1994). Synaptobrevin-2 (VAMP-2) is classified as a v-SNARE because of its anchoring in the vesicular membrane (Söllner et al., 1993b; Trimble et al., 1988). Similarly, syntaxin-1 and SNAP-25 are classified as t-SNAREs because of their residence in the target membrane (Bennett et al., 1992; Oyler et al., 1989). The SNARE proteins are characterized by a ~65-residue sequences, that consists of evolutionary conserved heptad repeat sequences, called the SNARE motifs, which tend to form coiled coils (Figure 1.1 B and C) (Rizo and Südhof, 2012). Synaptobrevin-2 and syntaxin-1 each contribute one SNARE motif preceding their C-terminal transmembrane region, whereas SNAP-25 contributes two SNARE motifs. The four SNARE motifs of the SNARE proteins assemble into a tight trans-SNARE complex, thereby linking the vesicular and plasma membranes (Söllner et al., 1993b). This complex contains sixteen layers (layer -7 to +8) of hydrophobic side chains and a core polar layer that mediate the interactions between the four helices (Söllner et al., 1993b; Sutton et al., 1998). At the central polar layer (layer 0) in the middle of the bundle, an arginine (R) residue from synaptobrevin-2 and glutamine residues (Q) from syntaxin-1 and SNAP-25 have been shown to ionically interact, hence have led to the recent classification into R- or Q-SNAREs, respectively (Fasshauer et al., 1998). Progressive amino- to the carboxyl-terminal zippering of a four-helical bundle formed by the SNARE motifs of SNARE proteins forces the phospholipid membranes into close proximity. The energy released upon formation of this stable 1:1:1 stoichiometric complex is believed to drive fusion by exerting a force on the membrane anchors. This causes destabilization of the lipid bilayer structure (caused by the repulsion between the negatively charged phospholipids) which ultimately leads to the fusion pore opening (Lin and Scheller, 2000; Rizo and Südhof, 2012; Sugita, 2008). Although how this energy is applied to the membranes remains unclear, it is now well
established that the SNARE complex plays a central role in membrane fusion. After fusion, the ternary SNARE complex resides in the plasma membrane in the low-energy cis configuration. The cis-complex is then disassembled by NSF and its SNAP cofactor which allows the SNAREs to be able to participate in the next round of exocytosis (Jahn and Fasshauer, 2012).

1.2.4 SNARE proteins as essential components of the fusion machinery

The importance of the SNARE proteins in membrane fusion has been clearly demonstrated by the use of the botulinum and tetanus neurotoxins which selectively cleave SNARE proteins and block presynaptic neurotransmission without affecting the morphological structure of the presynaptic terminal (Pellizzari et al., 1999; Südhof and Rizo, 2011). Tetanus toxin and botulinum toxin types B,D,F and G were shown to selectively cleave synaptobrevin-2 (Link et al., 1994; Schiavo et al., 1992). Moreover, SNAP-25 has been shown to be a proteolytic substrate of botulinum toxins types A,C, and E (Blasi et al., 1993a; Link et al., 1994), whereas syntaxin-1 is a target of botulinum toxin type C (Blasi et al., 1993b; Link et al., 1994). In addition, the light chains (the proteolytic subunits) of clostridial neurotoxins were shown to block DCV exocytosis in permeabilized adrenal chromaffin cells and PC12 cells (Ahnert-Hilger et al., 1989; Bittner et al., 1989a; Bittner et al., 1989b; Lomneth et al., 1993; Lomneth et al., 1991; McInnes and Dolly, 1990; Xu et al., 1998). Likewise, applying SNARE-directed antisera or peptides was shown to inhibit DCV exocytosis in chromaffin cells, further confirming the findings from the use of the clostridial neurotoxins (Gutiérrez et al., 1995; Gutierrez et al., 1995). Despite these findings that support the idea that the SNAREs are essential components of the membrane fusion machinery, the exact mechanisms underlying the SNARE-mediated membrane fusion still remains to be clarified.

The idea that SNARE complexes are essential for membrane fusion in vivo is further supported by genetic studies in a wide variety of organisms. In yeast (Saccharomyces cerevisiae),
genetic screening for temperature-sensitive mutants has identified at least 23 complementation groups of Sec genes whose products are essential for protein transport at different stages along the secretory pathway (Novick et al., 1980). Among these 23 complementation groups, Sec9 gene has been identified as one of the ten late-acting Sec genes that is required for post-Golgi transport (Novick et al., 1981). Sec9 is the yeast homolog of SNAP-25 and is localized to the plasma membrane in yeast (Brennwald et al., 1994). Meanwhile, two multicopy suppressors of mutations in the Sec1 gene in addition to several other Sec genes that act in the late stage of secretory pathway have been cloned (Aalto et al., 1993). These duplicated genes are Sso1 and Sso2 whose protein products are homologous to mammalian syntaxin and are localized to the plasma membrane in yeast (Aalto et al., 1993; Brennwald et al., 1994). In the absence of Sso1 and Sso2, transport vesicles were accumulated within the bud, the region of the plasma membrane that is active in exocytosis, suggesting defective transport of secretory vesicles from Golgi complex to the plasma membrane. Moreover, loss of Sso1p and Sso2p were shown to result in impaired secretion of invertase in yeast (Aalto et al., 1993). Likewise, Snc1 and Snc2 genes encode homologues of synaptobrevin which localizes to post-Golgi vesicles in yeast. Snc1 and Snc2 are thought to be important for post-Golgi transport as Snc1 and Snc2 deficiency in yeast results in accumulation of large quantities of post-Golgi invertase-containing vesicles. This phenotype is accompanied by defective secretion and growth in addition to conditional lethal phenotypes (Protopopov et al., 1993). Like their neuronal counterparts, the Sec9, Sso1 and 2, and Snc1 and 2 proteins have been shown to be associated with each other and co-precipitated as a complex from detergent extracts of yeast (Brennwald et al., 1994; Rossi et al., 1997).

In mice, genetic ablation of synaptobrevin-2 or SNAP-25 was shown to selectively abolish evoked synaptic transmission with minimal effect on the spontaneous transmission (Schoch et al., 2001; Washbourne et al., 2002). Likewise, deletion of synaptobrevin or syntaxin-1A led to
complete abolishment in evoked transmission in Drosophila melanogaster (Deitcher et al., 1998; Schulze et al., 1995). Furthermore, in Caenorhabditis elegans, hypomorphic mutations of synaptobrevin result in severe deficits in synaptic transmission whereas complete removal of this gene led to death just after completing embryogenesis (Nonet et al., 1998). Together, these findings strongly support the universal requirement of neuronal SNARE proteins and their homologues in membrane fusion in all eukaryotic cells.

1.3 Stages of dense-core vesicle exocytosis

SNARE-complex mediated fusion between vesicular membrane and plasma membrane is a multistage process. Each stage is highly regulated by various proteins leading to efficient exocytosis (Figure 1.2).

Figure 1.2 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 each stage. Adapted and modified from Sugita, 2008.
1.3.1 Docking

Docking is generally defined as a stage of exocytosis in which vesicles appear to be within a minimal distance from the plasma membrane by electron microscopy (EM) (Lin and Scheller, 2000). The concept of docked vesicles initially emerged when synaptic vesicles in close apposition to the plasma membrane (within 10nm) could be counted in fixed brain samples by electron microscopy (Gray, 1959; Verhage and Sørensen, 2008). In mouse embryonic adrenal chromaffin cells, dense-core vesicles (DCVs) are regarded as being in a ‘docking’ state if they are localized within 50 nm of the plasma membrane (Sugita, 2008). However, the docked vesicles observed by

orphologically docked” as not all of these morphologically docked vesicles are released upon Ca\(^{2+}\)-stimulation. Docking is generally considered as a necessary first step before vesicles gain fusion-competence. It is believed that a variety of proteins play a role in this process but exact mechanism of docking is poorly understood. Deletion of syntaxin-1,2, and 3 by applying light chain of Botulinum neurotoxin serotype C resulted in a robust reduction of docking in chromaffin cells, demonstrating the importance of syntaxin isoforms in dense-core vesicle docking (de Wit et al., 2006). However, deletion of syntaxin isoforms did not change the docking frequency in synapses of central nervous system (CNS). This may be due to the presence of other proteins that have redundant functions in these highly specialized zones. Contribution of other SNAREs such as synaptobrevin-2 and SNAP-25 in vesicle docking is not well supported. Docking of DCVs was shown not to be affected in SNAP-25 deficient chromaffin cells despite the strongly inhibited DCV secretion (Sørensen et al., 2003). Similarly, a docking phenotype was not observed in synaptobrevin-2 and 3 double knockout chromaffin cells while secretion was abolished (Borisovska et al., 2005). Another protein involved in vesicle docking is Munc18-1. In Munc18-1 deficient chromaffin cells, the number of docked DCVs is reduced by 10-fold (Voets et al., 2001).
Moreover, DCV docking was reported to be reduced in the heterozygous knockout of Munc18-1 while enhanced upon over-expression of Munc18-1, further implicating the positive contribution of Munc18-1 in docking (Toonen et al., 2006a). However, vesicle docking was not affected in the Munc18-1 deficient neurons. These findings imply that Munc18-1 and syntaxin-1 are both critical for docking of DCVs although their contributions to synaptic vesicles docking remain obscure.

Another protein implicated in vesicle trafficking and docking is a family of Rab proteins, a member of the Ras superfamily of small GTPases. Individual Rab proteins have specific subcellular localization, guiding vesicles to their specific target during a particular membrane transport event by interacting with specific effectors. For example, Rab3a is known to be mainly expressed in neurons and endocrine cells from tissues such as pituitary or adrenal medulla, therefore suggesting its role in regulated exocytosis (Burstein and Macara, 1989; Darchen et al., 1995; Darchen et al., 1990; Olofsson et al., 1988; Regazzi et al., 1992). In line with this, Rab3a knockout in C. elegans or mice was shown to result in defective synaptic vesicle targeting to the site of fusion (Castillo et al., 1997; Geppert et al., 1994a; Geppert and Südhof, 1998; Lin and Scheller, 2000; Nonet et al., 1997). Likewise, Rab3a has been shown to be involved in DCV docking in bovine chromaffin cells and rat PC12 cells (Holz et al., 1994; Tsuboi and Fukuda, 2006). One study has reported that the effect of Rab3a on vesicle docking is Munc18-1 dependent in chromaffin cells as overexpression of wild-type Rab3a in wild-type chromaffin cells, but not in Munc18-1 null chromaffin cells, increased the amount of DCVs in direct contact with the membrane (van Weering et al., 2007). Additionally, an interaction between Munc18-1 and Rab effector protein, Slp4, in PC12 cells has been reported to be important for vesicle docking although mechanism is unknown (Tsuboi, 2009). Interestingly, in yeast, Rab proteins were shown to regulate vesicle docking through genetic interaction with Sec1/Munc18 proteins (Nielsen et al., 2000; Tall et al., 1999).
1.3.2 Priming

Priming is generally defined as a process in which the docked vesicles undergo one or more preparatory reactions to become fusion competent such that they can rapidly fuse with the plasma membrane when the trigger arrives. However, the priming stage is not accurately characterized, largely due to the limited knowledge in the molecular mechanisms underlying this process. Priming is thought to occur through both ATP-dependent and ATP-independent mechanisms.

1.3.2.1 ATP-dependent priming

The ATP-dependent priming was initially characterized as the cytosol- and ATP- requiring step preceding Ca\(^{2+}\)-dependent triggering of vesicle fusion through the work done on permeabilized neurosecretory cells such as chromaffin cells and PC12 cells (Bittner and Holz, 1992; Hay and Martin, 1992; Holz et al., 1992; Klenchin et al., 1998). In these studies, decreased secretion competence in cytosol-free permeabilized cells was restored upon supplementing with cytosolic proteins and Mg-ATP. ATP-dependent priming, requiring ATP hydrolysis, involves the following events: modification of the SNARE proteins by NSF/\(\alpha\)-SNAP and maintaining the levels of polyphosphoinositides required for continued exocytosis (Burgoyne and Morgan, 2003). One of the key events of ATP-dependent priming involves generation of phosphatidylinositol 4,5-bisphosphate (PIP\(_2\)) at the plasma membrane. Likewise, phosphatidylinositol transfer protein (PITP) and type I phosphatidylinositol 5-kinase (PIP5KI) which are involved in PIP\(_2\) generation and regulation were identified as essential cytosolic factors that reconstitute exocytosis after ATP depletion (Hay et al., 1995; Hay and Martin, 1993). The transfer of phosphoinositide by PITP to plasma membrane followed by ATP-dependent phosphorylation of phosphatidylinositol 4-phosphate (PIP) to generate PIP\(_2\) by PIP5KI seem to constitute one of the major components of
ATP-dependent priming. The ability of PIP$_2$ to bind to synaptotagmin-1 and calcium-dependent activator protein for secretion 1 (CAPS1), both of which are the Ca$^{2+}$ sensors for membrane fusion, may underlie its important role in exocytosis. The evidence that the size of releasable vesicle pool in chromaffin cells depend on PIP$_2$ level (Milosevic et al., 2005) and that DCV secretion is reduced in PIP5KI$\gamma$ knockout mice (Gong et al., 2005) further supports the important role of PIP$_2$ in exocytosis.

NSF (N-ethylmaleimide sensitive factor) which itself is an ATPase is another cytosolic factor identified to be important for ATP-dependent priming. ATP-dependent binding of NSF/\(\alpha\)-SNAP to the SNARE complex is known to disrupt exocytosis-incompetent \(\text{cis-SNARE}\) complexes in which SNARE proteins are localized on the same membrane, while active \(\text{trans-SNARE}\) complexes are resistant to NSF action. Therefore, NSF may play a major role in the priming stage by encouraging exocytosis-competent \(\text{trans-SNARE}\) complex formation. In permeabilized PC12 cells, NSF and \(\alpha\)-SNAP have been shown to stimulate secretion when supplemented during the priming stage, implicating their importance in the priming of DCVs (Banerjee et al., 1996).

Similarly, Sec17p and Sec18p (the yeast homologs of \(\alpha\)-SNAP and NSF, respectively) have been shown to be required at the priming stage in an \textit{in vitro} assay measuring the homotypic fusion of isolated yeast vacuoles (Mayer et al., 1996).

1.3.2.2 ATP-independent priming

The \(\text{trans-SNARE}\) complex assembly is another key event that should occur prior to actual membrane fusion. Mutating the N-terminal region of synaptobrevin-2 which interferes with initial formation of N-terminal half of SNARE complex was shown to impair priming. This suggested that priming involves the initial formation of only the N-terminal end of the SNARE complex (Walter et al., 2010). In this aspect, proteins that aid in initial \(\text{trans-SNARE}\) complex formation
would be the major players in ATP-independent priming. The most characterized candidates are: Munc13, CAPS and Munc18 family of proteins.

Efficient SNARE complex assembly requires syntaxin-1 to be in an open conformation, which exposes its H3 domain, SNARE motif, for interaction with other SNAREs. In regulated exocytosis, syntaxin-1 is initially stabilized in its closed conformation through its interaction with Munc18-1. This interaction needs to be released in order for syntaxin-1 to adopt an open conformation. This conformational transition of syntaxin-1 is thought to be mediated by Munc13 proteins which are 200-kDa neuron-specific peripheral membrane proteins. Deletion or point mutations in Munc13-1 that abolish syntaxin-1 interaction were shown to impair its function in DCV priming (Stevens et al., 2005). Initially, Munc13-1 was thought to displace Munc18-1 from closed syntaxin-1 through competitive binding to allow syntaxin-1 to open and facilitate the formation of the SNARE complex (Betz et al., 1997; Liu et al., 2010; Sassa et al., 1999). More recently, MUN domain of Munc13-1 has been shown to mediate opening of syntaxin-1 by extracting SNARE motif from the closed syntaxin-1 and providing a template to assemble the SNARE complex through direct or indirect interaction with SNARE and/or Munc18-1, thereby contributing to the priming stage (Basu et al., 2005; Guan et al., 2008; Ma et al., 2011).

CAPS1 (calcium dependent activator protein for secretion 1) is another protein that has been suggested to play an essential role in vesicle priming. CAPS1 (~1400 amino acids) was initially identified as a cytosolic factor critical for reconstituting calcium-dependent regulated secretion in permeabilized PC 12 cells (Walent et al., 1992). CAPS1 knockout in PC12 cells was shown to cause strong reduction in secretion without any effect on DCV docking. Likewise, CAPS1 knockout was shown to cause a reduction in the primed pool of vesicles, leading to impaired secretion in adrenal chromaffin cells (Liu et al., 2008). In addition, CAPS-deficient neurons were shown to have a severe reduction in fusion-competent synaptic vesicles, which leads
to a selective impairment in fast phasic neurotransmitter release (Jockusch et al., 2007). CAPS1 is thought to contribute to vesicle priming by promoting and/or stabilizing the open conformation of syntaxin-1 by binding to H3 domain of syntaxin-1 (Liu et al., 2010). This is further supported by the finding that the open conformation mutant of syntaxin-1 (L165E/E166A) is capable of rescuing the readily releasable pool phenotype observed in the CAPS1/CAPS2 double knockout mice (Liu et al., 2010). However, the observation that over-expression of Munc13-1 cannot rescue CAPS-deficient phenotype and vice versa indicate that these two proteins act through different mechanisms during the priming stage of exocytosis.

In addition to Munc13-1 and CAPS1, accumulating evidence supports the importance of Munc18-1 in priming of exocytosis. Munc18-1 is thought to play a role in priming of vesicles by assisting or stabilizing SNARE complex assembly through direct interaction with the SNARE complex. However, the exact mechanisms remain to be elucidated. This will be further deliberated in section 1.7.2 of Chapter 1.

1.3.3 Ca\(^{2+}\)-triggered membrane fusion

In the final step of exocytosis, the vesicle membrane fuses with the plasma membrane leading to the release of its contents. Upon depolarization of the plasma membrane by an action potential, voltage gated Ca\(^{2+}\)-channels open and Ca\(^{2+}\) flows in to the cytosol triggering exocytosis of the vesicles. Although the exact mechanism of membrane fusion is unclear, complete zippering of the trans-SNARE complex from N-terminal to the C-terminal end of the SNARE bundle seems to be the key event. The recently established concept in membrane fusion suggests the following: an initial N-terminal assembly of SNARE complex which is thought to occur during the priming stage is clamped in the half-zippered state until the proper trigger is provided (Krishnakumar et al., 2011). This clamping of the fusion machinery in a “ready-to-go” state only needs a small
triggering stimulus to burst forward which is thought to be necessary for rapid and synchronous fusion (Südhof and Rothman, 2009). The key players in this complete zippering of SNARE complex are complexin and synaptotagmin.

Complexin (134 residues) is a cytosolic protein that consists of N-terminal domain, accessory helix, central α-helix and C-terminal domain (Weninger, 2011). Complexin is known for its dual function as both inhibitor and activator of membrane fusion (Giraudo et al., 2006; Giraudo et al., 2009; Giraudo et al., 2008; Maximov et al., 2009; Südhof and Rothman, 2009; Xue et al., 2010; Xue et al., 2009; Xue et al., 2007). Complexin first binds to the N-terminal membrane-distal region of the SNARE motifs of synaptobrevin-2 and syntaxin-1 in the SNARE complex through its central α-helix. This is known to stabilize the initial state of v-/t-SNARE interactions in which they are only partially zippered while simultaneously acting as a clamp that prevents the full zippering of the SNARE complex by competing with synaptobrevin-2 for binding to the C-terminal part of the SNARE complex through its accessory helix (Jahn and Fasshauer, 2012; Kümmel et al., 2011; Yang et al., 2010). This results in the stabilization of the partially assembled SNARE complex while blocking further zippering of the SNARE complex. This increases the level of half-zippered SNARE complexes in a frozen state that are ready to be activated upon Ca\(^{2+}\) signal for synchronous release of neurotransmitters. This clamp is thought to be released when Ca\(^{2+}\) enters and binds to synaptotagmin.

Synaptotagmins are the most well characterized Ca\(^{2+}\) sensor in eukaryotes and are known to be critical for tightly regulated, synchronous synaptic exocytosis (Chapman, 2002; Geppert et al., 1994b; Tucker and Chapman, 2002). The neuronal synaptotagmins are anchored to synaptic vesicles by a single TMR and consist of two protein kinase C-like C2 domains, C2A and C2B, which contain calcium-binding loops. C2A and C2B domains can accommodate three and two calcium ions, respectively. The C2B domain also contains highly conserved polybasic residues
that bind to membranes enriched in phosphatidylinositol (4,5) bisphosphate (PI(4,5)P$_2$) (Rickman et al., 2004). Upon Ca$^{2+}$ binding, synaptotagmin is thought to trigger fusion by the following mechanisms: 1) displaces complexin from the SNARE complex by binding to SNARE complex, thus enabling full zippering of the SNARE complex (Jahn and Scheller, 2006; Krishnakumar et al., 2011; Kümmel et al., 2011; Malsam et al., 2012); 2) lowering the activation energy barrier that is produced from counteracting electrostatic forces as membranes are brought into close proximity (Jahn et al., 2003). The latter role of synaptotagmin is thought to be mediated through direct interaction with the membrane that is adjacent to the partially complexed SNAREs, thereby destabilizing the bilayer at the fusion site (Hui et al., 2006; Jahn and Scheller, 2006; Martens et al., 2007; Paddock et al., 2011; Shahin et al., 2008).

The energy released upon zippering of the four-helical bundle brings the vesicular and plasma membranes in close proximity. Then the ultimate merger of two lipid bilayers creates the opening of an aqueous channel, termed a fusion pore (Jahn et al., 2003). The fusion pore is a channel-like structure connecting the vesicle and plasma membranes, which is formed during exocytosis (Westerink and Ewing, 2008). The stiff linker region connecting SNARE motifs and transmembrane regions of SNARE proteins has been suggested to be essential for transferring energy from the core complex to the membrane for fusion pore formation as mutating these regions reduced fusion efficacy (Jahn and Scheller, 2006; Kesavan et al., 2007; Knecht and Grubmüller, 2003). Likewise, an insertion of a linker between the TMR and SNARE domain decreased fusion efficiency with increasing length (McNew et al., 2000). The effect of full zippering of SNARE complex on the fusion pore formation is further supported by the finding that mutations in C-terminal region of synaptobrevin-2 that impairs C-terminal zippering of the SNARE complex reduces the fusion pore duration as evident by a reduction in duration of the prespike foot signal in amperometric traces (Walter et al., 2010). Moreover, mutations within the
transmembrane domain of syntaxin-1 were shown to impair the flux of neurotransmitter through the fusion pore as well as its conductance, indicating its contribution to fusion pore formation (Han et al., 2004). However, it remains unclear how the fusion pore is created during exocytosis and what its molecular composition is.

1.4 Sec1/Munc18 (SM) Family

Reconstitution of the SNARE proteins into artificial lipid bilayers has demonstrated that they are sufficient to drive membrane fusion, although unphysiologically slow (Weber et al., 1998). This implies that SNARE complex-mediated membrane fusion relies on other regulators that coordinate rearrangement of SNARE complexes into efficient fusion machinery since regulated exocytosis requires a high degree of spatial and temporal control.

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. 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 yeast (Saccharomyces cerevisiae), nematodes (Caenorhabditis elegans), drosophila (Drosophila melanogaster) and mice (Mus musculus). 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 (Mus musculus), 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 (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 (Garcia et al., 1994; Halachmi and Lev, 1996; Hata and Südhof, 1995; Katagiri et al., 1995; Pevsner et al., 1994; Riento et al., 1996; 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 and -3, whereas Munc18-3 binds to syntaxin-2 and -4 (Halachmi and Lev, 1996; Hata and Südhof, 1995; Hu et al., 2007; Kauppi et al., 2002; Latham et al., 2006; Riento et al., 1998; Tamori et al., 1998; Tellam et al., 1997b; Tellam et al., 1995). Munc18-1 is essential for synaptic vesicle and DCV fusion with its knockout leading to a lethal phenotype (Verhage et al., 2000; Voets et al., 2001). Munc18-2 has been implicated in regulating exocytosis in platelets and mast cells as well as in apical membrane trafficking in epithelial cells (Bin et al., 2013; Houng et al., 2003; Riento et al., 1998; Riento et al., 2000; Schraw et al., 2003; Tadokoro et al., 2007). Munc18-3 has been reported to play a critical role in glucose transporter (GLUT) 4 translocation in adipocytes and skeletal muscles in addition to insulin secretion from pancreatic β-cells (Kanda et al., 2005; Khan et al., 2001; Oh et al., 2005; Thurmond et al., 1998; Thurmond et al., 2000). Munc18-3 knockout leads to embryonic lethality (Kanda et al., 2005; Oh et al., 2005).

Mammalian Vps33 protein (mVps33p) is a homologue of the yeast vacuolar protein sorting protein (Vps33p) that is involved in vacuolar biogenesis and Golgi to vacuole traffic (Gissen et al., 2005). Likewise, mVps33 proteins are thought to play a role in organelle biogenesis. In mammals, two Vps33p homologues have been identified: mVps33A and mVps33B. The presence of two homologues that exhibit non-redundant function is believed to reflect the evolution of organelle/tissue-specific functions of each isoform. Mammalian Vps33A has been shown to play a role in intracellular vesicle trafficking with its mutation causing δ-granule deficiency in mice (Kim
Moreover, mVps33B has been implicated in α-granule biogenesis in megakaryocyte and platelets (Lo et al., 2005).

Mammalian Vps45p (mVps45p) is the mammalian homologue of the yeast Sec1p-like protein, Vps45p (Tellam et al., 1997a). Yeast Vps45 is known to play a role in the fusion of Golgi-derived transport vesicles with a post-Golgi/pre-vacuolar compartment through the interaction with t-SNARE Pep12p (Tellam et al., 1997a) in addition to its role in membrane traffic at the trans-Golgi network and early endosomes through interaction with Tlg2p (Dulubova et al., 2002). The mVps45p was characterized as a ubiquitously expressed peripheral membrane protein that is associated with Golgi/endosomal membranes (Tellam et al., 1997a). Although the exact role of the mVps45p in membrane traffic still remains unclear, the observation that the mVps45 is able to bind to syntaxin-6 (mammalian homologue of Pep12p) and syntaxin-16 (mammalian homologue of Tlg2p) suggests that the function of yeast Vps45p may be conserved in mammals (Dulubova et al., 2002; Tellam et al., 1997a). Mammalian Sly1p (mSly1p) is a homologue of yeast Sly1p. Consistent with the role of Sly1p in yeast, mSly1p has been reported to play a role in endoplasmic reticulum to Golgi transport by positively regulating its t-SNARE partner, syntaxin-5 (Dascher and Balch, 1996; Ossig et al., 1991; Yamaguchi et al., 2002).

In this thesis, the focus is on a member of SM family, Munc18-1, which plays essential roles in synaptic vesicle and dense-core vesicle trafficking and fusion.

1.5 Munc18-1 and its orthologues in exocytosis

The importance of Munc18-1 and its orthologues in synaptic transmission has been clearly demonstrated by the severe impairment in neurotransmitter release observed in null mutants (Hosono et al., 1992; Novick and Schekman, 1979; Schulze et al., 1994; Verhage et al., 2000; Weimer et al., 2003). Unc-18 (59% homology to Munc18-1) was the first identified SM protein to
be discovered through genetic screening for uncoordinated phenotypes in *C. elegans* (Brenner, 1974; Rizo and Sudhof, 2002). Deletion of Unc-18 in *C. elegans* resulted in severe locomotory defects and truncation of this protein severely decreased evoked and spontaneous activity at the ventral nerve cord neuromuscular junction (Brenner, 1974; Hosono et al., 1992; Weimer et al., 2003). The yeast orthologue, Sec1p (25% homology to Munc18-1), was the first secretory mutant identified in yeast through genetic screening for temperature sensitive mutants (Hashizume et al., 2009; Novick et al., 1980; Novick and Schekman, 1979). Deletion of Sec1p was shown to result in severe secretion defects with accumulation of intracellular membrane-bound vesicles containing acid phosphatase and invertase at the non-permissive temperature of 37°C (Novick and Schekman, 1979). Moreover, a null mutation in Rop (63% homology to Munc18-1) in *Drosophila melanogaster* was shown to result in loss of synaptic response to a light stimulus due to impaired synaptic transmission (Harrison et al., 1994). Consistent results were obtained in the mammalian system in which neurotransmitter release was completely abolished in Munc18-1 knockout mice (Verhage et al., 2000; Voets et al., 2001). Interestingly, Munc18-1 knockout exhibited a more severe phenotype than any of the SNARE null mutants (Deitcher et al., 1998; Nonet et al., 1998; Schoch et al., 2001; Schulze et al., 1995; Washbourne et al., 2002), implying that this protein is an essential component of the fusion machinery. Despite the general consensus on the indispensable role of Munc18-1 in exocytosis, the mechanism underlying its function in exocytosis remains unclear.

### 1.5.1 Homozygous Munc18-1 knockout phenotype

Genetic ablation of Munc18-1 in mice was shown to result in a complete paralysis of the animal which leads to lethality (Verhage et al., 2000). Munc18-1 null embryos were found to be alive until birth, but die immediately after birth, largely due to the inability to breathe. The
Munc18-1 null mutant completely lacked both spontaneous and evoked synaptic transmission despite the presence of functional postsynaptic receptors and ion channels at embryonic day 18 (E18) (Verhage et al., 2000). However, the null mutant showed apparently normal initial brain assembly including normal neuronal proliferation, migration, and differentiation into specific brain areas. Furthermore, synaptogenesis and all the aspects of the ultrastructure of the synapses including the amount of total and docked synaptic vesicles of the null mutant were comparable to that of the wild-type (Verhage et al., 2000). However, massive neuron apoptosis and degeneration was evident after the initial brain assembly in Munc18-1 null mice. Neuronal death seemed to start from the part of the brain that develops first, such as brain stem, followed by degeneration in the midbrain and basal forebrain (Verhage et al., 2000). This lethal phenotype of Munc18-1 supports the fundamental requirement of this protein in neurotransmitter release.

1.5.2 Heterozygous Munc18-1 knockout and Munc18-1-overexpression phenotype

An autapse is a synaptic connection formed by a neuron onto itself (Van der Loos and Glaser, 1972). Autaptic cultures from Munc18-1 heterozygous knockout mice displayed intact morphology, exhibiting normal dendrite length and number of synapses compared to the cultures from wild-type. Furthermore, excitatory and inhibitory post-synaptic currents upon single depolarization were comparable between Munc18-1 heterozygous knockout neurons and wild-type neurons (Toonen et al., 2006b). Moreover, spontaneous miniature excitatory and inhibitory postsynaptic events were not altered in these neurons compared to the wild-type, suggesting that reducing the level of Munc18-1 does not affect synaptic physiology under basal conditions. However, upon repeated stimulation, more pronounced rundown of evoked response, reflecting synaptic depression, was observed in both glutamatergic and GABAergic synapses in Munc18-1 heterozygous neurons (Toonen et al., 2006b). Moreover, the size of the readily releasable pool
(RRP) as measured by hypertonic sucrose stimulation was reduced in these neurons compared to the wild-type. Furthermore, Munc18-1 heterozygous mice were shown to have impaired neuromuscular synaptic function.

In contrast, over-expression of Munc18-1 resulted in a larger RRP and showed less synaptic depression during high-frequency stimulation without any effect on neuronal morphology or total number of synapses. Moreover, the frequency of spontaneous vesicle release in both glutamatergic and GABAergic neurons were increased by more than two-fold (Toonen et al., 2006b). However, evoked post-synaptic response remained unchanged which may be due to the saturation of post-synaptic receptors. Furthermore, Munc18-1 overexpressing mice exhibited enhanced synaptic functions in neuromuscular junctions. Lastly, electron microscopic analyses on Munc18-1 heterozygous knockout neurons, wild-type neurons, and Munc18-1 over-expressing neurons revealed that the number of morphologically docked vesicles at the presynaptic active zone membrane increases in parallel with increasing level of Munc18-1 (Toonen et al., 2006b). Together, this evidence suggests that the Munc18-1 contributes to regulating the size of readily releasable pool and synaptic efficacy without affecting the general morphology of the synapse.

1.6 Structure of Munc18-1

Polypeptide chains of Munc18-1 (67 kDa) are characterized by multiple domains: domain-1, -2, -3a, and -3b (Figure 1.3 A). Crystal structure analysis revealed that Munc18-1 is an arch-shaped protein with a central cavity that is ~15 Å wide (Misura et al., 2000). Domain-1 consists of residues 4-134. Domain-2 is comprised of residues 135-245 and 480-592. Domain-3 consists of residues 246-479 which is inserted between two parallel β-strands of domain-2 (Figure 1.3 B). Domain-3 is subdivided into domain-3a and -3b according to its location with respect to the rest of the protein (Misura et al., 2000). Another aspect of the structural specificity of Munc18-1 reported
in this study is that the domain-3a makes relatively few contacts with other parts of the protein. This means that this secondary structure of domain-3a may have an ability to move with respect to the rest of the protein. Moreover, domain-1 and -3a of Munc18-1 have been shown to provide a binding cleft for syntaxin-1 (Misura et al., 2000). The specific residues within these two domains that make contact with syntaxin-1 include residues 38-71 of domain-1 and residues 271-280 and 331-338 of domain-3a. Domain-1 has been suggested to contact both $H_{abc}$ and H3 domains of syntaxin-1A while domain-3a makes a contact with the H3 domain. Hence, these two domains have been the main region of interest for investigating the structural specificity of this protein underlying various modes of interaction with syntaxin-1.

Figure 1.3 Structure of Munc18-1. (A) Ribbon representation of Munc18-1. Domain-1,-2, and -3 are represented in blue, green, and yellow, respectively. Adapted by permission from Macmillan Publishers Ltd: Nature, Misura et al., 2000. (B) Domain diagram of Munc18-1. The number of residues of each domain is indicated below each domain on the right.
1.7 Pleiotropic functions of Munc18-1

The essential function of Munc18-1 in vesicle exocytosis is supported by substantial evidence. However, the precise roles of Munc18-1 in this process remain unclear. At least three important functions of Munc18-1 have been supported with extensive experimental, although sometimes contradictory evidence: 1) Molecular chaperone of syntaxin-1 by stabilizing and properly trafficking syntaxin-1 to the plasma membrane; 2) priming of vesicles via a promotion of SNARE complex-mediated membrane fusion; 3) docking of large dense-core vesicles to the plasma membrane.

1.7.1 Molecular chaperone of syntaxin-1: stabilizing and trafficking syntaxin-1

Munc18-1 is often referred to as a “chaperone” of syntaxin-1 as it ensures syntaxin-1 stabilization and proper trafficking to its cellular destination, plasma membrane. Initial evidence of the chaperone function came from work done on normal rat kidney (NRK) fibroblast cells and other non-neuronal cells. In these cells, ectopically expressed syntaxin-1 that remains trapped in the Golgi or endoplasmic reticulum correctly localized to the plasma membrane only when co-transfected with Munc18-1 (Medine et al., 2007; Rickman et al., 2007; Rowe et al., 1999). This finding strongly suggested that Munc18-1 acts as a molecular chaperone that enables proper trafficking of syntaxin-1 to the plasma membrane. Likewise, in Munc18-1/-2 double knockdown (DKD) PC12 cells, syntaxin-1 was shown to remain accumulated in a perinuclear region. This mislocalization of syntaxin-1 was rescued upon re-expression of wild-type Munc18-1 (Arunachalam et al., 2008; Han et al., 2011; Han et al., 2009). Moreover, anterograde transport of Unc-64 (syntaxin homologue) in C. elegans was severely disrupted in Unc-18 (Munc18-1 homologue) mutants (McEwen and Kaplan, 2008). In addition, the expression level of syntaxin-1 was shown to be strikingly decreased in Munc18-1-deficient neurons and neuroendocrine cells.
(Verhage et al., 2000; Voets et al., 2001). This line of evidence strongly supported a chaperone function of Munc18-1 which allows both stabilization and specific trafficking of syntaxin-1 to the plasma membrane. This function is thought to be done at least in part by preventing the formation of inappropriate, ectopic SNARE complexes (Martinez-Arca et al., 2003; Medine et al., 2007). It has been shown that mislocalized syntaxin-1 at ER induces a parallel mislocalization of cognate SNAREs through direct interaction (Martinez-Arca et al., 2003). In line with this, it has been suggested that if syntaxin-1 encounters SNAP-25 in the Golgi complex before it binds to Munc18-1, which allows it to be stabilized in inactive closed conformation, syntaxin-1 may form misplaced t-SNARE complex with SNAP-25 that prevents proper trafficking of both proteins to the plasma membrane (Medine et al., 2007). Furthermore, it has become clear that complex formation between SNARE proteins is less specific than previously recognized. This means that unspecific SNARE complex formation can occur when noncognate SNAREs encounter one another during membrane recycling. However, this rarely happens in intact cells, which can be explained by the presence of regulatory proteins that bind to individual SNARE with a higher degree of specificity until SNAREs encounter their cognate partners (Fasshauer et al., 1999). Likewise, Munc18-1 seems to bind to syntaxin-1 to ensure its proper trafficking to the plasma membrane where it allows syntaxin-1 to form SNARE complex with its cognate partners and efficiently execute membrane fusion. However, the effect of Munc18-1 on syntaxin-1 localization in mammalian neurons still remains to be elucidated.

1.7.2 Priming of vesicles via promotion of SNARE complex-mediated membrane fusion

Priming is generally referred to a step in exocytosis in which the vesicles mature to become fusion-competent after the docking but prior to the actual fusion. Although priming is traditionally considered to be downstream of docking, due to high efficiency in vesicle recycling in synapses, it
is difficult to separate docking and priming temporally (Toonen and Verhage, 2007). In electrophysiological studies, priming is often defined as the process by which the pool of readily releasable vesicles is refilled (Verhage and Sørensen, 2008). The readily releasable pool is believed to consist of the “primed” vesicles that are ready to be rapidly released upon hypertonic sucrose stimulus (Rosenmund and Stevens, 1996). Therefore, the size of the readily releasable pool is often used as an indication of the priming activity in cells.

In Munc18-1 knockout neurons, synaptic release is completely abolished despite the presence of intact pool of docked synaptic vesicles (Verhage et al., 2000). Moreover, overexpression of SNAP-25 has been shown to rescue the docking but not the secretion phenotype of Munc18-1 deficient chromaffin cells (de Wit et al., 2009). This line of evidence strongly indicates a role of Munc18-1 downstream of vesicle docking. Moreover, the size of readily releasable pool was shown to decrease in Munc18-1 heterozygous knockout and increase upon Munc18-1 overexpression, further supporting the role of Munc18-1 in priming stage (Toonen et al., 2006a). However, the exact molecular mechanisms underlying Munc18-1-mediated vesicle priming is poorly understood.

Accumulating evidence suggest that Munc18-1 plays a role in the priming of vesicles by assisting or stabilizing SNARE complex formation through interactions with assembled SNAREs. In line with this, Munc18-1 has been shown to strongly accelerate SNARE-mediated liposome fusion and the fusion between large vesicles and giant membranes (Diao et al., 2010; Shen et al., 2007; Tareste et al., 2008). The observation that Munc18-1 only strongly stimulated liposome fusion when \(\text{trans}\)-SNARE complexes were pre-assembled (pre-incubation of t- and v-SNARE liposomes) suggested that Munc18-1 specifically acts on a transient, partially assembled intermediate of the SNARE complex and promotes the progression of the fusion process (Shen et al., 2007). However, whether the mechanisms suggested by an \textit{in vitro} assay can really represent
the scenario in vivo still remains unclear. Presumably, a specific interaction between Munc18-1 and the SNARE complex will be required for Munc18-1 to regulate this process. However, diverging evidence involving the mechanisms underlying Munc18-1-SNARE complex interaction further complicates our understanding of Munc18-1-mediated priming or stimulation of fusion. This aspect will be further discussed in section 1.8 of Chapter 1.

1.7.3 Docking of large dense-core vesicles to the plasma membrane

The essential function of Munc18-1 in the docking of dense-core vesicles (DCV) was first demonstrated by electron microscopic analysis of Munc18-1-deficient adrenal chromaffin cells in which ~90% of the secretory granules were undocked (Voets et al., 2001). Moreover, Munc18-1 deficiency was shown to result in a marked defect in DCV docking in somatotrophs of the anterior pituitary (Korteweg et al., 2005). Likewise, dense-core vesicle docking was shown to decrease in Munc18-1 heterozygous chromaffin cells while it was increased in Munc18-1 over-expressing chromaffin cells, further confirming the positive role of this protein in docking (Toonen et al., 2006a). However, how Munc18-1 regulates DCV docking still remains unclear.

One suggested mechanism by which Munc18-1 exerts its role in vesicle docking is through the regulation of the subplasmalemmal actin cortex. It has been reported that the density of filamentous actin constituting the actin cytomatrix beneath the plasma membrane of chromaffin cells determines the access of secretory vesicles to fusion sites; lowering its density leads to an increase in morphologically docked vesicles (Malacombe et al., 2006; Vitale et al., 1995). With respect to this, the thickness of subplasmalemmal filamentous actin has been reported to be affected by the level of Munc18-1 expression such that the actin cortex is thinner and more fenestrated in wild-type and Munc18-1-overexpressing cells compared to Munc18-1 null mutant (Toonen et al., 2006a). Disrupting the submembrane cytomatrix by the actin depolarizing drug
Latrunculin A (Lat A) was shown to completely restore the morphological docking phenotype of Munc18-1 null mutants although vesicle fusion remained severely impaired (Toonen et al., 2006a). Therefore, the defective docking phenotype observed in electron micrographs of Munc18-1 null mutants may be related to the association of Munc18-1 with the cytoskeleton (Bhaskar et al., 2004). This means Munc18-1 may influence cytoskeletal stability or penetrance and thus vesicle delivery, although the mechanism is unknown. However, the observation that not all remaining morphologically docked vesicles were fusion competent in the absence Munc18-1 suggests that Munc18-1 not only contributes to the physical linking of vesicles to the plasma membrane, but also biochemically tethers the vesicles to the plasma membrane so they can further advance to the priming state. However, this protein has no inherent affinity to membranes. This means that it is highly likely that Munc18-1 performs this action through interaction with plasma membrane protein such as syntaxin-1. In fact, the importance of syntaxin-1 in vesicle docking has been clearly demonstrated as cleavage of syntaxin-1 with botulinum neurotoxin serotype C resulted in a robust reduction of docking in chromaffin cell (de Wit et al., 2006). In line with this, Munc18-1 may interact with its partner, syntaxin-1, to aid with setting up or stabilizing the t-SNARE complexes at the plasma membrane to which vesicular proteins can bind and further tether the vesicles. The importance of specific interactions between Munc18-1 and syntaxin-1 in vesicle docking is further highlighted by the observation that the deletion of SNAP-25 or synaptobrevin-2 in chromaffin cells do not result in docking defects as seen for Munc18-1 or syntaxin-1 deficient mutants (Borisovska et al., 2005; Sørensen et al., 2003). Together, it can be postulated that Munc18-1 contributes to DCV docking through two sequential mechanisms. First, Munc18-1 regulates delivery of vesicles to plasma membrane by influencing the actin cytoskeleton. Then it induces the higher-affinity tethered state of vesicles by promoting biochemical linkage between vesicles and plasma membrane through specific interaction with plasma membrane protein,
syntaxin-1. Nonetheless, much more work needs to be done in order to clarify the molecular mechanisms underlying the role of Munc18-1 in vesicle docking.

1.8 Munc18-1-SNARE Interaction

It is generally agreed that the conserved function of SM proteins in membrane fusion involves the specific interaction with their cognate SNAREs (particularly syntaxin homologues) (Halachmi and Lev, 1996; Hata and Südhof, 1995; Hu et al., 2007; Kauppi et al., 2002; Latham et al., 2006; Riento et al., 1998; Riento et al., 1996; Riento et al., 2000; Tamori et al., 1998; Tellam et al., 1997b). Although the interaction between SM proteins and their cognate syntaxins is conserved through evolution, the modes of their interactions have diverged. These diverse binding modes may reflect different regulatory requirements of membrane trafficking in different systems. Likewise, multiple modes of interaction between Munc18-1 and SNAREs, particularly syntaxin-1, have further complicated our understanding of the mechanisms underlying the role of Munc18-1 in regulated exocytosis. At least three different binding modes have been proposed to date. Whether the different binding modes occur sequentially to regulate each stage of exocytosis finally leading to membrane fusion, or whether they independently contribute to different stages of exocytosis remains to be elucidated.

1.8.1 Mode 1: Binary interaction

The first binding mode discovered is the high affinity binary interaction between Munc18-1 and the “closed” conformation of syntaxin-1 (Figure 1.4 A). This mode of interaction is unique to Munc18-1 (and possibly Munc18-2), and may have evolutionarily emerged to accommodate the precise spatial and temporal requirements of neuronal exocytosis (Dulubova et al., 2007; Shen et al., 2007). In this interaction, the central cavity formed by domain-1 and -3a of Munc18-1
provides the binding cleft for syntaxin-1 in an auto-inhibited closed conformation state in which
H_{abc} domains of syntaxin-1 are folded back onto H3 domain. The residues 38-70 of domain-1 and
271-280 and 331-338 of domain-3a were suggested to make specific contacts with closed
syntaxin-1 (Misura et al., 2000). Importantly, this form of binding is incompatible with SNARE
complex assembly, which requires syntaxin-1 to be in an “open” conformation.

The importance of domain-1 in the binary interaction and consequently in secretion has
been characterized through the analyses of various Munc18-1 domain-1 mutations that have been
designed to disrupt this interaction. The functional importance of the binary interaction mediated
by domain-1 of Munc18-1 was best supported by the lack of secretion rescue activity of domain-1
K46E/E59K double mutants upon re-expression in Munc18-1/-2 DKD PC12 cells (Han et al.,
2009). Both Lys46 and Glu59 are highly conserved residues that are suggested to interact with
syntaxin-1 (Han et al., 2009; Misura et al., 2000). According to yeast two-hybrid analysis,
mutating these two residues independently (K46E or E59K) specifically reduced the binary
interaction between Munc18-1 and syntaxin-1 but binding was not completely impaired. However,
mutating these two residues simultaneously (K46E/E59K) completely abolished binding to
syntaxin-1A while maintaining intact binding to another binding partner of Munc18-1, Mint-1
(Munc18 interacting protein). Consequently, this double mutant also failed to rescue the
expression, localization of syntaxin-1 and the secretion ability of Munc18-1/-2 DKD cells (Han et
al., 2009). These findings emphasized the importance of domain-1 in the Munc18-1-syntaxin-1
binary interaction, which is consequently essential for both chaperone and neurosecretory
functions of Munc18-1 (Han et al., 2009).

A previous study has reported that the E59K single mutation in Munc18-2 loses the ability
to bind to syntaxins 1-4 (Kauppi et al., 2002). However, the result obtained by isothermal titration
calorimetry (ITC) and NMR spectroscopy have shown that the E59K mutation in Munc18-1 only
moderately affects the binary interaction between Munc18-1 and syntaxin-1 while severely impairing its interaction with the SNARE complex (Deák et al., 2009). This data appears to be inconsistent not only with the yeast two-hybrid result but also with the current structural model of the binary interaction between Munc18-1 and syntaxin-1 that identified Glu59 as a key interacting residue (Misura et al., 2000). Nonetheless, inability of the Munc18-1 E59K mutant to rescue secretion in Munc18-1 null neurons (Deák et al., 2009) still supports the importance of domain-1 of Munc18-1 in neurosecretion (Han et al., 2009).

The D34N/M38V mutant is another Munc18-1 mutation that was shown to significantly reduce the Munc18-1-syntaxin-1 binary interaction (Gulyás-Kovács et al., 2007). In line with other studies, this domain-1 mutant also exhibited impaired docking and secretion rescue ability, further confirming the importance of domain-1 in binary interaction and consequently in secretion. Surprisingly, however, this mutant was reported to be able to significantly restore syntaxin-1 pool in Munc18-1 deficient chromaffin cells, which is known to mainly rely on the binary interaction. This is also at odds with the previous finding that suggested a defect in vesicle docking is accompanied by reduced syntaxin-1 levels in these cells (Voets et al., 2001). Nonetheless, the impaired ability of this mutant to restore docking and secretion in Munc18-1 null cells further emphasized the importance of binary interaction in docking and secretion of dense-core vesicles.

In complement to the mutational analysis of the Munc18-1 that does not bind to “closed” syntaxin-1, there are studies that have examined the phenotypes of a syntaxin-1 mutant that impairs the binary interaction with Munc18-1. In mice, the syntaxin-1B mutant (L165A/E166A) which prefers to adopt an “open” conformation was shown to abolish its binding to Munc18-1. Consequently, trafficking of this syntaxin-1B mutant to the plasma membrane was reduced (Dulubova et al., 1999; Gerber et al., 2008). The reduced localization of the syntaxin-1B “open” conformation mutant along the plasma membrane appeared to be compensated by the accelerated
SNARE complex formation ability of this mutant, as normal synaptic transmission was exhibited (Gerber et al., 2008). Nonetheless, this finding not only confirms that the binary interaction between Munc18-1 and syntaxin-1 requires syntaxin-1 to be in a closed conformation but also that this binding is important for syntaxin-1 trafficking.

1.8.2 Mode 2: N-terminal interaction

N-terminal interaction refers to the interaction between an outer surface of the hydrophobic pocket of Munc18-1 and an N-terminal peptide sequence of their cognate syntaxin-1. This interaction mode is conserved in variety of SM proteins as Sly1, Vps45, and Munc18-3 are also known to bind to the N-terminal peptide of their respective cognate syntaxins independently of whether they are part of SNARE complexes or not (Carpp et al., 2006; Dulubova et al., 2003; Dulubova et al., 2002; Kosodo et al., 1998; Latham et al., 2006; Nichols et al., 1998; Yamaguchi et al., 2002). This means that this interaction is compatible with either a closed conformation of syntaxin that is not part of a SNARE complex or an opened syntaxin-1 within a SNARE complex. This binding mode is also conserved for the Munc18-1-syntaxin-1 interaction. (Bracher and Weissenhorn, 2002; Burkhardt et al., 2008; Hu et al., 2007; Rickman et al., 2007). Nonetheless, functional significance of this binding mode is highly debated.

One school of thought proposed that the N-terminal binding is required to further secure the binary interaction between Munc18-1 and closed syntaxin-1 (Figure 1.4 C) (Burkhardt et al., 2008). In this view, binding of Munc18-1 to the closed conformation and to the N-peptide of syntaxin-1 represents two sequential steps of the same function which is stabilizing syntaxin-1 in its inactive form (Figure 1.4 A and C). In this aspect, syntaxin-1 in its closed formation would be further secured by additional binding through its N-peptide until it reaches the plasma membrane where Munc18-1 bound N-peptide is released allowing syntaxin-1 to open up and assemble into
the SNARE complex (Burkhardt et al., 2008). This is supported by experimental evidence that showed that the inhibitory role of Munc18-1 in the formation of SDS-resistant SNARE complexes depends on the presence of syntaxin-1 N-terminal peptide (Burkhardt et al., 2008). In this model, what triggers the release of the N-peptide is yet to be determined, although the phosphorylation of syntaxin-1 Ser14 or Mint-1, which seems to share the same interactive site within Munc18-1 as N-peptide, has been suggested to play this role (Han et al., 2009; Rickman and Duncan, 2010). Although experimental evidence to directly support this model is limited, a recent analysis of Munc18-1 mutants (F115E, E132A, F115E/E132A), designed to abolish N-terminal binding, demonstrated that the ability of these mutants to restore syntaxin-1 expression and localization in Munc18-1/-2 double knockdown cells is slightly reduced compared to the wild-type (Han et al., 2009). This may be explained at least partially by the weaker binary interaction with syntaxin-1 in the absence of the additional contribution from N-terminal binding which is thought to strengthen the binary interaction in this model (Burkhardt et al., 2008; Han et al., 2009).

Another school of thought suggests that the N-terminal binding mode is required for the Munc18-1 interaction with the assembled SNARE complex (Figure 1.4 B1 and B2) (Shen et al., 2010; Shen et al., 2007; Toonen and Verhage, 2007). This appears to be a common feature of almost all SM proteins, including Munc18-1 (Dulubova et al., 2007; Latham and Meunier, 2007). However, the exact mechanism of how N-terminus binding contributes to mediate the interaction between Munc18-1 and the SNARE complex is unclear. One proposed mechanism is that Munc18-1 that is initially bound to the closed conformation of syntaxin-1 stays bound to the N-terminal peptide of syntaxin-1 while it changes its conformation from closed to open upon participating in SNARE complex formation (Figure 1.4 B1 and B2). This interaction has been suggested to assist SNARE complex assembly. However, whether Munc18-1 dissociates from the
complex upon SNARE complex formation (Zilly et al., 2006) or it stays bound to assembled
SNARE complex to further exert its regulatory role remains to be elucidated.

Although the exact sequence of events is unclear, Munc18-1 binding to the N-terminus of
syntaxin-1 seems to be critical for its interaction with the SNARE complex, as deletion or
mutation in syntaxin-1 N-terminus dramatically impairs the ability of Munc18-1 to bind to
assembled SNARE complexes (Dulubova et al., 2007; Rickman et al., 2007; Shen et al., 2007;
Zilly et al., 2006). In support of this, mutations at Phe115 and/or Glu132 residues in the N-peptide
binding hydrophobic pocket of Munc18-1 have been shown to abrogate Munc18-1 interaction with
the SNARE complex (Malintan et al., 2009). However, whether Munc18-1 interacts with an
assembled SNARE complex solely through N-terminal binding or not remains as a question.
Isothermal titration calorimetry (ITC) data has indicated that Munc18-1 has a similar affinity for
the SNARE complex and the syntaxin-1 N-terminal region, suggesting that this region is solely
responsible for the SNARE complex binding to Munc18-1 (Burkhardt et al., 2008). However,
other mutations (E59K, Y337L) in Munc18-1 residues that do not to interact with the N-peptide of
syntaxin-1 were also shown to abolish binding to the SNARE complex with limited effect on the
binary interaction with syntaxin-1 (Boyd et al., 2008; Deák et al., 2009). This suggests that
Munc18-1 binding to the SNARE complex may involve other binding sites in addition to the N-
terminal peptide of syntaxin-1. This is further supported by a recent finding that Munc18-1 can
bind to synaptobrevin-2 and to the SNARE four-helix bundle (Xu et al., 2010).

The function of the N-terminal binding mode in membrane fusion/exocytosis is also
controversial. The experimental evidence that the stimulatory effect of Munc18-1 on fusion is
abolished upon removing or mutating N-terminal residues of syntaxin-1 both in reconstituted
system and in vivo suggest that N-terminal interaction is functionally required for Munc18-1
mediated membrane fusion (Shen et al., 2007; Tareste et al., 2008; Zhou et al., 2013). However,
this was challenged by another study that showed Munc18-1 binding to the SNARE core in the absence of N-peptide is sufficient for Munc18-1 stimulated SNARE-dependent lipid fusion (Diao et al., 2010). Likewise, genetic evidence is also highly debated. In C. elegans, the F113R mutant in UNC-18 (corresponds to F115R mutation in Munc18-1) 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 (Han et al., 2010; Johnson et al., 2009). This was further supported by another study suggesting that a synthetic N-terminal peptide of syntaxin-1 inhibits neuronal secretion from neurons and neuroendocrine PC12 cells, by competitively binding to Munc18-1 (Han et al., 2010; 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 (Han et al., 2010; Rickman and Duncan, 2010). 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 dense-core vesicle exocytosis in Munc18-1 single (Malintan et al., 2009) and Munc18-1/-2 double knockdown (Han et al., 2009) PC12 cells. In line with this, 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). The different phenotypes observed from mutating the same residue may reflect distinct physiology of the various model system or different techniques used in each study. Therefore, resolving these conflicting observations is the key in understanding the functional importance of N-terminal binding in exocytosis.
Figure 1.4 Interactions between Munc18-1 and syntaxin-1. Schematic representation of two possible mechanisms underlying N-terminal binding modes of Munc18-1. (A) High affinity binary interaction between Munc18-1 and closed conformation of syntaxin-1. (B₁, B₂) The binding between Munc18-1 and N-terminal peptide of syntaxin-1, which enables an interaction with the SNARE complex. (C) The binding between Munc18-1 and N-terminal peptide of syntaxin-1 further stabilizes the binary interaction. Domain-1, -2, -3b, and -3a of Munc18-1 are represented in purple, blue, green, and yellow, respectively. Adapted from Han et al., 2010.
1.8.3 Mode 3: SNARE complex interaction

Binding to assembled SNARE complexes was first reported for the yeast orthologue, Sec1p (Carr et al., 1999). In fact, Sec1p is the only SM protein that does not bind to monomeric syntaxin (Sso1p). The Sec1p-SNARE interaction does not require the N-terminal peptide, which suggests that Sec1p uses a distinct SNARE binding mode and that, at least in yeast, SM proteins can promote fusion without N-terminal interactions. Likewise, increasing evidence supports a direct interaction between Munc18-1 and assembled SNARE four helical bundle (Dulubova et al., 2007; Shen et al., 2007). However, whether Munc18-1-SNARE complex interaction exclusively depends on Munc18-1 interaction with N-terminal peptide of syntaxin-1 (Figure 1.4 B) or direct interaction with the four-helical SNARE bundle (Figure 1.5) or involves sequential interactions of both still remains highly debated due to inconsistent experimental evidence.

Munc18-1 has been shown to only selectively stimulate liposomes reconstituted with cognate neuronal t- and v- SNAREs. This reflects the ability of Munc18-1 to recognize the specificity of an assembled SNARE complex (Shen et al., 2007; Toonen and Verhage, 2007; Xu et al., 2010). This means Munc18-1 has to be able to distinguish its cognate SNARE complex through a highly specific interaction. However, a recent study reported that Munc18-1 binds to both cognate and non-cognate N-peptide of syntaxin-1 with similar affinity (Hu et al., 2010). This lack of specificity in N-terminal binding suggests that there must be another mechanism that enables Munc18-1 to distinguish its cognate SNARE complex, perhaps by binding directly to the core SNARE bundle. With respect to this, a few studies have highlighted interaction between Munc18-1 and synaptobrevin-2 within the SNARE complex as an underlying mechanism of the direct Munc18-1-SNARE bundle interaction. It has been suggested that Munc18-1 functionally interacts with the membrane proximal region of synaptobrevin-2 to stimulate liposome fusion, as mutating the residues within this region of synaptobrevin-2 markedly decreased Munc18-1-
stimulated fusion (Shen et al., 2007). In line with this, Munc18-1 was shown to directly bind the SNARE motif of synaptobrevin-2 and to the SNARE four-helix bundle (Xu et al., 2010). The sites at which Munc18-1 and synaptobrevin-2 interact were mapped to be residues 333-339 (KMPQYQK) of Munc18-1 and residues 87-91 (KYWWK) of synaptobrevin-2 (Xu et al., 2010). These residues of Munc18-1 reside within the cavity that has been reported to bind closed syntaxin-1. In fact, syntaxin-1 Habc domain has been shown to compete with the SNARE four helical bundle and synaptobrevin-2 for Munc18-1 binding. The residues 87-91 of synaptobrevin-2 are located at the very C-terminus of the SNARE motif. These findings led to the hypothesis that this Munc18-1 interaction with the very C-terminus of synaptobrevin-2 may place Munc18-1 right at the site of membrane fusion where it can cooperate with SNARE complexes to mediate membrane fusion (Xu et al., 2010). Moreover, a recent structural analysis has revealed that domain-3a (residues 295-358) of Munc18-1 can undergo a conformational change where it can adopt both bent and extended conformations (Hu et al., 2010). It has been suggested to exhibit a bent hairpin structure when bound to closed syntaxin-1 while its extended conformation is incompatible with this binary interaction. Therefore, it could be postulated that Munc18-1 undergoes a conformational change to release its binding with closed syntaxin-1 and to adopt another mode of interaction, perhaps with SNARE complex (Hu et al., 2010). This is further supported by an observation that the isolated domain-3a peptide corresponding to the residues 326-359 interacts with the SNARE ternary complex as analyzed by CD spectroscopy (Hu et al., 2010). These biochemical and structural analyses together highlights the importance of domain-3a in SNARE complex interaction.

Although the ability of GST-Munc18-1 to co-precipitate SNARE complexes was shown to be dramatically reduced in the absence of N-peptide motif of syntaxin-1, Munc18-1 was found to bind assembled SNARE complexes even in the absence of N-peptide in liposome flotation assay,
suggesting that the core domains of assembled SNARE complexes constitute an efficient binding target of Munc18-1 when present on the surface of membrane (Shen et al., 2010). Together, these results suggest that Munc18-1 may have multiple contact sites on SNARE complex, including core regions as well as the N-terminal peptide of syntaxin-1. This multiple site interaction may be necessary to ensure the proper positioning of Munc18-1 for both spatial and temporal regulation of the specificity of a fusion reaction. Nonetheless, the mechanism underlying this Munc18-1-SNARE complex interaction needs to be further investigated.

Figure 1.5 Interaction between Munc18-1 and assembled SNARE complex. Schematic representation of the direct interaction between Munc18-1 and the four helical bundle of SNARE complex. Domain-1, -2, -3b, and -3a of Munc18-1 are represented in purple, blue, green, and yellow, respectively. Adapted and modified from Han et al., 2010.
1.9 Other binding partners of Munc18-1

Although the predominant binding partner of Munc18-1 are SNAREs, Munc18-1 has also been reported to interact with other proteins such as Mint (Munc18 interacting) protein and Doc2 (double C2 like domain containing protein).

Mint proteins (Mint1 and Mint2) were identified by yeast two-hybrid screening of a rat brain cDNA library with full-length Munc18-1 as bait (Okamoto and Südhof, 1997). Mint proteins (839 amino acids) consist of an N-terminal sequence that binds to Munc18-1, a middle phosphotyrosine-binding (PTB) domain, and two C-terminal PDZ domains that are believed to attach proteins to the plasma membrane (Okamoto and Südhof, 1997). Interaction between Mint proteins and Munc18-1 is supported by the following evidence: 1) Mint proteins are co-expressed with Munc18-1 at high levels only in neurons; 2) Mint proteins specifically bind to Munc18-1 as detected by yeast two-hybrid assays, GST-pull down methods, and co-immunoprecipitation (Okamoto and Südhof, 1997). However, the functional significance of this interaction is unclear. Based on the structural characteristic of Mint proteins, it has been speculated that Mint proteins may play a role in synaptic vesicle docking/fusion as a part of a multimeric complex containing Munc18-1 and syntaxin-1(Okamoto and Südhof, 1997). The PTB domain of Mint proteins was shown to be able to interact with PIP while the presence of the PDZ domain could allow it to interact with the cytoplasmic tails of membrane proteins, thereby providing a linkage between the plasma membrane and synaptic vesicles allowing synaptic vesicles to be docked (Okamoto and Südhof, 1997). However, this speculative model needs to be further clarified. Moreover, a ubiquitous Mint isoform, Mint3, was found not to bind to Munc18-1 or -2. This implies that neuron-specific Mint-1 and Munc18-1 interaction may reflect their specific requirement in more specialized synaptic transmission (Okamoto and Südhof, 1998). More recently, the importance of
Mint-1 has been demonstrated by impaired GABAergic synaptic transmission in Mint1 knockout mice (Ho et al., 2003).

Doc2 is another protein that binds to Munc18-1. Doc2 isoforms (Doc2a and Doc2b) were isolated by cloning from a rat brain cDNA library (Verhage et al., 1997). Doc2 contains two C2 domains and are associated exclusively with synaptic vesicles. The Ca^{2+}-independent, direct binding of Doc2 to Munc18-1 has been demonstrated by yeast two-hybrid assay, co-immunoprecipitation, and affinity chromatography (Verhage et al., 1997). Doc2b was reported to bind Munc18-1 more efficiently than Doc2a. It was further shown that the first C2 domain is sufficient for Munc18-1 interaction. Moreover, Doc2 and syntaxin-1 was shown to compete for Munc18-1 binding although syntaxin-1-Munc18-1 binding was observed to be consistently stronger (Verhage et al., 1997). It has been also suggested that the Doc2-Munc18-1 interaction is relatively weak compared to the high affinity Mint-Munc18-1 interaction (Okamoto and Südhof, 1997). Based on these observations, it has been speculated that Doc2 removes Munc18 from syntaxin-1 as soon as the SNARE complex is ready to assemble, thus regulating SNARE complex formation and synaptic vesicle docking (Verhage et al., 1997). Doc2 was recently reported as a high-affinity Ca^{2+}-sensor for spontaneous neurotransmitter release and asynchronous neurotransmitter release (Groffen et al., 2010; Yao et al., 2011).

1.10 Munc18-1: Implication in human disease

De novo mutations in Munc18-1 were reported to be associated with a wide spectrum of early infantile epileptic encephalopathy (EIEE) such as Ohtahara Syndrome, West Syndrome, and mental retardation with or without epilepsy in humans (Hamdan et al., 2011; Hamdan et al., 2009; Milh et al., 2011; Otsuka et al., 2010; Saitsu et al., 2008; Saitsu et al., 2010). Specifically, all missense mutations in Munc18-1 reported to date are implicated in Ohtahara Syndrome, which is
one of the most severe and earliest forms of epilepsy that is characterized by early onset of tonic spasms, intractable seizure, and severe psychomotor retardation. Initially, V84D, C180Y, M443R, and G544D mutations in Munc18-1 were screened in unrelated individuals with Ohtahara syndrome (Saitsu et al., 2008; Saitsu et al., 2010). A missense mutation, C180Y, showed structural instability and impaired binding to syntaxin-1A leading to the hypothesis that synaptic vesicle release may be greatly impaired in the individuals with the C180Y substitution. However, biochemical approaches did not analyze how the expression of these proteins would affect cellular phenotypes. More recent studies have identified novel missense mutations in Munc18-1 that are implicated in Ohtahara syndrome and non-syndromic mental retardation. These mutations include L183R, R406H, P480L, C552R, and T574P (Hamdan et al., 2011; Mignot et al., 2011; Milh et al., 2011; Otsuka et al., 2010; Saitsu et al., 2010). Nonetheless, biological aspects of these mutants have never been assessed.

1.11 PC12 cells as a model system

1.11.1 Characterization of PC12 cells

The adrenal phaeochromocytoma (PC12) cell line is a clonal line isolated in 1976 from a tumour in the adrenal medulla of a rat (Greene and Tischler, 1976). PC12 cells contain two types of vesicles: large dense core vesicles (DCVs; ~100nm) that store monoamines (catecholamines and neuropeptides) and smaller vesicles of endosomal origin termed small synaptic vesicle-like microvesicles (SLMVs; ~40nm) that contain acetylcholine (Greene and Rein, 1977b; Greene and Tischler, 1976; Rebois et al., 1980; Schubert and Klier, 1977; Travis and Wightman, 1998). The majority of catecholamine contained in PC12 cells is dopamine, but addition of ascorbic acid can lead to production of limited amounts of noradrenalin in some PC12 subcultures (Schubert and Klier, 1977; Tischler et al., 1983). The ability of PC12 cells to synthesize and store
neurotransmitters such as dopamine and norepinephrine resembles noradrenergic and cholinergic adrenal chromaffin cells and sympathetic neurons (Westerink and Ewing, 2008).

Moreover, the presence of both nicotinic and muscarinic receptors in PC12 cells enables one to trigger exocytosis through two different mechanisms upon activation. Application of nicotine causes opening of sodium channels, which depolarizes the cell membrane to open voltage-sensitive Ca\(^{2+}\) channels allowing rapid calcium influx and subsequent exocytosis (Greene and Rein, 1977a; Stallcup, 1979). On the other hand, activation of muscarinic receptors by muscarine initiates the activation of intracellular secondary messengers which leads to the release of Ca\(^{2+}\) ions from intracellular stores triggering exocytosis (Berridge and Irvine, 1984). In addition to these triggering methods, KCl can be used to directly depolarize the membrane to promote rapid exocytosis. These different stimulating methods do not affect the average amount of catecholamine released, but significantly vary the latencies of exocytosis (Martin and Grishanin, 2003; Zerby and Ewing, 1996b).

1.11.2 PC12 cells as a model cell line for neurons and neuroendocrine cells

The PC12 cells can be grown and cultured indefinitely with a well-defined secretory cell phenotype. Furthermore, these cells are not only extremely versatile for genetic manipulation, but also maintain a robust exocytotic phenotype in response to Ca\(^{2+}\) influx, providing a set of important experimental advantages for cell biological and biochemical studies of the secretory pathway. Interestingly, PC12 cells can exhibit the cholinergic neuronal phenotype upon NGF and retinoic acid treatment (Greene and Tischler, 1976; Matsuoka et al., 1989), while the endocrine adrenergic phenotype is maintained in undifferentiated cells and can be strengthened by dexamethasone treatment (Anderson, 1993).
The ability of PC12 cells to respond to nerve growth factor (NGF) allows them to serve as a model system for primary neuronal cells. Like adrenal chromaffin cells, PC12 cells resemble the phenotype of sympathetic ganglion neurons upon differentiation with NGF (Martin and Grishanin, 2003; Westerink and Ewing, 2008). NGF-induced differentiated PC12 cells can be characterized by ceased proliferation, extended neurites, and changes in cellular composition and organization (Ng et al., 2002). In these cells, varicosities (bulbous regions, 1-2 μm in diameter) containing aggregates of small vesicles (20-70 nm in diameter) form along the extended neurites (Greene and Tischler, 1976; Westerink and Ewing, 2008). Most release is observed to come from the varicosities with no release from the cell body and only very infrequent release from the smooth regions of the neurites (Zerby and Ewing, 1996a). However, the amount of vesicular catecholamine released from the varicosities in differentiated cells is not significantly different from that observed for exocytosis at the soma of undifferentiated cells (Westerink and Ewing, 2008). This indicates that upon differentiation, the sites of exocytosis are relocated (from soma to varicosities) without affecting the overall amount of catecholamine release. PC12 cells are thought to more closely resemble neurons with smaller vesicles (75-120nm radius vs. 170nm radius) and quantal size than that of chromaffin cells (Clark and Ewing, 1997; Greene and Tischler, 1976; Schubert et al., 1980; Travis and Wightman, 1998).

PC12 cells are neuroendocrine in origin and exhibit a well-developed DCV secretory pathway (Anderson, 1993). PC12 cells can differentiate into neuroendocrine chromaffin-like cells upon treating with dexamethasone, a potent synthetic glucocorticoid that is used to treat a wide variety of conditions such as an anti-inflammatory agent or immunosuppressant. Enhanced endocrine adrenergic phenotype involves dramatically increased quantal size, release frequency, the amount of releasable vesicles and coupling between calcium channels and vesicle release sites, leading to rapid exocytosis and endocytosis. This is consistent with an observation that
approximately 90% of dexamethasone-differentiated PC12 cells released vesicular neurotransmitter compared to ~50% in undifferentiated cells upon KCl-induced depolarization (Elhamdani et al., 2000; Westerink and Ewing, 2008).

### 1.11.3 Studying secretion using PC12 cells

Exocytosis of DCVs in intact as well as permeable PC12 cells can be readily studied by assaying the release of preloaded radioactive norepinephrine, $[^3$H] NE (Martin and Grishanin, 2003). Dense-core vesicles in PC12 cells can be pre-labeled with $[^3$H] NE (radioactive labeled norepinephrine) which will be taken up via noradrenaline transporter (NAT) at the plasma membrane and vesicular monoamine transporter (VMAT) at the vesicular membrane (Mahata et al., 1993; Whim, 2006). Then the secretion ability of wild-type or genetically manipulated PC12 cells can be studied by measuring the amount of released $[^3$H] NE upon stimulating the cells with high KCl solution to depolarizes the membrane.

Another advantage of PC12 cells for studying the regulated exocytosis is that many (40-60%) of the DCVs are docked and retain competence for Ca$^{2+}$-triggered fusion even following mechanical homogenization of cells (Martin, 1989; Martin and Grishanin, 2003; Martin and Kowalchyk, 1997). Cytosolic proteins required for exocytosis can be removed by washing the permeabilized cell. Then Ca$^{2+}$-dependent exocytosis can be reconstituted in vitro by incubating permeabilized cells with Mg-ATP and cytosolic factors required for secretion (Martin, 1989; Martin and Grishanin, 2003; Martin and Kowalchyk, 1997; Walent et al., 1992). The secretory ability of the reconstituted cells can be assayed by measuring the amount of pre-labeled content released from the cells. This assay is useful for determining the important cytosolic proteins/factors that are required for secretion.
1.11.4 Munc18-1/-2 double knockdown PC12 cells

A stable Munc18-1/-2 double knockdown PC12 cell line was initially established in the Sugita lab by RNA interference (RNAi) system. This system involves usage of a double stranded short hairpin RNA (shRNA) that is designed to be complementary to a specific region of the target mRNA (Munc18-1 and Munc18-2 in this case). Upon introducing the shRNA into the cells, these short dsRNA are incorporated into a multiprotein RNA-inducing silencing complex (RISC). The antisense strand of unwounded short dsRNA then directs the RISC complex to the target mRNA which ultimately induces the target gene silencing (Arunachalam et al., 2008; Han et al., 2009). Phenotypic analyses of Munc18-1/-2 double knockdown cells revealed that the Ca\textsuperscript{2+}-dependent secretion was prominently inhibited (~90%) (Han et al., 2009). Moreover, syntaxin-1 expression level was dramatically reduced (~75%) and majority of syntaxin-1 was found to be accumulated in the perinuclear region in Munc18-1/-2 double knockdown cells (Han et al., 2009). Through extensive phenotypic confirmation, Munc18-1/-2 double knockdown clones (DKD7 and DKD16) that show the strongest knockdown phenotypes were selected to be used for further experiments. In this thesis, the phenotypes of various Munc18-1 mutations were assessed upon re-expression in these Munc18-1/-2 double knockdown clones.

1.12 Rationale, Hypothesis and Specific Aims

Multiple lines of evidence suggest the pleiotropic functions of Munc18-1 in exocytosis. However, there is no apparent consensus on its function due to inconsistent evidence. Moreover, multiple binding modes between Munc18-1 and syntaxin-1 have further complicated our understanding of the mechanisms underlying the essential roles of this protein. Therefore, the objective of this research has been to elucidate the molecular mechanism underlying the
pleiotropic functions of Munc18-1 in exocytosis. The main focus has been to investigate the structural/functional specificity of Munc18-1 in relation to syntaxin-1 interaction and regulation.

The study presented in the thesis has been conducted based on the hypothesis that the pleiotropic functions of Munc18-1 in exocytosis are mediated through its distinctive binding modes with SNARE proteins which involve specific regions of this protein. Based on the previous crystal structure that has suggested domain-1 and domain-3a consist of highly conserved residues that make specific contact with syntaxin-1 (Misura et al., 2000) in addition to the data from the previous studies, the following hypotheses have been generated:

**Hypothesis 1:** Domain-1 of Munc18-1 critically contributes to the high affinity binary interaction with monomeric syntaxin-1. This mode of interaction underlies an important role of Munc18-1 in syntaxin-1 chaperoning which involves both stabilization and proper trafficking of syntaxin-1 to the plasma membrane.

**Hypothesis 2:** Domain-3a plays a crucial role at the priming stage of exocytosis beyond syntaxin-1 trafficking to the plasma membrane.

These hypotheses have been assessed through following specific aims:

**Specific Aim 1:** To determine the functional specificity of domain-1 of Munc18-1 in dense-core vesicle exocytosis.

**Specific Aim 2:** To determine the functional specificity of domain-3a of Munc18-1 in dense-core vesicle exocytosis.
Chapter 2: Materials and Methods

2.1 General Materials

Parental pLVX-IRES-puro plasmid for lentivirus-mediated Munc18-1 expression was purchased from Clontech Laboratories (Mountain View, CA). pLVX-EmGFP-IRES-blast was generated by replacing the puromycin gene with the blasticidin resistance gene. pLVX-EmGFP-IRES-hygro was generated by replacing the blasticidin resistance gene with the hygromycin resistance gene. 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 monoclonal antibodies against syntaxin-1 (clone HPC-1) (Barnstable et al., 1985) from Sigma Chemical (ON Canada); Munc18-1 from BD Biosciences (Mississauga, ON, Canada), GFP from Clontech (Mountain View, CA) and GAPDH (clone 6C5) from Millipore (Billerica, MA).

2.2 Cell culture

Two clonal lines of Munc18-1/2 double-knockdown cells (D7, D16) 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), puromycin (2.5 g/ml), and G418 (700 g/ml).

Human embryonic kidney-fibroblast (HEK293FT) cells were maintained in DMEM medium with 110mg/L 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 PCR-based site directed mutagenesis

Bacterial expression vectors to express full-length rat Munc18-1 point mutants (K46E, E59K, K46E/E59K, K63E, E66A, D34N/M38V, F115R, E132K, F115R/E132K, E278K, K314L/R315L, K332E, K333E, K332E/K333E, Q336A/Y337L, Y337L/Q338A, Q336A/Y337L/Q338A, KE/39I, KE/5I, WT5I) were generated from the pCMV-Munc18-1 WT (SNM)-EmGFP or pBluescript-Munc18-1 WT (SNM) construct using the QuickChange Site-Directed Mutagenesis kit (Strategene) and custom-designed primers. Each mutation was verified by sequencing.

2.4 Munc18-1 variant expression constructs

The lentivirus-mediated expression constructs of various Munc18-1 mutants were generated so that the encoded proteins can stably express in the Munc18-1/2 double knockdown cells. The parental expression plasmid was developed by modifying pLVX-IRES-puro. First, the puromycin-resistance gene was replaced by a blasticidin-resistance gene so that the infected cells could be selected with blasticidin. Second, cDNA sequence for EmGFP was subcloned into the BamHI site, generating pLVX-EmGFP-IRES-blast. To protect the mRNA transcripts transcribed from the Munc18-1 expression plasmid from being degraded by the anti-Munc18-1 RNA interference machineries already induced within the Munc18-1/-2 double knockdown cells, six silent nucleotide mutations (SNM) (GTCCGTGCACAGCCTGATC, underlines indicate SNM) were introduced into the target sequence in rat Munc18-1 gene. The resulting Munc18-1 (SNM) gene produced mRNA transcripts expressing Munc18-1 proteins that lacked the siRNA target sites.
The Munc18-1 (SNM) gene (WT or its indicated mutant) digested from pLCMV-Munc18-1 (SNM)-EmGFP-blast (Han et al., 2009) with EcoRI/XbaI was subcloned into the same site of the pLVX-EmGFP-IRES-blast plasmid.

2.5 Lentivirus-mediated expression of Munc18-1 variants in Munc18-1/2 Double Knockdown Cells

The Munc18-1 expression plasmid was cotransfected with psPAX2 and pMD.G into HEK-293FT cells to generate recombinant lentiviruses that express Munc18-1 WT or its variant fused with EmGFP. The Munc18-1/-2 double knockdown cells (D7 and/or D16 clone) were then infected with lentiviruses expressing rescue proteins. The stable cell line expressing the protein of interest was isolated through continuous selection with blasticidin (5 µg/ml).

2.6 Yeast Two-Hybrid Assays

Full-length WT Munc18-1 with SNM (see above) or indicated mutant Munc18-1 (SNM) was subcloned into the SmaI-PstI site of a bait vector, pLexN. A cytoplasmic domain (residues 1–264) of rat syntaxin-1A was subcloned into the EcoRI-BglII site of a prey vector, pVP16-3 (Okamoto and Südhof, 1997). Yeast strain L40 (Vojtek et al., 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 d 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 d.

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 [DTT], 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 15min 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, pH7.0). 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 0-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 7min], where OD₄₂₀ is the optical density of the product 0-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 0-nitrophenol. The unit of β-galactosidase–specific activity is therefore expressed as nano-moles per minute per milligram of protein.
2.7 Isothermal Titration Calorimetry (ITC)

Recombinant proteins Syntaxin1a2-243 and Munc18-1-WT or mutants used for the ITC experiments were prepared as previously described (Deák et al., 2009). All proteins were further purified by gel filtration chromatography using 20 mM sodium phosphate buffer (pH 7.5), 150 mM NaCl and 1 mM DTT (ITC buffer). Isothermal titration calorimetry was carried out at 298 K using a MicroCal iTC200 (GE Healthcare, United Kingdom), with 16 x 2.4 µl injections of 70 µM Syntaxin1a2-243 into 7 µM Munc18-1-WT or mutants. Integration of the titration curves was performed using the ORIGIN software (OriginLab, Northampton) to extract thermodynamic parameters, stoichiometry \( N \), equilibrium association constant \( K_a = K_d^{-1} \) and the binding enthalpy \( \Delta H \). The Gibbs free energy of binding \( \Delta G \) was calculated from the relation \( \Delta G = -RT \ln(K_a) \) and the binding entropy \( \Delta S \) was deduced from the equation \( \Delta G = \Delta H - T \Delta S \). Experiments were performed with protein concentrations well within the recommended range for the c-value (concentration of protein in cell/ \( K_d \sim 200 \)). Binding parameters were calculated as the average of at least three independent experiments ± standard deviation (SD).

2.8 Cell Preparation for Confocal Immunofluorescence Microscopy

Sterilized circular glass cover slips (0.25 mm in width, 1.8 cm in diameter) were placed in 2.2-cm wells within 12-well cell culture plates. The cover slips were then coated for 1 h with poly-d-lysine (0.1 mg/ml) at room temperature. Cells were allowed to adhere to the cover slips overnight and then differentiated on the cover slips for 3–4 d in DMEM that contained 100 ng/ml nerve growth factor (NGF) (Sigma Chemical), 1% horse serum, 1% calf serum, and penicillin/streptomycin. The cells were washed with phosphate-buffered saline (PBS), 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 times wash with PBS. Non specific sites were blocked for 1 h at room temperature in PBS containing 0.3% BSA. Primary antibodies against syntaxin-1 (HPC-1 diluted 1:1000) were applied to the cell for 1 hr. After three washes in blocking buffer, rhodamine red-x-conjugated anti-mouse antibodies (diluted 1:1000) (Jackson ImmunoResearch Laboratories, West Grove, PA) were applied for 1 h. Samples were washed again three times in blocking buffer and mounted in Fluoromount-G reagent (Southern Biotechnology, Birmingham, AL). Immunofluorescence staining was recorded with a laser confocal scanning microscope (LSM510; CarlZeiss, Jena, Germany) with an oil immersion objective lens (63x).

2.9 Electron Microscopy and Analysis of Docking of Dense-core Vesicles

The initial fixation was performed within the 10-cm dishes for 1 h using a 3.2% glutaraldehyde, and 2.5% paraformaldehyde fixative mixture (Karnovsky’s Fixative) in 0.1 M cacodylate buffer (pH adjusted to 7.6). Cells were then pelleted in microcentrifuge tubes and fixed overnight with new fixative. The following day, the pellets were fixed in 1 mg/ml (1%) osmium tetroxide for 1 h and En bloc staining was then performed by incubating with 1% uranyl acetate for 1 h in dark conditions. Washed pellets were incubated successively in increasing concentrations of ethanol for dehydration and then infiltrated overnight with Spurr’s resin (23.6g NSA, 16.4g ERL-4221, 5.72g DER-736, and 0.4g DMAE). After transferring the cell pellets to Beem capsules the capsules were incubated for 48 h at 65°C. The plasticized pellets were sliced to ultrathin 80-nm sections, which were then mounted on copper grids for subsequent staining and viewing.

Grids mounted with the ultrathin cell sections were first etched by exposing the grids to 3% uranyl acetate for 45 min at room temperature. Grids were then washed and stained with lead...
citrate for 20 min. Grids were washed and dried again before loading onto Hitachi H7000 transmission electron microscope for viewing. Electron micrographs were taken of individual Munc18-1/-2 double knockdown cells expressing EmGFP (negative control), wild-type Munc18-1-EmGFP (positive control) or KE/5I-EmGFP. These images were then used for analyzing the docking of dense-core vesicles in the control or the Munc18-1 variants expressing PC12 cells. Dense-core vesicles were identified within the single-cell electron micrographs as dark spots of radius between 60-120 nm. The distance of each vesicle from the plasma membrane was then calculated for each individual cell. The data from multiple single cell images (n = 46 - 48) within each control or Munc18-1 variants has been collated.

2.10  $[^3]H$ Noradrenaline (NA) Release Assays from PC12 cells

PC12 cells were plated in 24-well plates; 3–4d after plating, the cells were labelled with 0.5 µCi of $[^3]H$ NA in the presence of 0.5 mM of ascorbic acid for 12–16 h. The labelled 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 physiological saline solution (PSS) containing 145 mM NaCl, 5.6 mM KCl, 2.2 mM CaCl$_2$, 0.5 mM MgCl$_2$, 5.6 mM glucose, and 15 mM HEPES, pH 7.4, 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 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.

2.11  hPLAP Secretion Assay from PC12 cells

Munc18-1/-2 double knockdown PC12 cells that stably express wild-type Munc18-1 or Munc18-1 variants were transfected with 3 µg of a reporter plasmid, pCMV-neuropeptide Y
(NPY)-hPLAP by using electroporation (Arunachalam et al., 2008; Fujita et al., 2007; Li et al., 2007), when the cells were at 70-80% confluency in 10-cm dishes. After 48 h, the cells were harvested and replated in 24-well plates. 6 or 7 d after electroporation, the plated cells were washed once with PSS. Then NPY hPLAP secretion was stimulated with 200 µl of PSS or highK\(^+-\)PSS. Secretion was terminated after a 25-min 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 solubilised in 200 µl of PSS containing 0.1% TritonX-100. The amounts of NPY-hPLAP secreted into the medium and retained in the cell were measured by the Phospha-Light Reporter Gene Assay System (Applied Biosystems, Foster City, CA). The samples are treated at 65°C for 30 min to inactivate non placental alkaline phosphatases and assayed an aliquot (10 µl) for placental alkaline phosphatase activity with the kit. The heat-stable (65°C) alkaline phosphatase activity of hPLAP permits easy quantification of both the levels of NPY-hPLAP secreted from the cells and those retained in the cells by using a quantitative secretory alkaline phosphatase kit. The total volume of the assay was 120 µl. After 5–10 min, chemiluminescence was quantified by FB12 luminometer (Berthold Detection Systems, Zylux Corporation, Oak Ridge, TN).

2.12 Lentivirus-mediated Expression of syntaxin-1A and Munc18-1 variants in Munc18-1/-2 Double Knockdown Cells

Munc18-1/-2 double-knockdown cells (D7, D16) were infected with lentivirus containing syntaxin-1A expression plasmid in pLVX-IRES-blast. Infected cells were selected by blasticidin to establish stable syntaxin-1A overexpressed D7 or D16 clones. Expression of syntaxin-1A was confirmed by immunoblotting. Syntaxin-1A overexpressed DKD cells were further infected by the lentivirus expressing Munc18-1 K46E/E59K-EmGFP, KE/39I-EmGFP or EmGFP alone from pLVX-IRES-hygro plasmids followed by hygromycin B selection.
2.13 Binding between Munc18-1 Variants and Monomeric syntaxin-1A

pGex-KG-rat syntaxin1A (1 – 264) was transformed into BL21 (DE3) cells and the bacteria were grown at 37°C until confluent. Recombinant protein expression was induced by adding 50 μM isopropylthio-β-D-galactoside (IPTG) at 30°C for 3 hrs. Cells were lysed using sonications in PBS containing 0.1% Triton X-100, 1 mM EDTA and protease inhibitors (10 µg/ml leupeptin and 10 µg/ml aprotinin). Supernatant portion was 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 Munc18-1-EmGFP variants. EmGFP, wild-type Munc18-1-EmGFP, K46E-EmGFP, K46E/E59K-EmGFP, K332E/K333E-EmGFP and KE/5I-EmGFP were transfected into HEK-293FT cells. After 3 days, cells were lysed with KGlu buffer (20 mM HEPES, pH 7.2, 120 mM KGlu, 20 mM potassium acetate, 2 mM EGTA) containing 0.1% Triton X-100 as a detergent. Portions were subjected to SDS-PAGE followed by western blot using anti-GFP antibody to confirm comparable expressions. After centrifugation, KGlu buffer plus detergent containing solubilized lysates were mixed with GST-syntaxin-1A overnight. Next day, mixtures were washed extensively with KGlu buffer containing 0.1% Triton X-100. Samples were then dried, and 2X SDS-PAGE sample buffer was added and subjected to SDS-PAGE followed by Coomassie Blue staining.

2.14 Binding between Munc18-1 Variants and the SNARE Complex

The expression plasmid called pET-strep was generated as follows. cDNA encoding GST and the following polylinker region of pGex-KG (Guan and Dixon, 1991) was amplified by PCR. The resulting PCR product (digested with NdeI and XhoI) was ligated to the pET-21a (Novagen) that was digested with NdeI and Sall, generating pET-GST plasmid. Then a NdeI-BamHI
fragment encoding GST was replaced with the annealed oligos that encode strep-tag (WSHPQFEK) following the initial methionine, generating the pET-strep plasmid. cDNA encoding full-length human SNAP-25B was subcloned into the NcoI-SalI site of pET-Strep. cDNAs encoding rat synaptobrevin-2 (1-94), rat syntaxin-1A (1-264) and full-length rat Munc18-1 variants were subcloned into the pGex-KG expression vector. The fusion proteins were expressed and purified as follows: A 50-mL preculture of BL21 (DE3) cells transformed with plasmids encoding recombinant proteins were grown overnight at 37°C in LB medium containing ampicillin. After overnight growth, these cultures were used to seed 500 mL of LB medium containing ampicillin. The cells were further grown for 3h at 37°C then were induced with 100 µM IPTG at 30°C for 3h. After centrifugation, the cell pellets were resuspended in PBS containing 0.25mM EDTA, PMSF, 10 µg/ml leupeptin and 10 µg/ml aprotinin. The cells were lysed by sonication. Cell debris was removed by centrifugation in a JA-20 rotor for 30 min at 15,000 rpm. To these lysates, 0.5mL of strep-tactin sepharose (IBA, GmbH, Germany) or glutathione-agarose (Pierce) were added and were rotated overnight at 4°C. Recombinant proteins immobilized onto the strep-tactin sepharose or glutathione agarose were washed with PBS containing additional 0.15M NaCl followed by final PBS wash. The GST-fused recombinant proteins were removed from the GST moiety by thrombin cleavage for 2-4h at room temperature. After centrifugation, the cleaved proteins were eluted in PBS containing PMSF.

The SNARE complex was produced by combining strep-SNAP-25 on immobilized on the strep-tactin sepharose and soluble cytosolic syntaxin-1A and synaptobrevin-2 proteins in a 1:1:2 molar ratio and incubating overnight at room temperature in PBS containing PMSF, protease inhibitors (10 µg/ml leupeptin and 10 µg/ml aprotinin). The complex was isolated on the strep-tactin sepharose via the strep-SNAP-25.
Binding experiments were performed by incubating molar excess of cleaved Munc18-1 variants or BSA (negative control) with SNARE complex immobilized on strep-tactin sepharose in PBS containing 0.1% Triton X-100, PMSF, and protease inhibitors for 2-4h at room temperature or overnight at 4°C. After extensive washing with PBS containing 0.1% Triton X-100, proteins bound to beads were analyzed by SDS-PAGE using standard procedures. The proteins were visualized by Coomassie Blue staining or Ponceau S staining. Immunoblot blot analysis was performed to detect Munc18-1 using anti-Munc18-1 antibody.

2.15 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 or one-way analysis of variance (ANOVA) followed by post hoc analysis, as indicated in the text or the figure legend. Statistical significance was determined at the level of p<0.01.
Chapter 3: Munc18-1 domain-1 controls vesicle docking and secretion by chaperoning syntaxin-1 to the plasma membrane through high affinity binary interaction

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3.1 Rationale of the study

Multiple lines of evidence support the pleiotropic roles of Munc18-1 in neurosecretion although the underlying mechanisms still remain highly debated due to inconsistent evidence. One of the most puzzling conclusions from the previous studies include the idea that similar or identical residues in the domain-1 cleft of Munc18-1 play critical roles in both the chaperone and priming functions of Munc18-1. Specifically, three different mutations (D34N/M38V, E59K and E66A) within the domain-1 cleft have been proposed to represent “priming” mutants. These mutants are believed to specifically impair their ability to promote vesicle priming/exocytosis. The D34N/M38V mutant has been shown to impair binary binding to syntaxin-1 and disrupt the docking of dense-core vesicles. Importantly, this mutant was capable of restoring syntaxin-1
distribution to the plasma membrane, which suggested that the D34N/M38V mutant was specifically a priming mutant (Gulyás-Kovács et al., 2007). It was, however, unclear how a mutant that loses its binary binding to syntaxin-1 could still maintain its chaperone function that is known to mainly rely on this interaction (Gulyás-Kovács et al., 2007). Unlike D34N/M38V, the E59K mutation was shown to maintain the binary binding to syntaxin-1 to a significant extent, while abolishing the binding to the SNARE complex (Deák et al., 2009). In addition, the E66A mutant was also shown to have intact binary binding to syntaxin-1 but decreased binding to the SNARE complex, although not as severely impaired as that of E59K mutant. The differential effects of these mutations on SNARE complex binding were suggested to underlie their defects in synaptic vesicle priming and neurotransmitter release (Deák et al., 2009). However, syntaxin-1 chaperone activity of E59K or E66A mutants of Munc18-1 was never assessed. Therefore, it remained unclear whether the impaired secretory phenotype observed for E59K or E66A solely reflect their perturbed priming function or is a consequence of reduced level of properly localized syntaxin-1 in these mutants. In addition, a homologous mutation to E59K in Vps33, a yeast SM protein, was proposed to cause a defect in fusion pore opening (Pieren et al., 2010), although the role of Munc18-1 in the opening of the fusion pore is highly controversial (Voets et al., 2001).

In an attempt to clarify the specific function of the domain-1 cleft of Munc18-1 in neurosecretion and to further characterize the phenotypes of previously proposed priming mutants, we have investigated various domain-1 cleft mutants (K46E, E59K, K46E/E59K, K63E, E66A, D34N, D34N/M38V) in detail (Figure 3.1) by employing a combination of biochemical and physiological analyses.

The significance of the alleged residues within domain-1 is highlighted by the specific contacts that they make with syntaxin-1 according to the previous crystal structure analysis (Misura et al., 2000). Lys46 of Munc18-1 specifically contacts residues Asp231 and Arg232 in
the H3 helix of syntaxin-1 and Glu59 of Munc18-1 forms a buried salt-bridge with Arg114 within the H3 domain of syntaxin-1 when in the closed conformation (Figure 3.1). Lys63 of Munc18-1 contacts SNARE motif of syntaxin-1 and Glu66 residue of Munc18-1 contacts both H_{abc} and SNARE motif of syntaxin-1 (Deák et al., 2009). Although Asp34 was suggested to form evolutionarily conserved hydrogen bond interactions with the syntaxin N-terminal H_{abc} domain and thus necessary for syntaxin-1 binding (Gulyás-Kovács et al., 2007), specific syntaxin-1 residues that interact with Asp34 of Munc18-1 are not observed in the crystal structure. It is important to note that Asp34 residue makes two intramolecular hydrogen bonds with backbone amide groups of Munc18-1 (Misura et al., 2000) (Figure 3.1). The residue Met38 forms a hydrophobic contact with C-terminal regions of the syntaxin-1 protein, specifically interacting with aliphatic regions of syntaxin-1 residues Glu234, Glu238, Val237 and Ile233 (Figure 3.1).
Figure 3.1 Structure of the Munc18-1/syntaxin-1 complex (Burkhardt et al., 2008) highlighting domain-1 cleft mutations studied in this study. Each domain of Munc18-1 is represented in different colours. Domain 1 is displayed in green, and the mutated residues are indicated in orange. Syntaxin-1 is represented in dark blue and purple (left). Enlarged structure within dash-lined box represented as ribbon (right, top) and surface (right, bottom) models. Key residues involved in the interaction between domain-1 cleft of Munc18-1 and syntaxin-1 are indicated in orange and blue, respectively.
Munc18-1 domain-1 also contains residues that have been implicated in N-terminal binding interactions. Residues F115 and E132 within the outer surface of hydrophobic pocket of Munc18-1 has been suggested to interact with N-peptide of syntaxin-1 (Burkhardt et al., 2008; Han et al., 2009; Hu et al., 2007; Malintan et al., 2009). Therefore, mutating these residues has been considered to disrupt the interaction between Munc18-1 and N-terminal peptide of syntaxin-1. However, the importance of these residues in this binding and the functional significance of these residues have remained controversial due to inconsistent evidence. In C. elegans, the F113R mutant in UNC-18 (corresponds to F115R mutation in Munc18-1) 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). In contrast, the mutations in the Munc18-1 hydrophobic pocket (F115E, E132A, F115E/E132A) that abolish N-terminal binding were shown to exhibit limited impact on dense-core vesicle exocytosis in Munc18-1 single (Malintan et al., 2009) and Munc18-1/-2 double knockdown (Han et al., 2009) PC12 cells. This discrepancy could reflect different mechanisms underlying synaptic vesicle secretion and dense-core vesicle secretion but also could be due to the nature of mutations as mutating F115 residue into arginine may exhibit more detrimental phenotype compared to when this residue is mutated into glutamic acid. Therefore, we re-evaluated the functional importance of this residue upon mutating to arginine residue (F115R). Furthermore, in previous studies, E132 was mutated to alanine which is neutral in charge. Therefore, we mutated this residue to the oppositely charged lysine to test whether this more dramatic change in charge results in more severe phenotype.
3.2 Results

3.2.1 Mutations in domain-1 cleft of Munc18-1 result in varying degree of reductions in the binding to syntaxin-1

Three mutants (D34N/M38V, E59K, E66A) in the domain-1 cleft of Munc18-1 have been proposed to selectively abrogate Munc18-1’s priming function with limited or no effects on its ability to transport syntaxin-1 to the plasma membrane (Deák et al., 2009; Gulyás-Kovács et al., 2007). Surprisingly however, these mutants have been shown to exhibit different characteristics in their ability to bind to syntaxin-1; the D34N/M38V mutant binds poorly to syntaxin-1 when measured by GST pull-down (Gulyás-Kovács et al., 2007) whereas the E59K and E66A mutants have been shown to have a limited (E59K) or no effect (E66A) on binding to syntaxin-1 as measured by isothermal titration calorimetry (ITC) (Deák et al., 2009). We therefore re-assessed the syntaxin-1 binding ability of these mutants together with other single or double domain-1 cleft mutants (D34N, K46E, K46E/E59K, K63E).

The binding between Munc18-1 mutants and the cytoplasmic domain of syntaxin-1 was examined by yeast two-hybrid assays. This assay is an unbiased method to examine protein-protein interactions in a more in vivo-like environment in which yeast cell is used as a living host that exhibits a greater resemblance to higher eukaryotic systems compared to bacterial systems (Han et al., 2009; Vojtek et al., 1993). To ensure that domain-1 mutants specifically lose their ability to bind to syntaxin-1, the interaction between Munc18-1 mutants and Mint-1 (Munc18-interacting protein) (Okamoto and Südhof, 1997) was also measured as a control. We found that single mutants of K46E or E59K significantly reduced binding to syntaxin-1 whereas a double mutation of K46E/E59K completely abolished the binding to syntaxin-1 (Figure 3.2 A). Despite reduced syntaxin-1 binding, all three mutants retained their ability to interact with Mint-1 (Figure
3.2 C). These results implied that K46E, E59K single mutants and K46E/E59K double mutant selectively lose their ability to bind to syntaxin-1. In contrast, the K63E and E66A single mutants retained their ability to bind to both syntaxin-1 and Mint-1 (Figure 3.2 B and D).

The D34N mutant exhibited slightly reduced binding to syntaxin-1 while D34N/M38V double mutations caused a dramatic reduction in binding to syntaxin-1 (Figure 3.2 A), which is consistent with the previous GST-pulldown experiments (Gulyás-Kovács et al., 2007). Unexpectedly however, the D34N/M38V mutant also abolished its binding to Mint-1 (Figure 3.2 C), suggesting that the D34N/M38V double mutant appears to generally lose its interactive ability not only towards syntaxin-1 but also towards other interactors as well. This inferred that the D34N/M38V mutation may affect the folding or expression level of Munc18-1. Therefore, we initially focused our efforts to re-evaluate the E59K mutant, together with K46E and K46E/E59K mutants.
Figure 3.2 Mutations in domain-1 of Munc18-1 result in reductions in the binding to syntaxin-1. The binding between these Munc18-1 mutants: D34N, D34N/M38V, K46E, E59K, K46E/E59K and (A) syntaxin-1A or (C) Mint-1 was analyzed by yeast two-hybrid assays. The binding between Munc18-1 mutants: K63E, E66A and (B) syntaxin-1A or (D) Mint-1 was also analyzed by yeast two-hybrid assays. 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 Munc18-1 was set to 100%. Error bars indicate SEM (n = 11-12 for binding to syntaxin-1; n = 11-12 for binding to Mint-1).
3.2.2 Lys-46 and Glu-59 are critical for the binary interaction between Munc18-1 and syntaxin-1

Previous ITC experiments have reported that the E59K mutation reduces binding affinity to syntaxin-1, although this effect was not statistically significant. The thermodynamic parameters were also shown to be significantly altered, with a much reduced enthalpic contribution to the association compared to the wild-type (Deák et al., 2009). Nonetheless, it has been suggested that the ability of the E59K mutant to interact with monomeric syntaxin-1 is largely retained. In contrast, our yeast two-hybrid data revealed that the E59K mutant significantly reduces binding to syntaxin-1 (Figure 3.2 A). Therefore, to further characterize the interaction between Munc18-1 domain-1 mutants and syntaxin-1, we performed our own ITC experiments to measure the affinity between a cytoplasmic domain of syntaxin-1A and domain-1 cleft mutants: K46E, E59K, and K46E/E59K (Figure 3.3, Table 1). The raw ITC data for binding of Munc18-1 variants to syntaxin-1A is presented in Figure 3.3 A. The raw signal represents the power (µcal/sec) applied to the control heater that is required to keep the calorimeter cell from changing temperature so the constant temperature is maintained between the reference and sample cells (Pierce et al., 1999). This means, if more heat is released upon higher affinity binding reaction, less power is applied to maintain the temperature of the calorimeter cell constant. For example, for binding between K46E/E59K mutant and syntaxin-1, there is no heat produced from the reaction due to abolished binding. Consequently, more power needs to be applied to the control heater to maintain the temperature. In contrast, interaction between wild-type Munc18-1 and syntaxin-1 exhibits the highest affinity thus produces the most heat from the reaction among the mutants tested. This means that there is no requirement for power compensation. Accordingly, binding of K46E or E59K single mutants to syntaxin-1 resulted in intermediate power supply. Integrated ITC data representing the heat produced for 1:1 complex formation between the syntaxin-1 and the
Munc18-1 variants was demonstrated in Figure 3.3 B. Each data point represents the amount of heat produced as an aliquot of titrant, syntaxin-1A, is added to the reaction (Pierce et al., 1999). The initial amount of heat released is larger than the heat released for the subsequent additions since there is a large excess of empty or unpopulated binding sites at the beginning of the titration which allows complete binding of the added ligand. As the titration proceeds, binding reaction between Munc18-1 and syntaxin-1 saturates, which results in less heat production as shown by the line graphs reaching plateau as amount syntaxin-1 in reaction increases (Figure 3.3 B).

In brief, our data from ITC experiments were consistent with our yeast two-hybrid data. Table 1 summarizes the thermodynamics of reaction. We found significant reductions in the binding enthalpy ($\Delta H$) for both K46E and E59K single mutants (-19.0 ± 1.98 kCal/mol, n =3, for the wild type (WT) vs. -13.7 ± 0.21 kCal/mol, n = 3, for K46E and -11.5 ± 1.17 kCal/mol, n = 4, for E59K; p<0.01 for both mutants (Figure 3.3 C, Table 1). The dissociation constants ($K_d$) of K46E and E59K mutants binding to syntaxin-1 were significantly increased compared to that of the corresponding wild-type (181.2 ± 46.4 nM for K46E:Syx1a$_{2-243}$ and 83.9 ± 34.3 nM for E59K:Syx1a$_{2-243}$ vs. 23.3 ± 11.1 nM for WT:Syx1a$_{2-243}$; p<0.01 for K46E, p<0.05 for E59K), which indicates reduced binding affinities of the single mutants (Table 1). When these two residues were mutated simultaneously (K46E/E59K), no binding was observed under the sample conditions, further confirming the data from the yeast two-hybrid assay (Figure 3.2, Figure 3.3, Table 1). These results indicate that the single point mutations in the domain-1 cleft of Munc18-1 (K46E and E59K) significantly affect the thermodynamics and affinity of association, while the combined mutation K46E/E59K essentially abolishes the association. The consistency between the results from ITC experiments (Figure 3.3, Table 1) and the yeast two-hybrid assay (Figure 3.2) validates that these mutations reduce the binding affinity to monomeric syntaxin-1 in order of WT > E59K ~ K46E >> K46E/E59K.
Figure 3.3 ITC analysis of the binding of Sx1a_{2-243} to Munc18-1. (A) Representative raw ITC data for binding of Munc18-1 WT, K46E, E59K and K46E/E59K variants to Sx1a_{2-243}. (B) Integrated and normalised ITC data for the same experiments as shown in (A). (C) The thermodynamic parameters of Munc18-1 binding (Table 1) are plotted to highlight the changes in binding enthalpy of the different Munc18-1 mutants. (D) Coomassie stained SDS polyacrylamide gel of the proteins used in ITC experiments. This experiment was performed by Drs. Malintan, Meunier and Collin from University of Queensland, Australia.

<table>
<thead>
<tr>
<th>Munc18 protein</th>
<th>ΔH (kcal mol(^{-1}))</th>
<th>-TΔS (kcal mol(^{-1}))</th>
<th>ΔG (kcal mol(^{-1}))</th>
<th>K(_d) (nM)</th>
<th>N</th>
</tr>
</thead>
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<tr>
<td>WT</td>
<td>-19.0±1.98</td>
<td>8.6±1.73</td>
<td>-10.5±0.25</td>
<td>23.3±11.1</td>
<td>1.0±0.01</td>
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<tr>
<td>K46E</td>
<td>-13.7±0.21**</td>
<td>4.5±0.11**</td>
<td>-9.2±0.11**</td>
<td>181.2±46.4**</td>
<td>1.0±0.06</td>
</tr>
<tr>
<td>E59K</td>
<td>-11.5±1.17**</td>
<td>1.8±0.94**</td>
<td>-9.7±0.26**</td>
<td>83.9±34.3*</td>
<td>1.1±0.10</td>
</tr>
<tr>
<td>K46E/E59K</td>
<td>No binding signal</td>
<td>1.0±0.01</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Values are expressed as means ± SD (n=3-4). All experiments were performed at 298K. Significant at *p<0.05, **p<0.01.

Table 1. Binding parameters of Munc18-1 interaction with Syntaxin-1A_{2-243}. The content of the table was generated by Drs. Malintan, Meunier and Collins from University of Queensland, Australia.
3.2.3 **Munc18-1** K46E, E59K, and K46E/E59K mutants restore cellular level of syntaxin-1 to various extents, which correlate with their ability to bind syntaxin-1

In Munc18-1/-2 double knockdown PC12 cells, the cellular level of syntaxin-1 is significantly reduced (Han et al., 2009). Nonetheless, reduced syntaxin-1 level can be restored upon re-expression of exogenous wild-type Munc18-1. We examined the abilities of K46E, E59K and K46E/E59K mutants to rescue syntaxin-1 expression and localization in the Munc18-1/-2 double knockdown cells. The domain-1 mutants were fused with Emerald GFP (Munc18-1-EmGFP) and stably expressed in the Munc18-1/-2 double knockdown PC12 cells (clones D7 and D16) using the lentivirus system (Han et al., 2009). The IRES-blasticidin resistance gene within the expression plasmid allowed the infected cells to be further selected using blasticidin (5 μg/ml).

In both D7 and D16 cells, Munc18-1 K46E, E59K and K46E/E59K mutants were expressed at a comparable level to the endogenous Munc18-1 in wild-type PC12 cells as verified by immunoblot analysis. Moreover, these mutants were expressed at similar level to the exogenous wild-type Munc18-1 fused with EmGFP in double knockdown PC12 cells (Figure 3.4 A and B). Importantly, in both lines of double knockdown clones (D7 and D16), the exogenous expression of wild-type Munc18-1-EmGFP resulted in a dramatic recovery of syntaxin-1 expression (Figure 3.4 A and B). The level of syntaxin-1 recovery upon re-expression of wild-type Munc18-1–EmGFP was comparable to that of endogenous syntaxin-1 in wild-type PC12 cells. Upon re-expression of the single mutants, K46E or E59K, syntaxin-1 expression was rescued, although less efficiently than in wild-type Munc18-1-EmGFP expressing cells. In K46E/E59K double mutant expression cells, no detectable level of rescue in syntaxin-1 expression level was observed as it remained at a low level that is comparable to the control EmGFP vector alone expressing cells (Figure 3.4 A and B). As the expression levels of these mutant proteins were comparable to the wild-type Munc18-1-EmGFP protein, their reduced ability to rescue syntaxin-1 expression reflects their loss-
of-function phenotype rather than any defects that could be affected from decreased levels of the proteins. This strongly advocates for a role of Munc18-1 and syntaxin-1 interaction in stabilizing the expression of syntaxin-1.

The rescue of syntaxin-1 expression by Munc18-1 mutants was observed to be in the order of WT > E59K ~ K46E >> K46E/E59K (Figure 3.4 A and B). This seemed to coincide with the order of their binding affinity to syntaxin-1 (Figure 3.2 and 3.3). Therefore, we examined the correlation between the binding affinity of Munc18-1 mutants to syntaxin-1 and their ability to restore syntaxin-1 expression. We used equilibrium association constant $K_a (= K_d^{-1})$ as an index of binding affinity and the value of each mutant was normalized to that of WT which was set to be 1. We quantified the recovery of syntaxin-1 expression from the chemiluminescence signal for the respective Munc18-1-EmGFP protein using Image J and normalized (set to 1 and 0 for syntaxin-1 expression in Munc18-1/-2 DKD PC12 cells expressing Munc18-1-WT-EmGFP and EmGFP respectively). We found a striking correlation ($r^2 = 0.93, n = 8$) between these two parameters, which is highly significant ($p < 0.001$) (Figure 3.4 C). This result has indicated that the binding affinity of Munc18-1 mutants to syntaxin-1 and their ability to restore syntaxin-1 expression are positively correlated.
Figure 3.4 Stable re-expression of Munc18-1 variants in Munc18-1/-2 double knockdown PC12 cells (D7 and D16) results in different degrees of recovery of syntaxin-1 expression. (A and B) Munc18-1/-2 double knockdown clones (D7 and D16) were infected with lentiviruses that express EmGFP, wild-type Munc18-1-EmGFP, or Munc18-1 mutants (K46E, E59K, K46E/E59K), and the infected cells were selected with blasticidin. A number on the left indicates the position of a molecular weight marker. (C) The correlation graph demonstrates that the degree of the binary interaction between Munc18-1 variants and syntaxin-1 is positively correlated with the ability of the mutants to rescue syntaxin-1 expression.
3.2.4 Munc18-1 K46E, E59K, and K46E/E59K mutants restore plasma membrane localization of syntaxin-1 to various extents, which reflect their ability to bind syntaxin-1

In Munc18-1/-2 double knockdown PC12 cells, not only the cellular level of syntaxin-1 is significantly reduced but the proper localization of syntaxin-1 along the plasma membrane is also severely impaired (Han et al., 2009). Therefore, the ability of the Munc18 mutants to rescue the mislocalization of syntaxin-1 in Munc18-1/-2 double knockdown cells (D7 and D16) has been examined by using confocal immunofluorescence microscopy (Figure 3.5 and 3.6). The plasma membrane localization of syntaxin-1 was recovered upon re-expression of the wild-type Munc18-1-EmGFP as previously demonstrated (Han et al., 2009; Malintan et al., 2009). Upon expression of empty vector containing only EmGFP, syntaxin-1 remained accumulated in the perinuclear region of the cells (Figure 3.5 and 3.6). Expression of either Munc18-1 K46E or E59K single mutants showed a limited level of rescue of syntaxin-1 along the plasma membrane, compared to the wild-type Munc18-1-EmGFP expressing cells. In these cells expressing the single mutants, the perinuclear accumulation and the apparent plasma localization of syntaxin-1 co-existed although perinuclear accumulation of syntaxin-1 seemed to be more prominent (Figure 3.5 and 3.6). In the case of K46E/E59K-EmGFP double mutant expressing cells, localization of syntaxin-1 was limited to the perinuclear region of the cells indicating the impaired ability of the double mutant to restore proper syntaxin-1 localization (Figure 3.5 and 3.6).

These results suggested that the K46E/E59K mutant not only loses its ability to support expression but also its ability to traffic syntaxin-1 to the plasma membrane. The differences in the extent to which these Munc18-1 variants restore syntaxin-1 expression and localization reflect their ability to bind to syntaxin-1. The results thus far strongly support the idea that the degree of interaction between Munc18-1 mutants and syntaxin-1 is positively correlated with their ability to
chaperone syntaxin-1 as measured by restored expression levels and plasma membrane localization of syntaxin-1 upon re-expression of Munc18-1 domain-1 mutants in Munc18-1/-2 DKD PC12 cells. This stresses the essential role of Munc18-1 domain-1 cleft in mediating high affinity syntaxin-1 binding and consequently in syntaxin-1 stabilization and trafficking.
Figure 3.5 Differential rescue of syntaxin-1 localization in Munc18-1/-2 double knockdown clone (D16) upon reintroduction of Munc18-1 variants. Confocal images of D16 cells infected with lentiviruses that express negative control EmGFP alone, WT Munc18-1-EmGFP, K46EmGFP, E59K-EmGFP and K46E/E59K-EmGFP (left). These cells were stained with anti-syntaxin-1 antibodies followed by rhodamine red-x-conjugated anti-mouse antibodies (middle). Right panels are merged pictures. Intensity and brightness were optimized for each picture to visualize localizations; thus, the resulting pictures do not contain quantitative information. Bar, 10 µm.
Figure 3.6 Differential rescue of syntaxin-1 localization in Munc18-1/-2 double knockdown clone (D7) upon reintroduction of Munc18-1 variants. Confocal images of D7 cells infected with lentiviruses that express negative control EmGFP alone, wild-type Munc18-1-EmGFP, K46E-EmGFP, E59K-EmGFP and K46E/E59K-EmGFP (left). These cells were stained with anti-syntaxin-1 antibodies followed by rhodamine red-x-conjugated anti-mouse antibodies (middle). Right panels are merged pictures. Intensity and brightness were optimized for each picture to visualize localizations; thus, the resulting pictures do not contain quantitative information. Bar, 10 µm.
3.2.5 The docking of dense-core vesicles correlates with Munc18-1 chaperone activity

The essential function of Munc18-1 in the docking of dense-core vesicles was first demonstrated by the electron microscopic analysis of Munc18-1-deficient adrenal chromaffin cells from Munc18-1 null mice in which the amount of docked vesicles were reduced by ~90% (Voets et al., 2001). Furthermore, in Munc18-1 knockdown PC12 cells, a significant decrease in the proportion of docked dense-core vesicles was observed (30 - 40% docking in the control PC12 cells vs. 15 - 20% docking in Munc18-1 single knockdown cells) (Arunachalam et al., 2008).

We examined the ability of Munc18-1 K46E, E59K and K46E/E59K mutants to restore dense-core vesicles docking in Munc18-1/-2 double knockdown D7 cells by using electron microscopy. Munc18-1/-2 double knockdown clones were rescued with either control protein (EmGFP), Munc18-1 wild-type or the indicated mutants as shown in representative examples of electron micrographs (Figure 3.7-3.11). Arrows within the electron micrograph indicate where the dense-core vesicles are located. The vesicles were defined as anatomically docked if they were located in close apposition (within 50 nm) to the plasma membrane (Arunachalam et al., 2008). Quantification of the dense-core vesicle distribution revealed a strong reduction in docked dense-core vesicles in Munc18-1/-2 double knockdown PC12 cells expressing empty-EmGFP vector (7.15 ± 1.06%) compared to that expressing wild-type Munc18-1-EmGFP (24.91 ± 2.32%) (Figure 3.12 A). Munc18-1/-2 double knockdown PC12 cells expressing either K46E or E59K showed an intermediate level of docked dense-core vesicles (13.45 ± 1.65% and 12.98 ± 1.73% respectively). The D7 cells expressing K46E/E59K double mutant showed no rescue activity for docking (8.30 ± 1.12%) as the proportion of docked vesicles was comparable to that of the negative control, EmGFP alone. The total number of dense-core vesicles was comparable in all Munc18-1 variants expressing cells (Figure 3.12 B), thus excluding the possibility that the differences in the percentage of docked vesicles are affected by the total number of vesicles. The
area (µm²) of the Munc18-1/-2 double knock-down PC12 cells expressing the Munc18-1 variants was also measured to confirm that the morphologies of the cells have not been significantly altered upon the introduction of the mutations (Figure 3.12 C).

The correlation between the syntaxin-1 chaperone function of Munc18-1 and the recovery of dense-core vesicle docking were assessed by plotting the ability of the Munc18-1 variants to restore dense-core vesicle docking against their ability to restore syntaxin-1 expression. The chaperone function of Munc18-1 should be evaluated by its effect on both the restoration of syntaxin-1 expression and the plasma membrane localization. However, the latter is difficult to accurately quantify. Therefore, the rescue of syntaxin-1 protein levels was used as an index to estimate the chaperone function of Munc18-1. We found a significant correlation ($r^2 = 0.90$) between the recovery of syntaxin-1 expression and that of dense-core vesicles docking defect in Munc18-1/-2 double knockdown PC12 cells upon re-expression of Munc18-1 domain-1 mutants (Figure 3.12 D). The results thus far clearly indicate that the efficiency in rescue of dense-core vesicles docking in cells expressing different Munc18-1 mutants positively reflect the abilities of each mutant to bind syntaxin-1 and to restore its expression level. This observation strongly supports the idea that the efficiency of dense-core vesicle docking is dependent on the intracellular expression and distribution of syntaxin-1 which is regulated by functional Munc18-1.
Figure 3.7 An example of an electron micrograph of Munc18-1/-2 double knockdown cells (D7) rescued with control protein, EmGFP. Dense-core vesicles are indicated by red arrows. Scale bar represents 500 nm.
Figure 3.8 An example of an electron micrograph of Munc18-1/-2 double knockdown cells (D7) rescued with WT Munc18-1-EmGFP. Dense-core vesicles are indicated by red arrows. Scale bar represents 500 nm.
Figure 3.9 An example of an electron micrograph of Munc18-1/-2 double knockdown cells (D7) rescued with K46E-EmGFP. Dense-core vesicles are indicated by red arrows. Scale bar represents 500 nm.
Figure 3.10 An example of an electron micrograph of Munc18-1/-2 double knockdown cells (D7) rescued with E59K-EmGFP. Dense-core vesicles are indicated by red arrows. Scale bar represents 500 nm.
Figure 3.11 An example of an electron micrograph of Munc18-1/-2 double knockdown cells (D7) rescued with K46E/E59K-EmGFP. Dense-core vesicles are indicated by red arrows. Scale bar represents 500 nm.
Figure 3.12 Electron microscopic analysis of dense-core vesicle distribution in the M unc18-1/-2 double knockdown cells (D7) expressing M unc18-1 variants. (A) The graph shows the mean percentage distribution of dense-core vesicles within individual PC12 cells, calculated from multiple single-cell electron micrographs. Dense-core vesicles were classed as being located within 50, 50-100, 100-200, 200-500, or 500-1000 nm or farther than 1000 nm from the plasma membrane. (B) The graph demonstrates mean number of dense-core vesicles present in each single-cell electron micrograph from the D7 clones expressing EmGFP alone or Munc18-1 variants. (C) The graph represents the mean area of each cell from D7 clones expressing EmGFP alone or Munc18-1 variants. Error bar, SEM (n = 37 - 40). (D) The correlation graph demonstrates the positive correlation between the ability of the mutants to rescue syntaxin-1 expression and to restore dense-core vesicles docking efficiency.
3.2.6 The degree of noradrenaline-secretion rescue correlates with Munc18-1 chaperone activity

The abilities of domain-1 mutants to bind syntaxin-1, to rescue syntaxin-1 expression and localization and to restore the dense-core vesicle docking were found to be positively correlated. Therefore, we further examined the role of these domain-1 residues in neurosecretion. We tested the ability of Munc18-1 K46E, E59K and K46E/E59K mutants to restore regulated secretion in Munc18-1/-2 double knock-down cells. Preloaded $[^3]H$-noradrenaline ($[^3]H$ NA) release was stimulated by 70 mM KCl for 15 min and a robust recovery of secretion was observed in Munc18-1/-2 double knock-down PC12 cells (D7 and D16) upon re-expression of wild-type Munc18-1-EmGFP. In contrast, the ability of K46E/E59K-EmGFP to rescue secretion was severely impaired, almost to the level of control EmGFP alone (Figure 3.13). Interestingly, Munc18-1 K46E-EmGFP and E59K-EmGFP single mutants exhibited an intermediate level of secretion rescue that was significantly different from both wild-type Munc18-1-EmGFP and EmGFP alone. These single mutants failed to rescue secretion as efficiently as that of wild-type Munc18-1-EmGFP but still were capable of restoring the secretion defect much more efficiently compared to that of the double mutant (K46E/E59K) (Figure 3.13). The ability of these Munc18-1 mutants to restore secretion was in the order of WT $>$ K46E $\sim$ E59K $>>$ K46E/E59K, which again reflects the abilities of these mutants to bind syntaxin-1 and to restore its expression, suggesting a strong positive correlations among these variables. More detailed correlation analyses of the secretory ability and syntaxin-1 chaperone activity of the K46E, E59K and K46E/E59K mutants, together with other domain-1 cleft mutants (K63E, E66A, D34N, and D34N/M38V) is illustrated in Figure 3.19.
Figure 3.13 Noradrenaline secretion defects are rescued to different degrees upon reintroduction of Munc18-1 variants (K46E, E59K, K46E/E59K) in Munc18-1/-2 double knockdown cells. NA release was stimulated by 70 mM KCl for 15 min in the rescued Munc18-1/-2 double knockdown cells (A) D7 clones and (B) D16 clones. Error bars indicate SEM (n = 12 for both D7 and D16).
3.2.7 Munc18-1 domain-1 cleft mutants (K46E, E59K, and K46E/E59K) rescue peptide-secretion defect in Munc18-1/-2 double knockdown PC12 cells to different extent

Although previous studies have suggested that the E59K mutant impairs the priming function (Deák et al., 2009), our data point to a partial effect of the E59K mutation in syntaxin-1 chaperone activity. The K46E and E59K single mutants display very similar phenotypes: both of them partially lose their functions in syntaxin-1 binding, chaperoning, dense-core vesicle docking and noradrenaline secretion. However, it was recently suggested that Vps33, a yeast SM protein, promotes fusion pore opening and this function is abolished upon introducing a homologous mutation to Munc18-1 E59K in Vps33 (Pieren et al., 2010). We therefore examined whether there was any difference between K46E and E59K in their ability to rescue peptidergic secretion, as peptide secretion is suggested to require a more complete opening of the fusion pore (Barg et al., 2002). We transfected the double knockdown clones (D7 and D16) engineered to stably express Munc18-1 mutants (WT, K46E, E59K, or K46E/E59K), with a plasmid that allows the expression of neuropeptide Y (NPY) fused with a soluble domain (residues 18-506) of human placental alkaline phosphatase (NPY-hPLAP) and measured the Ca$^{2+}$-dependent secretion of this transfected peptide (Figure 3.14) (Fujita et al., 2007; Han et al., 2009). We have found that the secretory phenotypes of the peptide in these mutants are remarkably similar to that observed with noradrenaline release. Both E59K and K46E mutants showed reduced ability to secrete the transfected peptide compared to the wild-type while the K46E/E59K mutant almost abolished its ability to rescue peptide secretion (Figure 3.14). The finding that the E59K does not exhibit a different phenotype from the K46E in rescuing peptide secretion suggests that the E59K mutant does not exhibit additional selective defects in secretion that requires a more complete opening of the fusion pore. Therefore, reduced secretion rescue ability of the E59K mutant seems to reflect its reduced syntaxin-1 chaperone activity that is caused by its reduced binding to syntaxin-1.
Figure 3.14 Peptidergic secretion defects are rescued to different degrees upon reintroduction of Munc18-1 variants (K46E, E59K, K46E/E59K) in Munc18-1/-2 double knockdown cells. Defects in peptide secretion in Munc18-1/-2 double knockdown clones (A) D7 and (B) D16 were rescued to different extent upon reintroduction of Munc18-1 variants (K46E, E59K, K46E/E59K). Secretion of transfected NPY-hPLAP from the double knockdown clones expressing Munc18-1 variants was stimulated by 70mM KCl for 25 min. Error bar indicates SEM (n = 11 for D7; n = 12 for D16).
3.2.8 *Munc18-1 E66A and K63E mutants restore syntaxin-1 level, localization and dense-core vesicle secretion as efficiently as the wild-type in Munc18-1/-2 double-knockdown cell*

Munc18-1 K63E and E66A single mutants were previously shown to retain strong binding to syntaxin-1 while the E59K mutant showed a limited reduction in its binding to syntaxin-1 (Deák et al., 2009). In agreement with the previous report, the results from our yeast-two-hybrid assay suggest that K63E and E66A mutants strongly retain the ability to bind to syntaxin-1 (Figure 3.2). However, the abilities of these mutants to stabilize and properly transport syntaxin-1 were never addressed previously. Therefore, we further examined the ability of the K63E and E66A mutants to restore syntaxin-1 expression level and localization. Both of these domain-1 cleft mutants were capable of restoring syntaxin-1 expression and localization as efficiently as that of wild-type Munc18-1 (Figure 3.15 A and C). This intact ability of the mutants to rescue syntaxin-1 chaperone activity seems to reflect strong binary interaction between the domain-1 mutants and syntaxin-1. Moreover, we observed that both K63E and E66A mutants of Munc18-1 were capable of restoring secretion defect in Munc18-1/-2 double knockdown cells as prominently as that of wild-type Munc18-1 (Figure 3.15 B). Taken together, these results strongly support the idea that the ability of Munc18-1 variants to retain the binary interaction with syntaxin-1 contributes to syntaxin-1 stabilization, trafficking, and consequently in dense-core vesicle secretion.
Figure 3.15 Syntaxin-1 expression level, syntaxin-1 localization and dense-core vesicle secretion defect are restored upon stable re-expression of Munc18-1 K63E or E66A mutants in Munc18-1/-2 double knockdown cells (D7). (A) Munc18-1/-2 double knockdown clones (D7) were infected with lentiviruses that express EmGFP, wild-type Munc18-1-EmGFP, or Munc18-1 mutants (K63E, E66A), and the infected cells were selected with blasticidin. A number on the left indicates the position of a molecular weight marker. (B) NA release was stimulated by 70mM KCl for 15 min in the rescued cells (D7 clones). Error bars indicate SEM (n = 9). (C) Confocal images of D7 cells infected with lentiviruses that express WT Munc18-1-EmGFP, K63E-EmGFP and E66A-EmGFP (left). These cells were stained with anti-syntaxin-1 antibodies followed by rhodamine red-x-conjugated anti-mouse antibodies (middle). Right panels are merged pictures. Bar, 10 µm.
3.2.9 Abilities of Munc18-1 D34N and D34N/M38V mutants to restore syntaxin-1 
chaperone activity or dense-core vesicle secretion reflect their expression level

In our yeast two-hybrid assays, the D34N single mutant was observed to modestly affect 
the binding to both syntaxin-1 while the D34N/M38V mutant was shown to abolish binding to 
both syntaxin-1 (Figure 3.2). Interestingly, these two mutants consistently reduced or abolished 
binding to another binding partner, Mint-1. Based on this observation, we initially suspected a 
perturbed stability of the D34N/M38V mutant (and potentially a less severe folding defect for the 
D34N single mutant) may have led to an inefficient expression of D34N/M38V protein. To 
confirm this hypothesis, the D34N and D34N/M38V mutants were re-expressed in Munc18-1/-2 
double knockout PC12 cells (Figure 3.16 A and B). In both D7 and D16 cells, the expression 
level of the D34N/M38V mutant was unusually low compared to that of the other mutants in PC12 
cells, suggesting that there is a possibility that this mutation has affected the stability of this 
protein. Albeit to a lesser degree, the D34N mutant also showed a reduced expression level.
Munc18-1 D34N mutant fused with EmGFP expressed at a comparable level to the endogenous 
Munc18-1 but slightly reduced compared to the expression level of exogenous wild-type Munc18-1-EmGFP. Consistently, this D34N mutant was able to rescue syntaxin-1 expression to certain 
degree although not as efficiently as that of wild-type Munc18-1-EmGFP. Moreover, the 
D34N/M38V-EmGFP double mutant was incapable of restoring syntaxin-1 expression level which 
was similar to that of Munc18-1/-2 double knock-down cells alone or control cells expressing 
EmGFP alone (Figure 3.16 A and B). The ability of the D34N single mutant to restore syntaxin-1 
expression level can largely be explained by its reduced binding to syntaxin-1 which has stemmed 
from its reduced expression. Additionally, the inability of D34N/M38V double mutant to restore 
syntaxin-1 level likely stems from its lack of expression.
Consistent with the abilities of the mutants to bind to and restore syntaxin-1 expression level, re-expression of the D34N-EmGFP mutant in D7 or D16 cells rescued syntaxin-1 localization significantly but not as efficiently as that of the WT Munc18-1-EmGFP (Figure 3.17 and 3.18). Although the plasma membrane localization of syntaxin-1 was prominent, intracellular accumulation of syntaxin-1 was also evident. In the case of D34N/M38V-EmGFP expressing cells, EmGFP fluorescent signal of this mutant was barely detectable further confirming inadequate expression of this fusion protein. Consequently, syntaxin-1 remained accumulated in the perinuclear region of D7 or D16 cells expressing this double mutant (Figure 3.17 and 3.18).

To further characterize the functional significance of D34N and D34N/M38V mutants, we studied the effect of these mutants on secretion by performing $[^3]$H NA-release assay (Figure 3.16 C and D). The D34N-EmGFP mutant prominently rescued secretion defect of Munc18-1/-2 double knock-down cells compared to EmGFP control alone but not as efficiently as that of wild-type Munc18-1-EmGFP. This secretory rescue ability of the D34N mutant reflects its relatively strong ability to bind syntaxin-1 and to stabilize and transport syntaxin-1. This once again stresses the importance of the binary interaction between Munc18-1 and closed conformation of syntaxin-1 on syntaxin-1 trafficking which is crucial for neurosecretion. Furthermore, the D34N/M38V-EmGFP mutant exhibited limited ability to restore the secretion defect in double knock-down cells which is likely due to its impaired expression (Figure 3.16 C and D). Interestingly, we noticed that the D34N/M38V mutant shows a more efficient recovery of secretion in D16 cells compared to that in D7 cells. This may reflect higher expression of the D34N/M38V mutant in D16 cells which may have led to a slightly better recovery of syntaxin-1 level in D16 cells compared to D7 cells. The significant recovery of secretion even in presence of a low expression of D34N/M38V mutant in D16 cells suggests that the phenotype of this mutant cannot be well explained by its specific impairments in the priming mutant as previously suggested (Gulyás-Kovács et al., 2007).
Figure 3.16 Stable re-expression of other domain-1 cleft (D34N, D34N/M38V) in Munc18-1/-2 double knockdown PC12 cells (D7 and D16) and their effects on restoring noradrenaline secretion. (A and B) Munc18-1/-2 double knockdown clones (D7 and D16) were infected with lentiviruses that express EmGFP, wild-type Munc18-1-EmGFP, or Munc18-1 mutants (D34N, D34N/M38V), and the infected cells were selected with blasticidin. A number on the left indicates the position of a molecular weight marker. (C and D) NA release was stimulated by 70mM KCl for 15 min in the rescued cells (C for D7 clones; D for D16 clones). Error bars indicate SEM (n = 9 for both D7 and D16).
Figure 3.17 Syntaxin-1 localization in Munc18-1/-2 double knockdown clone (D16) upon reintroduction of D34N and D34N/M38V Munc18-1 mutants. Confocal images of D16 cells infected with lentiviruses that express wild-type Munc18-1-EmGFP, D34N-EmGFP, and D34N/M38V-EmGFP (left). These cells were stained with anti-syntaxin-1 antibodies followed by red-x-conjugated anti-mouse antibodies (middle). Right panels are merged pictures. Bar, 10 µm.
Figure 3.18 Syntaxin-1 localization in M unc18-1/-2 double knockdown clone (D7) upon reintroduction of D34N and D34N/M 38V M unc18-1 variants. Confocal images of D7D16 cells infected with lentiviruses that express wild-type Munc18-1-EmGFP, D34N-EmGFP, and D34N/M38V-EmGFP (left). These cells were stained with anti-syntaxin-1 antibodies followed by rhodamine red-x-conjugated anti-mouse antibodies (middle). Right panels are merged. Bar, 10 µm.
3.2.10 The ability of Munc18-1 domain-1 cleft mutants to rescue noradrenaline-secretion defect in Munc18-1/-2 double knockdown PC12 cells is positively correlated with their ability to restore syntaxin-1 chaperone activity.

Finally, the correlation between the ability of all the domain-1 cleft mutants to restore syntaxin-1 expression and their ability to restore regulated secretion was examined (Figure 3.19). The restored syntaxin-1 protein level was used as an index to estimate the chaperone function of Munc18-1 in this study. Regulated secretion was calculated by subtracting NA release in PSS from High K⁺-induced NA release and normalizing these values to that of the wild-type, using the data shown in Figure 3.13, 3.15, and 3.16. The regulated secretion observed in EmGFP-expressing D7 or D16 cells was subtracted from the regulated secretion of each mutant to more accurately estimate the restored regulated secretion (Y-axis in Figure 3.19). In both D7 and D16 cells, there was a strong positive correlation between the restored syntaxin-1 expression and the restored regulated secretion, and these correlations were highly significant (D7: $r^2 = 0.93$, $p < 0.001$, D16: $r^2 = 0.73$, $p < 0.05$). Our results strongly indicate that the secretion defects exhibited by these mutants can largely be explained by their reduced ability to stabilize and to traffic syntaxin-1.
Figure 3.19 The ability of Munc18-1 mutants to restore syntaxin-1 and that to restore regulated secretion are positively correlated. The correlation graph demonstrates the positive correlation between the ability of the domain-1 cleft mutants to restore syntaxin-1 expression level and that to rescue regulated NA secretion defect.
3.2.11 Domain-1 hydrophobic pocket mutants of Munc18-1 results in reduced syntaxin-1 chaperone activity and secretion rescue ability upon re-expression in Munc18-1/2 double knockdown PC12 cells.

The phenotypes of the new domain-1 hydrophobic pocket mutants generated, F115R, E132K, and F115R/E132K, were investigated. These mutations have previously shown to disrupt interaction between Munc18-1 and N-terminal peptide of syntaxin-1. Firstly, the ability of these mutants to interact with monomeric syntaxin-1 was assessed by yeast two-hybrid assay. These mutants effectively retained binding to monomeric syntaxin-1 (Figure 3.20 A) but reduced binding to Mint-1 (Figure 3.20 B). This may be due to the possibility that Mint-1 binds to the same site that involves N-terminal peptide interaction. Then the ability of these hydrophobic mutants to restore syntaxin-1 expression and localization were assessed upon re-expression in Munc18-1/-2 double knockdown PC12 cells (D16). It was found that the new hydrophobic pocket mutants (F115R, E132K and F115R/E132K) of Munc18-1 were highly effective in restoring the expression level of syntaxin-1 although it seemed to be less efficient compared to the cells expressing wild-type Munc18-1 (Figure 3.20 C). Likewise, the ability of these mutants to restore the plasma membrane localization of syntaxin-1 appeared to be not as efficient as that of the wild-type Munc18-1 as both perinuclear accumulation and plasma localization of syntaxin-1 co-existed (Figure 3.21). Finally, when the ability of these mutants to restore secretion defects in Munc18-1/2 double knockdown cells was examined, it was found that the ability of these mutants to restore secretion defect was reduced compared to the wild-type Munc18-1 although it was restored prominently compared to the negative control, EmGFP alone infected cells (Figure 3.20 D).

These results together suggested that the interaction between the N-peptide of syntaxin-1 and the hydrophobic pocket of Munc18-1 contributes to efficient trafficking of syntaxin-1 to the plasma membrane which is consequently important for vesicle secretion.
Figure 3.20 Mutations in the hydrophobic pocket of Munc18-1 show significant rescue ability of NA release in Munc18-1/2 double knockdown (D16) cells. Interaction between the mutations in the hydrophobic pocket of Munc18-1 and syntaxin-1A (A) or Mint-1 (B) was analyzed by yeast-two hybrid assays. In each assay, β-galactosidase activities of the yeast clones were quantified and normalized so that the activity of the clones transformed with the wild-type Munc18-1 was set to 100%. Error bars indicate SEM (n = 9). (C) Immunoblot analysis of Munc18-1/-2 double knockdown clone (D16) which re-express Emerald-GFP, wild-type Munc18-1-Emerald GFP or mutant (F115R, E132K, F115R/E132A) Munc18-1-Emerald GFPs. A number on the left indicates the position of a molecular weight marker. (D) NA release was stimulated by 70 mM KCl for 15 minutes in the rescued cells. Error bars indicate SEM (n = 12).
Figure 3.21 Rescue of syntaxin-1 localization upon reintroduction of the M unc18-1 hydrophobic mutants in M unc18-1/-2 double knockdown (D16) clones. Confocal images of D16 cells that were infected with lentiviruses that express EmGFP alone, WT Munc18-1-EmGFP, F115R-EmGFP, E132K-Emerald GFP and F115R/E132K-EmGFP (left panels) and stained with anti-syntaxin1 antibodies followed by rhodamine red-x-conjugated anti-mouse antibodies (middle panels). Right panels are merged pictures. Scale bar = 10 µm.
3.3 Conclusion

The results from this chapter revealed striking correlations between the abilities of the domain-1 cleft mutants (K46E, E59K, K46E/E59K, K63E, E66A, D34N, and D34N/M38V) to bind to and chaperone syntaxin-1 and their abilities to restore dense-core vesicle docking and secretion. These results demonstrate that the domain-1 cleft of Munc18-1 is essential for high affinity syntaxin-1 interaction which is consequently critical for its syntaxin-1 chaperoning, dense-core vesicle docking and secretion. Furthermore, the data suggests that the effect of previously proposed priming mutants (E59K, D34N/M38V) on exocytosis can be explained by their reduced ability to stabilize and traffic syntaxin-1 to the plasma membrane or limited expression of the protein (Han et al., 2011).

Furthermore, the analysis of the domain-1 hydrophobic mutants (F115E, E132K, and F115E/E132K), which has been designed to interrupt N-terminal interaction mode of Munc18-1, suggests that this interaction contributes to syntaxin-1 stabilization, trafficking, and dense-core vesicle secretion.
Chapter 4: Domain-3a of Munc18-1 plays a crucial role at the priming stage of exocytosis

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4.1 Rationale of the study

In Chapter 3, the residues lining the domain-1 cleft of Munc18-1 were demonstrated to serve an important role in syntaxin-1 stabilization trafficking which is consequently critical for dense-core vesicle docking and exocytosis (Han et al., 2011; Han et al., 2009). The K46E/E59K mutant has been identified as the key “chaperone mutant” that essentially loses its abilities to bind to the “closed” conformation of syntaxin-1 and consequently to restore syntaxin-1 expression, localization, dense-core vesicle docking and secretion in Munc18-1/2 double knockdown PC12 cells. However, syntaxin-1 chaperone function of Munc18-1 itself seems to be insufficient to explain the detrimental secretory phenotype observed in Munc18-1 deficient neurons. For instance, Munc18-1 knockout in neurons causes a strong (~50 - 75%), but not complete reduction in syntaxin-1A level (Verhage et al., 2000). Nonetheless, neurotransmitter release was completely abolished in these neurons. Also, in contrast to severely perturbed dense-core vesicle docking in Munc18-1/2 double knockdown PC12 cells (Han et al., 2011; Han et al., 2009), synaptic vesicle
docking has been reported to be unaffected in Munc18-1 deficient neurons despite its severe defect in neurotransmitter release (Verhage et al., 2000). This evidence suggests that Munc18-1 additionally plays an essential role in regulating secretion downstream of syntaxin-1 chaperoning and vesicle docking. Moreover, over-expression of SNAP-25 has been shown to rescue the docking phenotype in Munc18 deficient chromaffin cells. However, despite the restored docking of dense-core vesicles in these cells, the secretion defect was not rescued in the absence of Munc18-1 (de Wit et al., 2009). This strongly indicates a post-vesicle docking role of Munc18-1 in regulating exocytosis. However, the direct evidence for the function of Munc18-1 downstream of syntaxin-1 trafficking or vesicle docking has been limited largely due to difficulty in identifying specific mutants that selectively impairs the priming function of Munc18-1. Although previous studies have suggested that the E59K and D34N/M38V mutations within domain-1 cleft of Munc18-1 specifically impair its priming function (Deák et al., 2009; Gulyás-Kovács et al., 2007), our analysis of these previously alleged “priming mutants” suggested that their phenotypes can be explained by their reduced syntaxin-1 chaperone activity or limited expression of the mutant (Han et al., 2011). Therefore, the Munc18-1 mutant that selectively loses its function during the priming stage has been yet to be identified.

Previous crystal structure analysis has revealed that both domain-1 and domain-3a of Munc18-1 consist of highly conserved residues that make specific contact with syntaxin-1 (Misura et al., 2000). Nonetheless, the domain-3a has been highly understudied compared to the domain-1 of Munc18-1. Therefore, we have thoroughly examined the functional significance of the domain-3a of Munc18-1 in syntaxin-1 chaperoning, dense-core vesicle docking and exocytosis. During this process, we discovered mutants that selectively abolish the exocytotic function of Munc18-1 while preserving its syntaxin-1 chaperone activity and the ability to support dense-core vesicle docking. These mutants represent the long-sought-after “priming mutant” of Munc18-1.
4.2 Results

4.2.1 Point mutations within domain-3a of Munc18-1 do not interfere with its function in exocytosis

The syntaxin-1 chaperone function of Munc18-1 is primarily mediated by the tight binary interaction between the “closed” syntaxin-1 and Munc18-1 cleft that is formed by domain-1 and domain-3a (Misura et al., 2000). In an attempt to investigate the functional significance of a relatively understudied domain-3a of Munc18-1, highly conserved residues within domain-3a that make specific contacts with syntaxin-1 according to the crystal structure have been mutated. The domain-3a mutations that were initially generated include: E278K, K314L/R315L (Kauppi et al., 2002), K332E, K333E, and K332E/K333E (Figure 4.1). Meanwhile, another group has reported that transient over-expression of a dominant negative Y337L mutation in domain-3a inhibits secretion from PC12 cells (Boyd et al., 2008). Based on this study, additional mutants flanking this Y337 residue have been generated. This series of mutations include Q336A/Y337L, Y337L/Q338A, and Q336A/Y337L/Q338A (Figure 4.1). As the mutated domain-3a residues have previously been suggested to make specific contact with syntaxin-1, it was expected that mutating these residues would disrupt the interactive property of Munc18-1. However, surprisingly all of these domain-3a point mutants retained intact abilities to interact with syntaxin-1 (Figure 4.2 and 4.3). Furthermore, when these Munc18-1 mutants were expressed stably as Emerald-GFP (EmGFP) fusion proteins in our Munc18-1/2 double knockdown (DKD) PC12 cells (D7, D16) (Han et al., 2011; Han et al., 2009), all the mutants effectively restored syntaxin-1 expression and dense-core vesicle secretion (Figure 4.2 and 4.3). None of the point mutations within domain-3a showed impaired phenotypes compared to the wild-type, implying that the domain-3a is highly resistant to the introduction of the point mutations.
Figure 4.1 Sequence alignment and structure of syntaxin-1 bound Munc18-1 highlighting domain-3a mutations used in this study. (A) Sequence alignment of domain-3a residues of rat Munc18-1, 18-2 together with Drosophila Rop and C. elegans Unc-18. Residues that are conserved in at least three sequences are highlighted. Mutated residues are shown in red: E278K, K314L/R315L, K332E, K333E, K332E/K333E, Q336A/Y337L, Y337L/Q338A, and Q336A/Y337L/Q338A. Location of inserted 39 residues (KE/39I) is marked by inverted triangle. (B) Ribbon representative of Munc18-1 structure bound to syntaxin-1 (Misura et al 2000). Each domain of Munc18-1 is represented in a different colour. Domain-1, 2, 3a, and 3b are shown in purple, blue, green, and yellow, respectively. Syntaxin-1 is represented in red. (C) Enlarged ribbon representative of domain-3a highlighting the residues that have been mutated in the study.
Figure 4.2 Series of point mutations within the domain-3a of Munc18-1 retain their abilities to interact with syntaxin-1, to stabilize syntaxin-1 and to rescue dense-core vesicle secretion. (A) The binding between Munc18-1 domain-3a point mutants (E278K, K314L/R315L, K332E, and K333E) and syntaxin-1 was analyzed by yeast-two-hybrid assays. In this 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 Munc18-1 was set to 100%. Error bars indicate SEM (n=11-12). This experiment was performed by Soo-Young Ann Kang from the Sugita Lab. (B) Secretion defects are rescued upon reintroduction of the domain-3a mutants in Munc18-1/-2 double-knockdown cells (D7). Error bars indicate SEM (n=5). (C, D) Stable reexpression of Munc18-1 domain-3a point mutants in Munc18-1/-2 double-knockdown PC12 cells (D7) results in efficient recovery of syntaxin-1 expression. A number on the left indicates the position of a molecular marker.
Figure 4.3 Series of point mutations within the domain-3a of Munc18-1 retains their abilities to interact with syntaxin-1, to stabilize syntaxin-1 and to rescue dense-core vesicle secretion. (A) The binding between Munc18-1 domain-3a point mutants (K332E/K333E, Q336A/Y337L, Y337L/Q338A, and Q336A/Y337L/Q338A) and syntaxin-1 was analyzed by yeast-two-hybrid assays. In this 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 Munc18-1 was set to 100%. Error bars indicate SEM (n=11-12). This experiment was performed by Soo-Young Ann Kang from the Sugita Lab. (B) Secretion defects are rescued upon reintroduction of the domain-3a mutants in Munc18-1/-2 double-knockdown cells (D7). Error bars indicate SEM (n=8-9). (C) Stable re-expression of Munc18-1 domain-3a point mutants in Munc18-1/2 double-knockdown PC12 cells (D16) results in efficient recovery of syntaxin-1 expression. A number on the left indicates the position of a molecular marker.
4.2.2 Discovery of domain-3a insertion mutants that selectively lose the ability to restore secretion in Munc18-1/-2 double knockdown cells while retaining the abilities to restore syntaxin-1 level and localization

During the process of generating one of the domain-3a point mutations, K332E/K333E, using PCR-based site-directed mutagenesis, we unintentionally generated an insertion mutant. This mutant contains 39 extra residues (MPQYQDLSQMLEEMPQYQDLSQMLEEMPQYQDLSQMLEE) following the intended mutation of K332E/K333E (Figure 4.1 A). Despite the presence of the large insertion, this mutant, KE/39I, expressed well and efficiently restored syntaxin-1A levels upon stable re-expression in the Munc18-1/-2 DKD cells. The level of restored syntaxin-1 can be compared with EmGFP alone (negative control), WT-EmGFP (positive control) and E278K mutant (Figure 4.4 A and B). Note that our rescue data are consistent between D7 and D16 cells, and thus we have been used these two clones of DKD cells interchangeably in some studies. Furthermore, the KE/39I mutant was able to restore syntaxin-1A localization at the plasma membrane almost as effectively as the wild-type in our immunofluorescence microscopic analysis (Figure 4.5). An intact ability of this insertion mutant to effectively restore syntaxin-1 expression level and localization represents its intact syntaxin-1 chaperone activity. Nonetheless, this insertion mutant completely failed to restore the secretion defect (Figure 4.4 C and D). This was the first indication that there is a potential Munc18-1 mutant that specifically loses the secretory ability while retaining the abilities to stabilize and properly transport syntaxin-1 to the plasma membrane.

We further examined whether the KE/39I mutant exhibits any dominant-negative effect by nonspecifically interfering with the secretory processes due to the presence of a large insertion. To test this, wild-type Munc18-1 or its variants (K46E/E59K or KE/39I) fused with EmGFP were stably over-expressed in the wild-type PC12 cells. As a negative control, EmGFP alone was stably
expressed in presence of endogenous Munc18-1. Firstly, stable over-expression of these Munc18-1 variants was confirmed by immunoblotting (Figure 4.4 E). We found that WT-EmGFP and KE-39I/EmGFP expresses ~2-3 times more than the endogenous Munc18-1 while the chaperoning mutant (K46E/E59K)-EmGFP expresses at a similar level to the endogenous Munc18-1. Importantly, the stable over-expression of the KE/39I had no deleterious effects on secretion from the wild-type PC12 cell (Figure 4.4 F). This suggests that this insertion mutant does not interfere with the function of endogenous wild-type Munc18-1. Therefore, it is clear that the KE/39I is not a dominant-negative mutant, but rather represents a pure loss-of-function mutant.
Figure 4.4 A K332E/K333E mutant with insertion of 39 residues (KE/39I) abolishes the rescue ability of exocytosis even though it restores syntaxin-1 level and localization. (A, B) Stable re-expression of Munc18-1 KE/39I in Munc18-1/-2 double-knockdown clones (D16 in A, D7 in B) restores syntaxin-1 expression. A number on the left indicates the position of a molecular weight marker. (C, D) Secretion defects are not rescued upon reintroduction of the KE/39I-EmGFP mutant in D16 (C) and D7 (D) clones. Error bars indicate SEM (n = 6 for D16; n = 24-27 for D7). (E) Over-expression of the K46E/E59K or KE39I mutant in presence of endogenous Munc18-1. A number on the left indicates the position of a molecular weight marker. (F) Overexpression of Munc18-1 K46E/E59K or KE/39I mutant in wild-type PC12 cells does not interfere with the secretion of the wild-type PC12 cells. Error bars indicate SEM (n = 15).
Figure 4.5 Rescue of syntaxin-1 localization in M unc18-1/2 DK D (D16) clones upon reintroduction of negative control EmGFP alone, wild-type M unc18-1-EmGFP, KE/39I-EmGFP and KE/5I-EmGFP. These cells were stained with anti-syntaxin-1 antibodies, followed by rhodamine red-X-conjugated anti-mouse antibodies (middle). Right panels are merged pictures. Bar, 10 μm.
4.2.3 Insertion of 5 residues in addition to K332E/K333E mutations is the minimal mutation that is required to impair the priming function of Munc18-1

To minimize the potential artifact that a large insertion of 39 residues may have on the overall structure of Munc18-1, the size of the insertion was reduced from 39 to 5 residues (MPQEE) in the presence of K332E/K333E mutation (Figure 4.6 A). The insertion of 5 residues with K332E/K333E mutation (KE/5I) also effectively retained its chaperone activity. This KE/5I mutant was able to restore syntaxin-1 expression level and localization along the plasma membrane as efficiently as that of the wild-type Munc18-1 (Figure 4.6 B and D). Nonetheless, the KE/5I mutant disrupted the secretory function of Munc18-1 (Figure 4.6 C). Therefore, it was confirmed that the KE/5I mutant exhibits the consistent phenotype as that of the KE/39I mutant.

To determine the minimum mutations that are necessary to selectively impair the secretory phenotype while retaining the syntaxin-1 chaperone activity, we further narrowed down the mutations. We examined whether the insertion of 5 residues alone, 5I (MPQEE) or 5I (MPQKK), without K332E/K333E mutations could exhibit the comparable phenotypes to that of the KE/39I and KE/5I mutants. Two different versions of the insertion mutants were generated: one version contains the five residues in which two of the five residues are negatively charged (MPQEE) while another version consists of two of the five residues that are positively charged (MPQKK). This was to assess whether difference in the composition of inserted residues would exhibit distinctive phenotypes. The mutants containing the insertion of 5 residues alone without double mutations were shown to retain their ability to restore syntaxin-1 level upon re-expression in Munc18-1/-2 double knockdown clone, D7 (Figure 4.6 E). However, the secretion rescue ability of these mutants was not as severely impaired as observed in the KE/39I or KE/5I mutants (Figure 4.6 F). No obvious difference in phenotypes was observed between the two versions of the insertion mutants.
The results from this part of the study suggest that although K332E/K333E point mutations alone do not cause functional impairment, they exacerbate the secretion rescue ability of the 5I mutants. Taken together, we concluded that the 5 residue insertion together with K332E/K333E mutation (KE/5I) is the minimal mutation that is necessary to completely abolish the secretion rescue ability of Munc18-1 among the mutations tested in the study. Therefore, we suggest that the KE/5I and KE/39I mutants represent the novel priming mutants that selectively impair secretion rescue ability while strongly retaining its ability to restore syntaxin-1 stabilization and proper trafficking to the plasma membrane.
Figure 4.6 The KE/5I mutant abolishes the rescue ability of exocytosis while restoring syntaxin-1 level (A) Sequence alignment of narrowed down KE/5I mutant and 5I mutants which contains insertion of 5 residues without K332E/K333E mutant, 5I(MPQKK) or 5I(MPQEE). (B) Stable reexpression of Munc18-1 KE/5I-EmGFP in D16 clones restores syntaxin-1 expression. (C) Secretion defects are not rescued upon reintroduction of the KE/5I-EmGFP mutant in Munc18-1/-2 double-knockdown cells (D7 and D16 clones). Error bars indicate SEM (n = 12 for both D7 and D16). (D) Stable reexpression of Munc18-1 KE/5I-EmGFP in D16 clones restores syntaxin-1 localization along the plasma membrane. These cells were stained with anti-syntaxin-1 antibodies, followed by rhodamine red-X-conjugated anti-mouse antibodies (middle). Right panels are merged pictures. Bar, 10 μm. (E) Stable reexpression of Munc18-1 5I mutants in D7 clones restores syntaxin-1 level. (F) Secretion defects are partially restored upon reintroduction of the 5I (MPQEE)-EmGFP or 5I (MPQKK) mutant in D7 clones although not as efficiently as wild-type Munc18-1. Error bars indicate SEM (n = 3-6).
4.2.4 The KE/5I mutant restores the docking of dense-core vesicles

The impaired dense-core vesicle (DCV) exocytosis in Munc18-1 deficient chromaffin cells has been tightly coupled to the perturbed docking of the DCVs (Voets et al., 2001). In Chapter 3 of this thesis, it has been demonstrated that the efficiency of DCV docking to the plasma membrane is positively correlated with Munc18-1-dependent syntaxin-1 localization through the analyses of Munc18-1 domain-1 mutants (Han et al., 2011). Based on the observation that the KE/5I mutant effectively restores syntaxin-1 at the plasma membrane, this mutant was expected to restore DCV docking as well. Using electron microscopy, we analyzed the effect of the KE/5I mutant on docking of DCVs in the Munc18-1/-2 double knockdown cells (D7) (Figure 4.7-4.10). The level of DCV docking was compared to the docking efficiency in the DKD (D7) PC12 cells rescued by EmGFP alone (negative control) or wild-type-EmGFP (positive control). Dense-core vesicles were defined as docked if they are localized within 50 nm from the plasma membrane.

In the D7 cells rescued with EmGFP alone, only 6.6 ± 0.9 % (n = 47) of vesicles were docked while 48.3 ± 2.5 % (n = 48) of vesicles were docked in D7 cells upon re-expression of wild-type Munc18-1-EmGFP, indicating the clear rescue of docking phenotype (Figure 4.10 A). In D7 cells expressing KE/5I-EmGFP, 35.3 ± 1.7 % (n = 46) of vesicles were localized within 50 nm from the plasma membrane. Our one-way ANOVA analysis has indicated the significant difference in the proportion of docked vesicles in these three groups. Furthermore, Post-hoc analysis has suggested the significant difference between each group at the level of p < 0.01 (Figure 4.10 A). These results clearly indicate that despite its complete loss in the ability to rescue secretion, the KE/5I mutant largely retains the ability to restore docking of DCVs. The size of the cells was not significantly different among three different rescue conditions (Figure 4.10 C) while the total number of DCVs was slightly (~20%) increased in the cells rescued by EmGFP compared to the cells rescued by the wild-type Munc18-1 or the KE/5I mutant (Figure 4.10 B).
These findings further confirm that the KE/5I mutant selectively loses the ability to stimulate or prime exocytosis downstream of the docking of dense core-vesicles. To our knowledge, this is the first mutant that demonstrates the selective effect of Munc18-1 on DCV secretion independently from its effect on docking. These results also support that Munc18-1 mediates the DCV docking through syntaxin-1 regulation, further confirming our previous findings with domain-1 cleft mutants.
Figure 4.7 An example of electron micrographs of M unc18-1/-2 double-knockdown cells (D7) rescued with EmGFP alone. Dense-core vesicles are indicated by black arrows. Scale bar, 500 nm.
Figure 4.8 An example of electron micrographs of Munc18-1/-2 double-knockdown cells (D7) rescued with wild-type Munc18-1-EmGFP. Dense-core vesicles are indicated by black arrows. Scale bar, 500 nm.
Figure 4.9 An example of electron micrographs of Munc18-1/-2 double-knockdown cells (D7) rescued with Munc18-1 KE/5I-EmGFP. Dense-core vesicles are indicated by black arrows. Scale bar, 500 nm.
Figure 4.10 Electron microscopic analysis of dense-core vesicle distribution in Munc18-1/-2 double-knockdown cells (D7) expressing EmGFP alone, wild-type Munc18-1-EmGFP, or KE/5I mutant-EmGFP. (A) The mean percentage distribution of dense-core vesicles within individual PC12 cells, calculated from multiple single electron micrographs. Dense-core vesicles were classed as being located within 50, 50 - 100, 100 - 200, 200 - 500, or 500 - 1000 nm or farther than 1000 nm from the plasma membrane. One-way ANOVA analysis indicates the significant difference in the proportion of docked vesicles in these three groups. Post-hoc analysis suggests the significant difference between each group at the level of p < 0.01. Error bar, SEM (n = 46-48). (B) The mean number of dense-core vesicles present in each single-cell electron micrograph from the D7 clones expressing EmGFP alone or Munc18-1 variants. (C) The mean area of each cell from the D7 clones expressing EmGFP alone or Munc18-1 variants. Error bar, SEM (n = 46-48).
4.2.5 Over-expressed syntaxin-1 partially localizes along the plasma membrane in the absence of Munc18-1

If the function of Munc18-1 can solely be explained by its chaperone function, which allows proper trafficking of syntaxin-1 to the plasma membrane, the secretory phenotypes caused by the loss of Munc18-1 should be at least partially restored in the presence of properly localized syntaxin-1A. To test this hypothesis, a system in which syntaxin-1 could properly localize along the plasma membrane even in the absence of Munc18-1 was needed. Therefore, we attempted to over-express syntaxin-1A in Munc18-1/2 DKD cells (D16) through the lentiviral infection followed by blasticidin selection. Firstly, we confirmed that syntaxin-1A could indeed be over-expressed in the DKD cells as shown by immunoblot analysis (Figure 4.11 A). Interestingly, although the majority of the over-expressed syntaxin-1A was accumulated in the perinuclear region, some successfully localized along the plasma membrane even in the absence of Munc18-1 (Figure 4.11). Nonetheless, over-expressed syntaxin-1A alone could not effectively restore the defective secretion of Munc18-1/2 DKD cells (D16 in the left panel of Figure 4.12 B, D7 in the left panel of Figure 4.12 C). This was another strong indication that the Munc18-1 function is extended beyond its role in syntaxin-1 trafficking.
Figure 4.11 Characterization of syntaxin-1 over-expressed Munc18-1/-2 PC12 cells. (A) Overexpression of syntaxin-1A in the Munc18-1/-2 double-knockdown cells (D16 clone). A number on the left indicates the position of a molecular marker. (B) Partial plasmalemmal localization of overexpressed syntaxin-1A in D16 clones. Cells were stained with anti-syntaxin-1 antibodies, followed by rhodamine red-X-conjugated anti-mouse antibodies. Bar, 10 μm.
4.2.6 The putative priming mutant, KE/39I, and chaperoning mutant, K46E/E59K, exhibit different properties in presence of over-expressed syntaxin-1

The partial localization of syntaxin-1A along the plasma membrane in syntaxin-1A over-expressed Munc18-1/-2 DKD cells provided an advantage to study whether the chaperone activity or the priming function of Munc18-1 could be further uncoupled. We have further expressed K46E/E59K (“chaperoning mutant”) or KE/39I (“priming mutant”) mutant in the presence of the over-expressed syntaxin-1A to distinguish the distinctive phenotypes of these two mutants (Figure 4.12). In Chapter 3, the K46E/E59K mutant was shown to impair the ability of Munc18-1 to properly traffic syntaxin-1 to the plasma membrane and thus was unable to restore the secretion defect upon re-expression in Munc18-1/-2 DKD cells. However, if this chaperoning mutant still retains the priming function, it should be able to rescue secretion to a certain extent in the presence of properly localized syntaxin-1 in contrast to the priming mutant which is unable to restore secretion defects under the same condition.

Upon introduction of the KE/39I mutant in syntaxin-1 over-expressed Munc18-1/-2 double knockdown PC12 cells, the syntaxin-1 localization along the plasma membrane was much more efficiently restored compared to when syntaxin-1 was over-expressed in the absence of Munc18-1 (Figure 4.12 D). In contrast, when the K46E/E59K mutant was introduced in these cells, the majority of syntaxin-1 remained accumulated in perinuclear region, confirming a lack of syntaxin-1 chaperone activity in this mutant (Figure 4.12 D). In line with this observation, syntaxin-1 expression levels seemed to be higher upon re-introduction of wild-type Munc18-1 or the KE/39I mutant compared to when the K46E/E59K was re-expressed (Figure 4.12 A). This may be due to further stabilization of over-expressed syntaxin-1 in the presence of its functional chaperone such as the wild-type Munc18-1 or the KE/39I mutant. However, in contrast to the poor ability of the K46E/E59K mutant to rescue syntaxin-1 localization in the syntaxin-1A overexpressed Munc18-
1/-2 DKD cells, this chaperoning mutant restored the secretion defect much more efficiently in these cells compared to the syntaxin-1A over-expressed cells infected with the EmGFP alone or the KE/39I mutant (D16 in the right panel of Figure 4.12 B, D7 in the right panel of Figure 4.12 C). This indicates that the chaperoning mutant (K46E/E59K) at least partially retains the ability to stimulate/prime exocytosis in presence of plasmalemmal syntaxin-1. In contrast, the KE/39I mutant failed to rescue the secretion defect despite its ability to efficiently escort syntaxin-1 to the plasma membrane. This indicates that domain-3a makes an important contribution to the post-syntaxin-1 chaperone role of Munc18-1 during exocytosis. Furthermore, the results from this experiment support the idea that the chaperone activity and the priming function are mediated through independent mechanisms which involve distinct region of Munc18-1.
Figure 4.12 Uncoupling the distinctive roles of the priming mutant, KE/39I, and the chaperoning mutant, K46E/E59K. (A) Overexpression of the chaperoning mutant (K46E/E59K-EmGFP) or the priming mutant (KE/39I-EmGFP) in D16 clones in which syntaxin-1A is overexpressed. A number on the left indicates the position of a molecular marker. (B, C) Secretion defects are not rescued upon overexpression of syntaxin-1 alone in D16 (the left panel of B, n = 7) and D7 clones (the left panel of C, n = 5 – 6), while they are partially, but significantly rescued upon reintroduction of the K46E/E59K-EmGFP mutant but not KE/39I-EmGFP mutant in syntaxin-1 overexpressed D16 clones (the right panel of B, n = 9, Independent t-test, **p<0.01) and in syntaxin-1 overexpressed D7 clones (the right panel of C, n = 6, Independent t-test, **p<0.01). Error bars indicate SEM. (D) Rescue of syntaxin-1 localization in syntaxin-1 overexpressed Munc18-1/2 DKD (D7) clone upon reintroduction of Munc18-1-K46E/E59K-EmGFP or KE/39I-EmGFP mutant. These cells were stained with anti-syntaxin-1 antibodies, followed by rhodamine red-X-conjugated anti-mouse antibodies (middle). Right panels are merged pictures. Bar, 10 μm.
4.2.7 KE/5I mutant retains the ability to interact with monomeric syntaxin-1A

To elucidate the underlying mechanisms by which the KE/5I mutation leads to the selective loss of the priming function, we examined the interactive property of the KE/5I mutant. We first investigated the ability of the KE/5I mutant to interact with cytoplasmic syntaxin-1A (residues 1-264) using yeast two-hybrid assays (Figure 4.13 A). We found that the KE/5I mutant is able to bind to syntaxin-1A as efficiently as the wild-type and the K332E/K333E mutant. This is consistent with our finding that these priming mutants are able to restore syntaxin-1A level and plasmalemmal localization (Figure 4.6). To further correlate syntaxin-1 chaperone activity of Munc18-1 variants in Munc18-1/-2 DKD cells with their syntaxin-1 binding ability, the ability of the KE/5I mutant to interact with monomeric syntaxin-1 was further analyzed together with previously alleged domain-1 mutant (K46E and K46E/E59K). In Chapter 3, the K46E single mutant was shown to significantly reduce binding to syntaxin-1A whereas the K46E/E59K double mutant abolishes the interaction as measured by using yeast two-hybrid analysis and isothermal titration calorimetry (ITC) (Han et al., 2011). In this experiment, the Munc18-1 variants (WT, K46E, K46E/E59K, K332E/K333E, and KE/5I) fused with EmGFP that have been expressed in mammalian cell line HEK-293 cells were pulled down by GST-syntaxin-1A (residues 1-264). This pull-down experiment using GST-syntaxin-1A mirrored the previous binding experiments, further validating the results from the both experiments; the pull-down of the K46E-EmGFP is reduced compared to the wild-type Munc18-1-EmGFP while the pull-down of the K46E/E59K-EmGFP is almost completely abolished (Figure 4.13 B). In contrast, the K332E/K333E mutant and the KE/5I mutant fused with EmGFP are quantitatively pulled down, which is comparable to the wild-type Munc18-1 fused with EmGFP (Figure 4.13 B).

The amount of Munc18-1 variants used for each pull-down reaction was confirmed to be comparable between the mutants as observed by Ponceau S staining and immunoblotting of the
lysate from each HEK-293 cell line expressing the Munc18-1 mutants (Figure 4.13 C and D).

These results demonstrate that the chaperoning mutant (K46E/E59K) loses the ability to bind to syntaxin-1A in a stoichiometric manner whereas the priming mutant (KE/5I) effectively retains it. This intact syntaxin-1 binding ability of the KE/5I mutant explains its effective syntaxin-1 chaperone activity that mainly relies on this binary interaction between Munc18-1 and syntaxin-1.
Figure 4.13 Munc18-1 KE/5I mutant retains binding to monomeric syntaxin-1A. (A) The binding between Munc18-1 domain-3a insertion mutants (KE/5I, 5I (MPQKK), 5I (MPQEE)) and syntaxin-1 was analyzed by yeast-two-hybrid assays. In this 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 Munc18-1 was set to 100%. Error bars indicate SEM (n=12-13). (B) Munc18-1 WT, K46E, K46E/E59K, K332E/K333E, and KE/5I mutants fused with EmGFP pulled down by GST-syntaxin-1A were analyzed by Coomassie-stained SDS-PAGE. Note that EmGFP-fused Munc18-1 variants expressed in mammalian HEK-293 cell line are pulled down by GST-syntaxin-1A to different degrees. (C) Amount of proteins contained in the lysates (20 µg) from each HEK-293 cell line expressing Munc18-1 variants was confirmed by Ponceau S staining. (D) Initial amount of Munc18-1 variants prior to pull down experiment was verified by immunoblotting the lysate from each cell line expressing Munc18-1 variants by probing with anti-GFP antibody. GST-pull down experiment was performed by Na-ryum Bin from the Sugita Lab.
4.2.8 KE/5I mutant impairs the ability to interact with the SNARE complex.

The binding of Munc18-1 to the SNARE complex has been suggested to be the potential mechanism underlying the priming of exocytosis (Südhof and Rothman, 2009). Therefore, we examined the ability of the priming mutant, KE/5I, to interact with the SNARE complex. To ensure the absence of monomeric syntaxin-1A or synaptobrevin-2, which can directly bind to Munc18-1 independently from the SNARE complex, the SNARE complex was generated centering the immobilized SNAP-25B on the column. Since a large tag such as GST can interfere with the formation of the SNARE complex (Dulubova et al., 2007), the full-length SNAP-25B was expressed as a strep (composed of 8 amino acids, ~ 1kDa) -tagged protein in E. coli, purified and immobilized onto the strep-tactin sepharose. Synaptobrevin-2 (residues 1 - 94) and syntaxin-1A (1 - 264) were expressed as GST-fusion proteins and GST-tag was removed by thrombin cleavage. Soluble synaptobrevin-2 and syntaxin-1A were incubated with SNAP-25B immobilized on the strep-tactin sepharose and unbound proteins were washed out from the sepharose. SDS-PAGE analysis of the unboiled sample on the strep-tactin sepharose showed the presence of the SNARE complex as well as the monomeric strep-SNAP-25 whereas the monomeric syntaxin-1A and synaptobrevin-2 were largely absent (Figure 4.14 A). Once the sample was boiled to disassemble the SNARE complex, the presence of syntaxin-1A and synaptobrevin-2 became evident. Please note that the boiling of the sample including immobilized SNAP-25B results in a partial elution of monomeric strep-tactin (~14 kDa band, indicated by ***) from the strep-tactin sepharose (Voss and Skerra, 1997) while the cytoplasmic synaptobrevin-2 is present as a ~13 kDa band in the SDS-PAGE gel stained with Coomassie (Figure 4.14 A). We then incubated the SNARE complex immobilized on sepharose with Munc18-1 variants or BSA (negative control) to initiate the interaction. We observed that only the wild-type Munc18-1, but not BSA, was pulled down with the preformed SNARE complex, demonstrating the specificity of binding. In this experiment, we
have also assessed the SNARE complex binding ability of an additional Munc18-1 mutant (F115E) aside from the KE/5I mutant. The F115E mutant, which is known to disrupt N-terminal binding with syntaxin-1, has previously shown to abolish its interaction with SNARE complex as measured by ITC (Meijer et al., 2012). Therefore, this mutant was initially used to represent an additional negative control aside from BSA. However, the binding of the F115E mutant to the SNARE complex was observed to be as almost effective as the wild-type which was unexpected (Figure 4.14 A). Under this condition, the KE/5I mutant exhibited dramatically reduced binding to the SNARE complex compared to the wild-type Munc18-1 or the F115E mutant (Figure 4.14).

These results suggest that the mutation in domain-3a may have more severe effect in binding to the SNARE complex than the mutation (F115E) in the outer surface of the hydrophobic pocket of Munc18-1. This result indicates that the domain-3a of Munc18-1 plays a significant role in binding to the SNARE complex.
Figure 4.14 Munc18-1 KE/5I mutant impairs binding to the SNARE complex. (A) Assembled SNARE complex consisting of comprising Strep-SNAP-25, syntaxin-1A, and synaptobrevin-2 was isolated on Strep-tactin sepharose, followed by incubation with BSA, Munc18-1 WT, KE/5I, or F115E for 2-4 hours at the room temperature. Following extensive washing, beads were analyzed for bound proteins using Coomassie-stained SDS-PAGE. Binding of Munc18-1 variants to assembled SNARE complex was confirmed by analyzing boiled (5min, denatured complex) samples (left). Asterisks (*** represents monomers of strep-tactin that is detached from the sepharose upon boiling. Flowthrough after the binding experiment was also visualized by Coomassie-staining (right). (B) Bound proteins to the SNARE complex were further analyzed by Ponceau S staining after the proteins were transferred to nitrocellulose membrane. (C) Western blot of the SNARE-complex mediated pull down of Munc18-1 WT, KE/5I, and F115E mutant probed with anti-Munc18-1 antibody.
4.3 Conclusion

The KE/5I and KE/39I mutants have been presented as novel priming mutants that selectively impair secretion rescue while strongly retaining its ability to restore syntaxin-1 stabilization, trafficking, and dense-core vesicle docking. The phenotype of these mutants suggest that the domain-3a of Munc18-1 plays a crucial role at the priming stage beyond syntaxin-1 chaperoning and dense-core vesicle docking through its interaction with the SNARE complex (Han et al., 2013).

Furthermore, it has been shown that the K46E/E59K mutant that selectively loses the chaperoning function can significantly restore the secretion defect in the presence of partially localized syntaxin-1. In contrast, the KE/39I mutant failed to restore the secretion defect despite much more prominent localization of syntaxin-1 at the plasma membrane. These observations suggest that the chaperone activity and priming role of Munc18-1 are mediated by independent mechanisms that involve distinctive binding modes.
Chapter 5: Domain-3a of Munc18-1 contributes to syntaxin-1 stabilization in addition to the priming function of Munc18-1

The content of the following chapter is unpublished.

5.1 Rationale of the study

Results from Chapter 3 and 4 have clearly indicated functional significance of domain-1 and domain-3a. It has been demonstrated that the domain-1 of Munc18-1 plays a critical role in syntaxin-1 stabilization and trafficking through high affinity binary interaction with monomeric syntaxin-1. The domain-1 mutant, K46E/E59K, that loses the chaperone activity could restore secretion defect in presence of partially localized syntaxin-1 in syntaxin-1 overexpressed Munc18-1/-2 double knockdown PC12 cells. This indicated that the priming activity of this domain-1 mutant is intact. Meanwhile, domain-3a of Munc18-1 has been demonstrated to play an important role at the priming stage of exocytosis beyond vesicle docking, perhaps via direct interaction with assembled SNARE complex. However, whether this domain additionally contributes to the chaperone activity of Munc18-1 has not been assessed. The findings that all the point mutations within domain-3a assessed do not exhibit any functional impairment were unexpected as these residues were highly conserved and were suggested to make specific contacts with syntaxin-1 according to the crystal structure analysis. Meanwhile, a more recent structural analysis on domain-3a of Munc18-1 raised the importance of P335 residue within the highly conserved region of Munc18-1 as this residue serves as a hinge point that allows domain-3a to undergo conformational change. It has been suggested that this conformation change within domain-3a allows Munc18-1 to release the binary interaction with closed syntaxin-1 and adopt N-terminal
binding mode that is compatible with SNARE complex interaction. We have previously confirmed that both K332E/K333E double mutant and Q336A/Y337L double mutant which are located adjacent to P335 residue do not have functional impairments. Therefore, to investigate whether mutating P335 residue along with these residues would result in functional impairments, we have generated a quintuple mutant in which K332, K333, P335, Q336, and Y337 residues were simultaneously mutated. To obtain the mechanistic insight into the importance of P335 residue, we assessed the phenotype of this quintuple K332E/K333E/P335A/Q336A/Y337L mutant.

5.2 Results

5.2.1 Mutating K332, K333, P335, Q336, and Y337 residues simultaneously impairs the binary interaction between Munc18-1 and syntaxin-1

In Chapter 4, we have confirmed that K332E, K333E, K333E/K333E, E278K/K332E/K333E, Q336A/Y337L, Y337L/Q338A, and Q336A/Y337L/Q338A single, double, or triple mutants all retained intact binary interaction with monomeric syntaxin-1. Based on this observation, we concluded that these residues are not important for specific interaction of Munc18-1 with monomeric syntaxin-1. However, when we simultaneously mutated K332, K333, Q336, and Y337 residues together with P335 residues, we observed that this quintuple mutant completely abolishes its ability to interact with monomeric syntaxin-1. In contrast, when K333, K333, Q336, and Y337 residues were mutated without P335 residue, this quadruple mutant retained intact binary interaction with monomeric syntaxin-1 (Figure 5.1). This result implies that P335 residue serves an important function during the binary interaction although the underlying mechanism is unknown. This could be due to the loss in direct contact with syntaxin-1 upon mutating this residue. Alternatively, it could be a consequence of impairment in structural flexibility of domain-3a in which this mutant perturbs the role of the P335 residue as a hinge point
during the conformational change of domain-3a from bent to extended and locks the structure in the extended form. The latter conformation is considered to be incompatible with the binary interaction with monomeric syntaxin-1. Although the mechanism is unknown, it is clear that simultaneously disrupting the five residues results in severe impairment in the binary interaction between Munc18-1 and syntaxin-1.

Figure 5.1 Interaction between Munc18-1 domain-3a quintuple mutant (K332E/K333E/P335A/Q336A/Y337L) or quadruple mutant (K332E/K333E/Q336A/Y337L) and syntaxin-1. Binding was analyzed by yeast-two-hybrid assays. 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 Munc18-1 was set to 100%. Error bars indicate SEM (n=12-13).
5.2.2 Munc18-1 K332E/K333E/P335A/Q336A/Y337L quintuple mutant fails to restore cellular level of syntaxin-1

In Chapter 3, we have clearly demonstrated that the ability of the domain-1 mutants of Munc18-1 to restore syntaxin-1 expression is positively correlated with their ability to retain their interaction with monomeric syntaxin-1. Therefore, we tested whether this is the case of this domain-3a mutant as well. We have confirmed that this quintuple, K333E/K333E/P335A/Q336A/Y337L, mutant expresses as efficiently as wild-type Munc18-1 upon re-expression in Munc18-1/-2 double knockdown PC12 cells (D7 and D16) (Figure 5.2). This suggests that this quintuple mutant is able to fold properly and express well. When the ability of the quintuple mutant to restore syntaxin-1 was assessed, we found this mutant failed to restore syntaxin-1 expression (Figure 5.2). This is in line with the previous observations with domain-1 mutants that the ability of Munc18-1 to restore syntaxin-1 expression relies on its ability to interact with monomeric syntaxin-1. This indicates the perturbed ability of Munc18-1 to stabilize syntaxin-1 upon mutating these five residues.
Figure 5.2 Impaired recovery of syntaxin-1 expression upon stable re-expression of the domain-3a quintuple mutant in Munc18-1/-2 double knockdown PC12 cells (D7 or D16). Munc18-1/-2 double knockdown clones (D7 or D16) were infected with lentiviruses that express EmGFP, wild-type Munc18-1-EmGFP, or the quintuple Munc18-1 mutants, and the infected cells were selected with blasticidin. A number on the left indicates the position of a molecular weight marker.
5.2.3 Munc18-1 K332E/K333E/P335A/Q336A/Y337L quintuple mutant fails to restore secretion defect in Munc18-1/-2 double knockdown PC12 cells

Based on the analysis of the domain-1 mutants in Chapter 3, we concluded that Munc18-1 mutants that lose the ability to stabilize syntaxin-1 consequently results in defective secretion since the initial stage of sequential events is perturbed. When we examined the ability of the quintuple mutant to restore secretion defect in Munc18-1/-2 double knockdown PC12 cells, we observed that this mutant failed to rescue the secretion defect (Figure 5.3). This was expected as the ability of this mutant to stabilize syntaxin-1 is impaired as demonstrated by lack of restored syntaxin-1 expression level upon re-expression in Munc18-1/-2 double knockdown PC12 cells.

![Figure 5.3 Impaired secretion rescue ability of the quintuple mutant. Secretion defects are not rescued upon reintroduction of the KE/5I-EmGFP mutant in Munc18-1/-2 double-knockdown cells (D7 and D16 clones). Error bars indicate SEM (n = 9).](image-url)
In Chapter 4, it has been clearly demonstrated that the domain-3a of Munc18-1 plays a critical role during the late stage of exocytosis beyond syntaxin-1 trafficking and dense-core vesicle docking. However, the results from this chapter indicate that the domain-3a additionally plays an important role in maintaining the high affinity binary interaction with monomeric syntaxin-1 which is critical for proper syntaxin-1 trafficking to the plasma membrane. These two different functions of domain-3a may be mediated by specific regions as mutating the specific residues lead to impairment in different roles of Munc18-1. The proline 335 residue seems to be a critical residue that allows domain-3a of Munc18-1 to exhibit this dual function. This could be due to the loss in direct contact within syntaxin-1 through this residue or it could be the consequence of the impairment in structural flexibility of domain-3a that underlies its ability to adopt different binding modes upon mutating the P335 residue. Therefore, the further research needs to be conducted in order to elucidate the mechanisms underlying the dual function of domain-3a of Munc18-1.
Chapter 6: Discussion

6.1 Resolving the discrepancy in the functional significance of domain-1 of Munc18-1 in exocytosis

In Chapter 3, the functional significance of the interaction between the domain-1 cleft of Munc18-1 and syntaxin-1 in regulated exocytosis has been investigated through phenotypic analyses of various domain-1 cleft mutants: K46E, E59K, K46E/E59K, K63E, E66A, D34N, and D34N/M38V. The mutant analyses included re-evaluation of the domain-1 mutants, E59K, E66A, and D34N/M38V, which were previously reported to impair Munc18-1 function in the priming of vesicles (Deák et al., 2009; Gulyás-Kovács et al., 2007). In the study presented in this thesis, inability of these mutants to restore secretion defects has been supported by their perturbed syntaxin-1 chaperone activity or inefficient expression. Moreover, the phenotypic analyses of all the domain-1 cleft mutants suggested the striking correlations between the abilities of these mutants to bind and chaperone syntaxin-1, and to restore the dense-core vesicle docking and secretion. Together, the results from this study strongly demonstrated that the domain-1 cleft of Munc18-1 is critical for high affinity binary interaction between Munc18-1 and syntaxin-1 and consequently in syntaxin-1 chaperone activity.

A previous study has reported that the E59K and E66A single mutants significantly impair their ability to rescue the neurotransmitter exocytosis defect in Munc18-1 deficient neurons compared to the wild-type. This impaired secretory ability has been correlated with their reduced binding to the SNARE complex that has been demonstrated through nuclear magnetic resonance (NMR) experiments (Deák et al., 2009). Consistently, the E66A mutant has been further shown to result in comparable decrease in lipid mixing (Ma et al., 2013). In our system, the E59K mutant
exhibited reduced ability to rescue secretion as observed in the previous study, however, the E66A mutant did not exhibit any defect in its ability to restore secretion. Moreover, unlike the previous study, the results from our study suggested that the secretory phenotype of E59K mutant can be explained by its reduced chaperoning of syntaxin-1 which stems from its reduced binary interaction with syntaxin-1 as measured by yeast two-hybrid assay and ITC. Discrepancy in the syntaxin-1 binding ability of the E59K mutant between different studies may reflect different sample preparation. Our result is in line with another previous mutagenesis study that showed the impaired binding between the E59K mutation in Munc18-2 and syntaxins-1-4 isoforms (Kauppi et al., 2002). Nonetheless, the results from our study cannot exclude the possibility that the E59K mutant also reduces interaction with assembled SNARE complexes in addition to monomeric syntaxin-1.

Although the mechanisms proposed by the two studies are different, it seems evident that this residue of domain-1 is playing an important role in exocytosis as mutating this residue led to impaired secretion in both cases. The importance of this residue in membrane fusion was further supported by the observation that the E59K mutant reduces the stimulatory activity of Munc18-1 on membrane fusion in in vitro liposome fusion assay (Shen et al., 2010). The E59 residue is located within the central cavity of Munc18-1 and is known to interact with the arginine 114 (R114) residue within H_{abc} domain of syntaxin-1 during the syntaxin-1-Munc18-1 binary interaction (Misura et al., 2000). However, disrupting this interaction by mutating the R114 residue of the syntaxin-1 into glutamate (R114E) exhibited a limited effect on Munc18-1 activation of liposome fusion. This interesting, but puzzling phenomenon could be at least partly explained by the possibility that unlike the case for the binary interaction, direct contact between E59 residue of Munc18-1 and R114 residue of syntaxin-1 may no longer be critical for Munc18-1-stimulated fusion. In fact, the H_{abc} domain of syntaxin-1 has been shown to be dispensable for
Munc18-1 stimulated fusion (Shen et al., 2010). Nonetheless, the observation that the E59K mutant still impairs the stimulatory activity of Munc18-1 on membrane fusion infers that this residue may be involved in interacting with another site of an assembled SNARE complex to exert its role during the later stage of exocytosis. In fact, the SNARE four helix-bundle has been shown to compete with the $H_{abc}$ domain of syntaxin-1 for binding to the central cavity region of Munc18-1 (Xu et al., 2010). Therefore, it could be postulated that the E59 residue lining the central cavity of Munc18-1 is initially important for its binary interaction with closed syntaxin-1 through specific contact with the R114 residue of the $H_{abc}$ domain of syntaxin-1 which allows proper syntaxin-1 trafficking. Then when syntaxin-1 adopts open conformation and assembles into the SNARE complex, the central cavity of Munc18-1 switches its interaction partner from monomeric syntaxin-1 to the four-helical bundles of the SNARE complex to further regulate membrane fusion process. This means that the residues lining Munc18-1 cavity may participate in dual mode of SNARE interaction, first with $H_{abc}$ domain of syntaxin-1 and later with the SNARE bundle (Shen et al., 2010). This is highly speculative. However, this could partially explain the different phenotypes observed for the E59K mutant in the two studies. This also fits well with our hypothesis that Munc18-1 sequentially contributes to different stages of exocytosis by adapting different modes of interaction.

This interpretation, however, is challenged by a recent finding that, the E59K mutant is capable of restoring vesicle docking and secretion phenotype in Munc18-1 null neurons despite its impaired affinity for both SNARE complexes and monomeric syntaxin-1 as measured by ITC (Meijer et al., 2012). This study led to the conclusion that the interaction between Munc18-1 and the SNARE complex is not essential for synaptic transmission in autaptic cultures of hippocampal neurons. This is at odds with the previous finding from networks of cortical neurons in which the E59K mutant that impairs SNARE complex binding is incapable of restoring synaptic
transmission (Deák et al., 2009). These results are contradictory. However, during recording from autapic neuronal cultures, neurons with higher expression level of the E59K mutant were reported to show normal synaptic transmission while it was abolished in low-expressing neurons. Based on these results, it has been suggested that the impaired neuronal response observed in the neuronal network from the previous study may reflect the reduced average response in mass culture. Therefore, the discrepancy in the phenotypes of the E59K mutant shown in different studies may reflect a wide range of model systems and techniques used in each of the studies to measure the secretory phenotypes and the interactive property of the mutant.

In yeast, Vps33, a yeast SM protein, has been reported to play an important role in promoting fusion pore opening via binding to Vam3, a syntaxin homologue. This study showed that weakening the Vps33-Vam3 interaction by introducing a homologous mutation to the Munc18-1 E59K in Vps33 (D88K) results in perturbed opening of the fusion pore (Pieren et al., 2010). To assess the possibility that the E59K mutation in Munc18-1 also have any effects on fusion pore opening, we employed hPLAP secretion assay which represent a peptidergic secretion that is known to require a more complete opening of the fusion pore (Barg et al., 2002). We confirmed that the peptidergic secretion ability of the E59K mutant is comparable to the K46E mutant and that the peptide secretion phenotypes of the both mutants are comparable to that observed with noradrenaline release. Therefore, no trace of evidence for defects that are specific to the E59K mutant that may have stemmed from impaired fusion pore opening was observed in our study. Based on these observations, we suggest that the impaired secretion rescue ability observed for the E59K mutant is mainly due to its reduced syntaxin-1 chaperone activity.

The D34N/M38V double mutant is another mutant that has been denoted as a priming mutant due to its selective impairment in vesicle priming (Gulyás-Kovács et al., 2007). Despite significantly impaired binding to the closed conformation of syntaxin-1 and limited rescue of both
secretion and docking phenotypes in Munc18-1 null cells, this double mutant was shown to be able to restore the syntaxin-1 pool. Our analysis of D34N/M38V, however, suggests that the inability of this mutant to rescue secretion of Munc18-1/-2 double knockdown cells is simply due to its inadequate expression which simply results in loss of the function of Munc18-1 in general. Therefore, it is difficult to conclude at which step of exocytosis this mutant loses its function. This observation is in line with the previous work done with Munc18-2, in which the same mutation in Munc18-2 resulted in destabilization of the protein leading to limited expression of Munc18-2 that is only about 20 - 25% of that of wild-type (Riento et al., 2000). Moreover, a low expression of D34N/M38V mutant in Munc18-1/-2 double knockdown (D16) clones could still rescue noradrenaline secretion to certain degree in our system. This excludes this double mutant from being a true priming mutant as it suggests the possibility that this mutant may be functional if expressed properly.

Taken together, our results suggest that the reduced secretion phenotypes observed in E59K and D34N/M38V mutants can be adequately explained by their reduced syntaxin-1 chaperone function and lack of expression, respectively. Nevertheless, our findings still do not completely rule out a possible role of these Munc18-1 domain-1 cleft mutants in priming stage of exocytosis in addition to the chaperone function.

6.2 The mechanism underlying syntaxin-1 chaperone activity of Munc18-1

The results shown in the Chapter 3 and 5 of the thesis clearly demonstrated the importance of Munc18-1 in proper syntaxin-1 trafficking to the plasma membrane. In the absence of Munc18-1, syntaxin-1 is mainly accumulated in the perinuclear region of Munc18-1/-2 double knockdown PC12 cells (Han et al., 2011; Han et al., 2009). This mislocalization of syntaxin-1 is restored upon re-expression of wild-type Munc18-1 in these cells. The observation that the degree of rescue in
syntaxin-1 localization positively reflects the degree of binding between Munc18-1 and syntaxin-1. Clearly, demonstrate that the syntaxin-1 chaperone activity of Munc18-1 largely depends on the high affinity binary interaction between Munc18-1 and syntaxin-1. This is in line with the previous study that showed that ectopically expressed syntaxin-1 that remains trapped in Golgi or endoplasmic reticulum correctly localized 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., 1999). Moreover, it has been suggested that this proper trafficking of syntaxin to the cell surface relies on binding of syntaxin-1 to Munc18-1 as this binding mode is thought to prevent the formation of ectopic SNARE complexes within cells (Medine et al., 2007; Rowe et al., 2001; Rowe et al., 1999). In the absence of Munc18-1 which holds syntaxin-1 in its inactive closed-conformation, syntaxin-1 is permitted to interact with SNAP-25 before they reach to the target membrane. This traps both proteins in intracellular compartments thereby preventing trafficking to the plasma membrane. Therefore, Munc18-1 should bind to syntaxin-1 shortly after syntaxin-1 synthesis before syntaxin-1 encounters other binding partners to prevent ectopic SNARE complex formation.

Furthermore, it has become clear that complex formation between SNARE proteins is less specific 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). This may be possible due to the high resemblance in their core sequence within SNARE motifs which participates in complex formation, even among distantly related SNAREs despite their remarkable specificity with their subcellular localization (Fasshauer et al., 1999). This means that unspecific SNARE complex formation can occur when noncognate SNAREs encounter one another during membrane recycling. However, this rarely happens in intact cells. This is highly likely due to the presence of regulatory proteins that bind to individual SNAREs with higher
degree of specificity until SNAREs encounter their cognate partners (Fasshauer et al., 1999). Likewise, Munc18-1 may bind to syntaxin-1 to ensure its proper trafficking to the plasma membrane where it allows syntaxin-1 to form SNARE complex with its cognate partners and efficiently execute membrane fusion. This hypothesis is well supported by the previous findings that the direct interaction between Munc18-1 and monomeric syntaxin-1 renders the syntaxin-1 unable to participate into the SNARE complex assembly thereby preventing syntaxin-1 from premature membrane fusion reactions on the way towards their cellular destination (Toonen and Verhage, 2003). Therefore, it seems that the binary interaction between Munc18-1 and syntaxin-1 serves an important role in proper syntaxin-1 trafficking by preventing inappropriate interaction of syntaxin-1 with non-cognate SNARE partners. However, this idea is challenged by the observation that the residual syntaxin-1 properly targets to the plasma membrane and forms SNARE complex in Munc18-1 knockout mice (Toonen et al., 2005). Nonetheless, due to lower syntaxin-1 protein level in the absence of Munc18-1, transport of syntaxin-1 to axonal projections could not been examined in a quantitative way. Therefore, whether this inconsistent evidence simply reflects different physiology in different cell types or not needs to be further clarified.

6.3 Munc18-1-dependent syntaxin-1 expression level

The results presented in the thesis clearly indicate that Munc18-1 plays an essential role in syntaxin-1 chaperoning through high affinity binary interaction. Additionally, the results suggest that Munc18-1 not only ensures the proper trafficking of syntaxin-1 to the plasma membrane but also is critical for stability of the protein as syntaxin-1 expression level is severely reduced in the absence of functional Munc18-1. This is in line with the evidence presented in other previous studies in which syntaxin-1 level is dramatically decreased in Munc18-1 knockout neurons, Munc18-1 deficient chromaffin cells, and Munc18-1/-2 double knockdown PC12 cells (Han et al.,
This phenotype seems to be conserved throughout the species as it has been reported that ablation of SM protein, Vps45p, in yeast strains also results in a marked reduction in the corresponding syntaxin, Tlg2p, as a result of proteasome-mediated degradation (Bryant and James, 2001). 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). Nonetheless, the mechanism underlying this down-regulation of syntaxin-1 protein level in the absence of Munc18-1 is unclear. There are two ways in which Munc18-1 can regulate syntaxin-1 expression level: either at transcription level by directly affecting syntaxin-1 synthesis or at the post-transcriptional level by ensuring the stability of syntaxin-1. The first possibility which suggests that Munc18-1 may be involved in syntaxin-1 transcription is difficult to support since this will require complex interaction between Munc18-1 and transcription factors whether it is direct or indirect. Evidence supporting such interaction is lacking up to date. Additionally, the lack of increase in syntaxin-1 level upon over-expression of ROP, Munc18-1 homologue in Drosophila, strongly indicates that the altered level of syntaxin-1 observed in the absence of Munc18-1 is most likely not due to the change in the production of syntaxin-1 (Schulze et al., 1994). In line with this, our data also suggest that syntaxin-1 expression is not greatly enhanced in presence of over-expressed functional Munc18-1. Therefore, it is highly unlikely that syntaxin-1 synthesis is decreased in the absence of functional Munc18-1. Then the remaining hypothesis suggests that Munc18-1 affects syntaxin-1 expression level by regulating the stability of the protein which leads to higher degradation of syntaxin-1 in the absence of Munc18-1.

A previous study has been shown that syntaxin-1 is able to form normal SNARE complexes without Munc18-1, consistent with an observation that syntaxin-1 is able to fold autonomously as shown in NMR studies (Fernandez et al., 1998; Sutton et al., 1998). However, in physiological system, syntaxin-1 requires the presence of Munc18-1 for its stabilization and proper
interaction with its cognate SNARE partners to form functional SNARE complexes. Nonetheless, how Munc18-1 contributes to the stability of syntaxin-1 remains as a question. Previously, it has been hypothesized that the decreased levels of secretory activity may lead to down regulation of proteins important for secretion. This may underlie decreased syntaxin-1 level in Munc18-1 deficient cells in which secretion is abolished (Sutton et al., 1999). 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 knockout mice despite the arrested neurotransmitter release (Varoqueaux et al., 2002). Therefore, it is highly likely that increased syntaxin-1 degradation is caused by the direct effect of Munc18-1 on syntaxin-1 stability. It could be simply postulated that the improperly localized syntaxin-1 in the absence of Munc18-1 would be highly unstable and does not serve any cellular functions thus would be naturally degraded by the cell via the ubiquitin-proteasome degradation pathway (Glickman and Ciechanover, 2002). From structural perspective, most of syntaxin-1 sequence except for the H_{abc} domain is unstructured in isolation. It is presumed that the unstructured region of syntaxin-1 becomes structured when encounter its binding partner such as Munc18-1. Therefore, in the absence of Munc18-1, the lack of stable tertiary structure of syntaxin-1 may facilitate its degradation. Likewise, it has been reported that in the absence of CSPα, which is known as a chaperone of SNAP-25, expression level of SNAP-25 decreases post-transcriptionally due to impaired stability of this protein (Sharma et al., 2011). As Munc18-1 and syntaxin-1 are also implicated in a similar chaperone–substrate relationship, they may behave in a similar pattern as CSPα and SNAP-25.

However, it has previously been shown that in Munc18-1 knockdown PC12 cells, mRNA level of syntaxin-1 is decreased by approximately 15% which correlates with the level of syntaxin-
1 protein down-regulation (Arunachalam et al., 2008). These results suggest that changes in expression level of syntaxin-1 could be regulated at least in part at the transcription level although it should be taken under consideration that change in mRNA level and protein level is not linearly correlated. In contrast, in Munc18-1/-2 double knockdown PC12 cells, despite more dramatic reduction in syntaxin-1 protein level compared to Munc18-1 single knockdown cells, no change in the level of syntaxin-1 mRNA was observed (Han et al., 2009). These findings are contradicting, thus needs to be clarified.

The mechanism underlying Munc18-1-dependent syntaxin-1 expression level is further complicated by the result shown in Chapter 4 of the thesis in which over-expressed syntaxin-1 could still be expressed in the absence of Munc18-1. This result implies that Munc18-1 deficiency has limited impact on the transcription of syntaxin-1 but it also suggests syntaxin-1 is not readily degraded even in the absence of Munc18-1 upon over-expression. This phenomenon could be interpreted in a way that under this condition, over-expressed syntaxin-1 level may be detectable because overly transcribed syntaxin-1 exceeds the quantity that can be processed by available ubiquitin/proteosome degradation system within a certain period of time. This means that protein turnover rate may be slower due to the overexpression. However, this is highly speculative and thus needs to be further confirmed. Clarifying the mechanism underlying Munc18-1-dependent syntaxin-1 expression level would provide significant insight into understanding how Munc18-1 regulates syntaxin-1 expression and localization.

6.4 The mechanism of Munc18-1-dependent dense-core vesicle docking

Another important finding from the study presented in this thesis is implication of Munc18-1 in dense-core vesicle (DCV) docking. Multiple lines of evidence from previous studies have suggested the positive contribution of Munc18-1 in DCV docking. However, the mechanism
underlying Munc18-1-regulated DCV docking has been unclear. The hypothesis for this study was that Munc18-1 indirectly contributes to DCV docking through regulation of syntaxin-1. This hypothesis was generated based on the previous findings that DCV docking is severely impaired in the absence of either Munc18-1 or syntaxin-1 and that these two proteins interact with high affinity. When we examined the ability of Munc18-1 K46E, E59K and K46E/E59K mutants to rescue vesicle docking through electron microscopic analysis in Chapter 3, we observed a strong correlation between the ability of the mutants to rescue syntaxin-1 expression and trafficking and to restore vesicle docking. This clearly indicated that these two components of the exocytotic process are closely related. Furthermore, the findings from the Chapter 4 that the abilities of the priming mutant, KE/5I, to restore syntaxin-1A expression, localization and dense-core vesicles docking without supporting exocytosis, further confirmed the notion that Munc18-1 regulates vesicle docking through syntaxin-1 regulation.

Based on the results presented in the thesis, we suggest that Munc18-1 contributes to vesicle docking by properly trafficking syntaxin-1 to the plasma membrane where syntaxin-1 can form a functional t-SNARE complex with SNAP-25 to serve as a docking site for vesicles. This means docking of secretory vesicles to the target membrane must involve vesicular proteins. The two main candidates that may serve as the docking factors include synaptobrevin-2 and synaptotagmin-1 as both these proteins are known to interact with t-SNAREs (de Wit et al., 2009). However, synaptobrevin-2 null mutants were shown not to exhibit any docking phenotype that is comparable to that of the Munc18-1 or syntaxin-1 null mutants (Borisovska et al., 2005). This lack of docking phenotype may be due to the presence of other isoforms although whether its isoform is able to functionally compensate for the loss of synaptobrevin-2 in vesicle docking needs to be further clarified. Another major candidate for vesicular docking phenotype is synaptotagmin-1. The importance of synaptotagmin-1 in vesicle docking is supported by strong docking defect
observed in synaptotagmin-1 null chromaffin cells (de Wit et al., 2009). This is also in line with the previous findings that demonstrated that synaptotagmin-1 provides a link between vesicles and syntaxin-1/SNAP-25 acceptor complexes through its specific interaction with SNAP-25 (de Wit et al., 2009). This study showed that over-expression of SNAP-25 can overcome docking defect in Munc18-1 deficient cells by increasing the amount of stable t-SNARE acceptor complex while SNAP-25 over-expression was no longer capable of restoring docking defect in synaptotagmin-1/Munc18-1 double knockout mutants (de Wit et al., 2009). These results led to the conclusion that all four proteins, syntaxin-1, SNAP-25, synaptotagmin-1, and Munc18-1 work together for efficient docking of vesicles. However, it still remains controversial which of the vesicular components contributes to vesicle docking. Nonetheless, our findings suggest that Munc18-1 plays a critical role in syntaxin-1 chaperoning which ensures proper localization of syntaxin-1 along the plasma membrane therefore enabling it to form stable t-SNARE acceptor complex with its cognate SNAP-25 partner for vesicles to properly dock at the plasma membrane. This chaperone activity of Munc18-1 relies on its high affinity binary interaction with syntaxin-1 which is mediated by domain-1 of Munc18-1.

Therefore, our results together with previous findings suggest that while docking is established through interaction between syntaxin-1/SNAP-25 acceptor complexes at the target membrane and synaptotagmin-1 or synaptobrevin-2 on the vesicular membrane, Munc18-1 is essential for promoting the formation or stability of the correct acceptor t-SNARE complex. However, it would be important to note that the findings from my study do not exclude the possibility that Munc18-1 directly contributes to vesicle docking through other means, perhaps by affecting the actin cortical network as previously suggested by other groups (Malacombe et al., 2006; Toonen et al., 2006a; Vitale et al., 1995). Therefore, more work needs to be done to
determine whether Munc18-1 contribution to dense-core vesicle docking is solely through its
effect on t-SNARE complex assembly or if it has any other direct contribution to vesicle docking.

The docking phenotype is more extensively studied with respect to dense-core vesicles due
to the specific characteristics. DCVs are more sparsely localized and high proportion (40-50%) of
DCVs is docked along the plasma membrane of neuroendocrine cells compared to synaptic
vesicles at the active zones. Therefore, docking phenotype is more evident for DCVs than SVs
upon genetic manipulations (de Wit et al., 2009). However, there has been discrepancy between
the effect of Munc18-1 on dense-core vesicle docking or synaptic vesicle docking despite the
consensus on the importance of this protein in secretion of both types of vesicles. In adrenal
chromaffin cells of Munc18-1 knockout mice, docking of DCVs was reduced by 90% while
synaptic vesicle docking was unaffected in neurons of the same mice (Verhage et al., 2000; Voets
et al., 2001). Therefore, it is of prime importance to clarify the role of Munc18-1 in both DCV and
synaptic vesicle docking as well.

6.5 The mechanisms underlying Munc18-1 mediated-priming of vesicle

In Chapter 3, we have demonstrated the important contribution of domain-1 of Munc18-1
in its syntaxin-1 stabilizing and trafficking activity which is mediated by high affinity binary
interaction. However, this syntaxin-1 chaperone activity alone is insufficient to explain the striking
phenotype of near complete loss of exocytosis observed in Munc18-1 deficient neurons,
chromaffin cells and Munc18-1/2 double knockdown PC12 cells (Han et al., 2011; Han et al.,
2009; Verhage et al., 2000; Voets et al., 2001). The observation that synaptic vesicle docking is
not affected despite complete abolishment in secretion (Verhage et al., 2000) or over-expression of
SNAP-25 can rescue defective docking phenotype but not secretion phenotype in Munc18-1
deficient chromaffin cells (de Wit et al., 2009) clearly indicates the post-docking role of Munc18-1.
In line with this, our findings in Chapter 4 that the secretion defect in Munc18-1/2 double knockdown PC12 cells cannot be restored by simple over-expression of syntaxin-1A, despite the partial localization of syntaxin-1A at the plasma membrane, further supports a critical role of Munc18-1 in exocytosis beyond its syntaxin-1 chaperone activity. Munc18-1 may play an essential role downstream of syntaxin-1 trafficking and vesicle docking, perhaps by directly stimulating SNARE-dependent membrane fusion/exocytosis. However, the direct evidence for Munc18-1 function in this late stage of exocytosis has been lacking largely due to difficulty in isolating the mutation that selectively impairs the priming function, therefore exocytosis, while retaining its function in syntaxin-1 trafficking and vesicle docking.

Domain-3a of Munc18-1 provides a binding cleft for syntaxin-1 together with domain-1. However, in contrast to domain-1, domain-3a of Munc18-1 is highly understudied. Therefore, in Chapter 4, we investigated the functional significance of the domain-3a by mutating the highly conserved domain-3a residues that have been suggested to make specific contact with syntaxin-1 according to the crystal structure of Munc18-1-syntaxin-1 binary interaction. Point mutations within domain-3a of Munc18-1 that have been investigated include: E278K, K314L/R315L, K332E, K333E, K332E/K333E, E278K/K332E/K333E, Q336A/Y337L, Y337L/Q338A, and Q336A/Y337L/Q338A. However, surprisingly none of these point mutations exhibited defective phenotype. It has been speculated that this functional resistance of the point mutants may be due to the structural flexibility of domain-3a of Munc18-1 in which this region appears to be unstructured in the unbound state thus is not greatly affected by the point mutations (Hu et al., 2010). However, this is at odds with the previous crystal structure analysis that demonstrated the importance of these residues in direct interaction with syntaxin-1. This may be due to the possibility that none of these residues make contact with syntaxin-1 through their side chains. In fact, it has been suggested that E278 does not make direct contact with syntaxin-1 through its side chain but rather
makes significant contact with E228 of syntaxin-1 through the main chain carbonyl group of E278. Likewise, other residues may not necessarily involve their side chains for direct interaction with syntaxin-1. However, whether this is really the case for other residues or not should be clarified.

Meanwhile, another study has reported the functional importance of domain-3a in the late stage of exocytosis by characterizing the Munc18-1 Y337L mutation (Boyd et al., 2008). Upon over-expression, Y337L was shown to act as a dominant negative mutant resulting in slowed and prolonged release of catecholamine from individual vesicles. However, this study has never assessed whether the abilities of this mutant to stabilize and traffic syntaxin-1 are retained. Nonetheless, in contrast to this study, the results from Chapter 4 suggest that Munc18-1 mutants that include Y337L mutation retain completely intact binding to the closed conformation of syntaxin-1 and that the abilities of the mutants to restore syntaxin-1 expression level and dense-core vesicle secretion defect in Munc18-1/-2 double knockdown cells are as efficient as that of wild-type. Therefore, a Munc18-1 mutation that selectively impairs the priming stage of exocytosis without affecting its syntaxin-1 chaperone function has yet been discovered to our knowledge at this point.

During the process of generating domain-3a point mutations using PCR-based site directed mutagenesis, we unexpectedly generated the novel insertion mutants, KE/5I and KE/39I, which represent the priming mutants that selectively impair secretion rescue ability while strongly retaining its abilities to restore syntaxin-1 chaperone activity and dense-core vesicle docking. We proposed that the impaired ability of the KE/5I mutant to restore secretion can be explained at least in part by its reduced binding to assembled SNARE complex compared to wild-type Munc18-1 or another Munc18-1 mutant, F115E, in our system.

Phe115 residue belongs to the hydrophobic domain of Munc18-1 that interacts with the N-terminal peptide of syntaxin-1. Therefore, mutating the F115 residue is expected to impair this N-
terminal binding between Munc18-1 and syntaxin-1. The interaction between Munc18-1 and N-peptide of opened syntaxin-1 has been suggested to underlie Munc18-1 interaction with the assembled SNARE complex which is important for Munc18-1 function during the membrane fusion. Unexpectedly, however, we did not observe a strong reduction in the binding of Munc18-1 hydrophobic pocket mutant (F115E) with the SNARE complex in our experimental conditions. This is at odds with the previous studies showing a striking reduction in binding of the same mutant to the SNARE complex (Malintan et al., 2009; Meijer et al., 2012). Nonetheless, syntaxin-1 N-peptide binding to Munc18-1 has been reported to be not highly selective as Munc18-1 binds to N-terminal peptides from both cognate and non-cognate syntaxin isoforms with the same binding affinity (Hu et al., 2010). This strongly suggests that other parts of the SNARE complex must be involved in binding between Munc18-1 and SNARE complex which seem to be highly specific (Hu et al., 2010). This alternative mechanism underlying Munc18-1-SNARE complex interaction may partially explain the observation that the mutation in Munc18-1 hydrophobic pocket (F115E) has a limited impact on the interaction between Munc18-1 and the SNARE complex as demonstrated in our study. This is also in line with the findings from other studies that showed the same mutant exhibits none or limited effect on its ability to rescue neurotransmitter exocytosis in Munc18-1 deficient neurons (Meijer et al., 2012) or Munc18-1/2 double knockdown PC12 cells (Han et al., 2009; Malintan et al., 2009).

The observation that the KE/5I mutant reduces its binding to the SNARE complex supports the hypothesis that the priming function of Munc18-1 may be mediated through the direct interaction with the SNARE complex. However, the ability of the mutant to bind to the SNARE complex was not completely impaired while its ability to support secretion was entirely abolished. This may be due to the presence of other domain-3a residues that participate in SNARE complex binding. Alternatively, this may be due to the presence of intact N-terminal binding mode in this
mutant. Currently, Munc18-1 is suggested to directly interact with the SNARE complex via two independent mechanisms: the first is through the N-terminal interaction with opened syntaxin-1 that is assembled into the SNARE complex and the second mechanism is via the binding between domain-3a of Munc18-1 and synaptobrevin-2 within SNARE complex (Xu et al., 2010). The finding that the KE/5I mutation within domain-3a of Munc18-1 impairs, although not abolishes, its binding to the SNARE complex is in line with the latter mechanism that supports the importance of domain-3a in the binding of Munc18-1 to the SNARE complex which may be crucial for directly stimulating SNARE-dependent exocytosis. Furthermore, the region of our mutation (insertion following K332E/K333E mutations) also coincides with the region of Munc18-1 (residues 333-339 (KMPQYQK)) that has been suggested to cross-link with the very C-terminal residues of synaptobrevin-2 (residues 87-91 (KYWWK))(Xu et al., 2010). Therefore, it can be postulated that the KE/5I mutant reduces its interaction with assembled SNARE complex, possibly due to loss of its contact with synaptobrevin-2 within the SNARE complex. The residual binding observed between the mutant and assembled SNARE complex may reflect its intact binding to the N-terminal peptide of syntaxin-1 which may also partly contribute to the SNARE complex interaction.

Moreover, a recent structural analysis of domain-3a suggested that this region can undergo structural changes from bent to extended upon binding of Munc18-1 with syntaxin-1 N-terminal peptide allowing for coiled-coil interaction with the SNARE complexes (Hu et al., 2010). The region within domain-3a that undergoes the conformation change has been mapped to include residues 295-358 with P335 residue as a hinge point that allows the domain to access both bent and extended structure. Considering that the site of the KE/5I mutation is in close proximity to this structurally significant residue of domain-3a of Munc18-1, it is possible that the KE/5I mutant not only reduces the direct binding to the SNARE complex but also interferes with the structural
flexibility of the domain-3a. This may interrupt this domain from undergoing the conformational change that is necessary for efficient interaction between Munc18-1 and syntaxin-1 N-peptide. This may in partly reflect a more severe reduction in SNARE complex binding upon mutating domain-3a compared to disrupting the N-terminal binding at least in our system. Although more research needs to be done to clarify the mechanism underlying Munc18-1-mediated priming of vesicle, the results presented in this thesis together with the previous findings clearly support the importance of domain-3a of Munc18-1 in this late stage of exocytosis through its interaction with SNARE complex. It could be postulated that the interaction of Munc18-1 with SNARE complex at multiple sites ensure proper positioning of Munc18-1 for it to exert its function during highly regulated fusion process.

6.6 Possible roles of Munc18-1 during the priming stage of exocytosis

Currently, the priming stage of exocytosis is not well defined largely due the abstruse mechanism underlying this process. In Chapter 4, “priming” was generally referred to a late stage of exocytosis that occurs after the docking but prior to the actual fusion although this may not be the most accurate term to describe this stage. The results from Chapter 4 clearly demonstrated that there is an important contribution of Munc18-1 at this late stage of exocytosis and this is at least partially mediated through the interaction between the domain-3a of Munc18-1 and assembled SNARE complex. However, it still remains unclear what kind of actions Munc18-1 is actually performing at this stage.

There are a few hypotheses regarding the role of Munc18-1 in the late stage of exocytosis beyond vesicle docking. Firstly, Munc18-1 may contribute to the priming stage by assisting the formation of the \( \text{trans-SNARE} \) complex that is formed by synaptobrevin-2, syntaxin-1, and SNAP-25. The reconstitution experiments have suggested that Munc18-1 facilitates \( \text{trans-SNARE} \)
complex assembly as it stimulates liposome fusion only when introduced after the t- and v-
SNARE liposomes were pre-incubated (Shen et al., 2007). The requirement for vesicle pre-
incubation suggests that Munc18-1 specifically acts on a transient, partially assembled
intermediate of the SNARE complex to promote the progression of the fusion process. This may
be mediated through Munc18-1 interaction with N-peptide of syntaxin-1. This is supported by the
evidence that Munc18-1 undergoes conformational change from a bent to extended hairpin
structure upon releasing its binding with the closed conformation and adopting N-terminal
interaction with syntaxin-1 (Hu et al., 2010). Therefore, it could be postulated that after Munc18-1
assists syntaxin-1 to the plasma membrane to form t-SNARE acceptor complex with SNAP-25 for
vesicle docking, Munc18-1 changes its structure to be compatible with binding to the N-terminal
peptide of syntaxin-1 to further assist in initial trans-SNARE complex formation. The possible
contribution of Munc18-1 at this step has been further supported by a recent in vitro reconstitution
assay (Ma et al., 2013). This study has demonstrated that Munc18-1 is capable of displacing
SNAP-25 from syntaxin-1 to protect syntaxin-1 from forming a non-productive t-SNARE complex
with SNAP-25 that does not lead to efficient fusion. Then, together with Munc13-1, it leads to
membrane fusion through an NSF-α-SNAP-resistant pathway by allowing the three SNAREs to
properly assemble together, initiating trans-SNARE complex formation (Ma et al., 2013).

Secondly, Munc18-1 may contribute to the stabilization of trans-SNARE complexes.
Direct evidence for this notion is limited. However, the observation that Munc18-1 only binds to
and activates SNARE complexes formed of cognate SNAREs reflect its ability to recognize the
specificity of the fusion reaction (Shen et al., 2010). This also suggests that Munc18-1 may have
the ability to distinguish between trans-SNARE complexes from cis-SNARE complexes, thereby
only protecting the trans-SNARE complex. This highly specific interaction between Munc18-1
and cognate SNARE complexes may be essential for efficient fusion processes. This idea could be
supported in respect to the highly conserved function throughout SM proteins despite their distinctive subcellular localization. For example, in yeast vacuolar fusion, tethering complex HOPS, which consists of SM protein called Vps33p, binds to and stabilizes trans-SNARE complexes to prevent their dissociation by NSF-SNAPs (Collins et al., 2005; Rizo and Südhof, 2012; Starai et al., 2008). This means Munc18-1 could play a similar role in which it keeps trans-SNARE complex stable until it is exposed to the following proper sequence of events leading to exocytosis. Indeed, a similar contribution of Munc18-1 in membrane fusion through NSF-α-SNAP resistant pathway has been demonstrated in the reconstitution system (Ma et al., 2013). This function may seem to be redundant to that of complexin which has been suggested to play a critical role in both activating and clamping trans-SNARE complex. However, it could be speculated that these two proteins both contribute to this process sequentially in precise time-dependent manner. For example, Munc18-1 may be responsible for stabilizing the trans-SNARE complex until complexin gets recruited to the site or these two proteins may simultaneously contribute to this step but through different interactive sites on SNARE complex. However, this is a highly speculative idea thus needs to be further investigated. Nonetheless, the ability of Munc18-1 to distinguish cognate SNARE complexes seems to rely on a direct interaction between Munc18-1 and the core SNARE helical bundle, especially as N-terminal interaction has been suggested to be non-selective. The interaction between Munc18-1 and the SNARE complex has been suggested to involve residues of domain-3a lining the central cavity of Munc18-1 (residues 333-339) and very c-terminal residues of synaptobrevin-2 (residues 87-91) (Xu et al., 2010). This infers that Munc18-1 could interact with synaptobrevin-2 within SNARE complex by embracing the four-helical bundle in its central cavity formed by the domain-1 and -3a in a similar configuration as to when it holds four helical bundles formed from closed syntaxin-1 during high affinity binary interaction (Shen et al., 2010; Xu et al., 2010). Indeed, the $H_{abc}$ domain of syntaxin-1 has been
shown to compete with four helical bundles of SNARE complex (Xu et al., 2010). Therefore, it could be hypothesized that this highly specific Munc18-1-SNARE core interaction enables the stabilization of trans-SNARE complex.

The binding of Munc18-1 to the membrane proximal region of the SNARE complex may place Munc18-1 right at the site where membrane fusion occurs so it can further regulate this process. This has led to another hypothesis that Munc18-1 plays an essential and more direct role in membrane fusion by exerting a force on the membrane together with the SNAREs (Rizo and Südhof, 2012). In this model, Munc18-1 would prevent vesicular and plasma membrane from being too close in distance by staying bound to the C-terminal region of SNARE complex. Meanwhile, the tendency of SNARE complex to try zippering at the C-terminal would attempt to pull the two membranes together. This may exert larger force on the membrane structure for efficient membrane fusion (Rizo and Südhof, 2012). This notion that Munc18-1 has a direct role in fusion is further supported by the previous finding that Sec1p, the yeast homologue of Munc18-1, has a function after SNARE complex assembly as Sec1p mutants were shown to inhibit secretion in the presence of assembled SNARE complex (Grote et al., 2000). However, this model is not supported by enough evidence therefore needs to be further investigated. Despite the various possible roles that Munc18-1 could be playing at this late stage of exocytosis prior to the actual membrane fusion, exact function still remains unknown. Therefore, continuous investigation is critically required to elucidate the mechanism underlying this indispensable function of Munc18-1 in exocytosis.
6.7 Uncoupling the mechanisms underlying the domain-specific role of Munc18-1 in syntaxin-1 chaperoning and dense-core vesicle priming

The results discussed in Chapter 3, 4 and 5 of the thesis, together with previous findings clearly support multiple roles of Munc18-1 in exocytosis. In addition to Munc18-1/-2 double knockdown and rescued phenotypes shown in this study, the observation that the secretion defect of Munc18-1/-2 double knockdown cells cannot be rescued at all upon syntaxin-1 overexpression despite its partial, although very low, localization along the plasma membrane clearly indicates post-syntaxin-1 trafficking activity of Munc18-1 during membrane fusion. At the same time, inefficient localization of over-expressed syntaxin-1 in the absence of Munc18-1 demonstrates the importance of Munc18-1 in syntaxin-1 trafficking. Nonetheless, the observation that syntaxin-1 could be at least partially localize at the plasma membrane also indicates that it is possible for syntaxin-1 to properly traffic to the plasma membrane even in the absence of Munc18-1. This is in line with the previous finding that suggested that despite dramatic reduction in syntaxin-1 level, the residual syntaxin-1 in Munc18-1 knockout mice can still correctly target to the synapse and efficiently form SNARE complexes (Toonen et al., 2005). Furthermore, it has been reported that overexpression of the yeast syntaxin homologs Sso1 or Sso2 can partially suppress a partial loss-of-function sec1 mutation (Aalto et al., 1993). Nonetheless, membrane fusion was completely absent even in presence of properly formed SNARE complexes with residual syntaxin-1 in Munc18-1 knockout mice, indicating that Munc18-1 either controls the spatially correct assembly of SNARE complexes for SNARE-dependent fusion, or acts as a direct component of the fusion machinery itself (Toonen et al., 2005). Based on the findings from current study together with previous observations, it is clear that Munc18-1 plays at least dual function during exocytosis. Firstly, it increases the efficiency of proper syntaxin-1 trafficking to the plasma membrane by
chaperoning syntaxin-1 then it makes important contribution to dense-core vesicle priming stage beyond dense-core vesicle docking.

The syntaxin-1 over-expressed Munc18-1/-2 double knockdown cells which exhibit partial localization of syntaxin-1 along the plasma membrane have clearly served as an advantageous tool to further dissect the chaperone activity and priming role of Munc18-1. Comparing the phenotypes of the priming mutants (KE/51 and KE/39I) with the chaperoning mutant (K46E/E59K) in presence of partial localization of syntaxin-1 allowed us to distinguish distinct functions of Munc18-1 that are independently regulated by specific domains. The finding that the chaperoning mutant can partially rescue exocytosis in the presence of over-expressed syntaxin-1A indicates that this mutant retains the priming activity despite the impaired chaperone activity. This suggests that the mutations (K46E/E59K) within the domain-1 of Munc18-1 selectively affect its binary interactive ability with monomeric syntaxin-1 which is critical for its syntaxin-1 chaperone function. However, it could be presumed that this mutation does not impair the ability of this protein to adopt a different binding mode that is important for the downstream function of Munc18-1, perhaps at the priming stage. Therefore, it has become more evident that the inability of the chaperoning mutant to restore the secretion defect is due to the interference in the earlier stage of secretory pathway (syntaxin-1 chaperoning) that consequently leads to perturbation in the following stages. In contrast, expressing the priming mutant (KE/39I) in presence of over-expressed syntaxin-1 resulted in much more efficient localization of over-expressed syntaxin-1. However, this mutant still failed to rescue secretion defect in these cells, further confirming the detrimental effect of the mutant in the later stage of exocytosis. Together, we have demonstrated that the mutations in different region of Munc18-1 resulted in selective impairment in specific stage of exocytosis. It seems that the chaperone activity largely depends on functional domain-1 and domain-3a residues that contribute to the high affinity binary interaction while priming role of
this protein is mediated through functional domain-3a residues that interact with assembled
SNARE complex. The fact that these two functions can be selectively manipulated clearly
indicates that the chaperone activity and priming function of Munc18-1 are mediated through
independent mechanism that involves specific region of the protein.

6.8 The proposed model: sequential action of Munc18-1

Based on the findings presented in this thesis together with the results from the previous
studies, the following model for Munc18-1 action in exocytosis could be postulated: Munc18-1
binds to the closed conformation of syntaxin-1 to both stabilize and properly traffic syntaxin-1 to
the plasma membrane. This interaction is highly dependent on functional domain-1 and domain-3a
residues lining the central cavity formed by these two domains of Munc18-1. In this binding mode,
Munc18-1 embraces the four helical bundles of syntaxin-1 in its horseshoe shaped central cavity.
At this stage, whether Munc18-1 further interacts with N-terminal peptide of syntaxin-1 to further
secure this binary interaction or not still remains highly debated. When Munc18-1- syntaxin-1
complex reaches the plasma membrane, Munc18-1 releases its binding to closed syntaxin-1 and
allows syntaxin-1 to adopt an open-conformation which is compatible with SNARE complex
formation. At this point, domain-3a of Munc18-1 may undergo a conformational change to release
its binding with monomeric syntaxin-1 and adopt an N-terminal binding mode which allows
Munc18-1 to stay bound to syntaxin-1 while syntaxin-1 assembles into SNARE complex along
with its cognate SNARE partners, SNAP-25 and synaptobrevin-2. Therefore, this N-terminal
interaction between Munc18-1 and syntaxin-1 may serve to assist in initial trans-SNARE complex
formation bringing the vesicular and plasma membrane in more proximity. Then Munc18-1 may
switch its interaction site to four helical bundles of SNARE-complex. Whether Munc18-1
simultaneously maintains its interaction with N-peptide of syntaxin-1 at this point or it
sequentially transits its interaction site from N-terminal peptide to the core SNARE complex remains unclear. Nonetheless, binding of Munc18-1 to C-terminal region of synaptobrevin-2 within SNARE complex seem to underlie the function of Munc18-1 in the late stage of exocytosis in which Munc18-1 stabilizes trans-SNARE complex formation to prepare it for the final fusion stage. The highly specific interaction between Munc18-1 and the core SNARE bundles may both spatially and temporally regulate membrane fusion for efficient exocytosis.

6.9 Strengths/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.

6.9.1 Strengths/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 pleiotropic function of Munc18-1 were studied using Munc18-1/-2 double knockdown PC12 cell line. The use of this cell line over primary neuronal 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 neurons. 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, PC12 cells maintain a robust exocytotic phenotype in response to \( \text{Ca}^{2+} \) influx, providing a set of important experimental advantages for cell biological and biochemical studies of the secretory pathway. Moreover, sparse localization of DCVs and high proportion of docked DCVs in the neuroendocrine cells allow this cell line to serve as a better model to study the docking phenotype compared to the neurons. Furthermore, the ability of these cells to differentiate into neuronal like cells in response to nerve growth factor (NGF) (Huff and Guroff, 1979) enables to visualize the localization of syntaxin-1 more readily and clearly compared to relatively small active zone of neurons (Ng et al., 2002).

Despite all these advantages, this cell line has several limitations that must be recognized to accurately interpret experimental results and avoid drawing premature conclusions. The PC12 cells represent a more important model for neuroendocrine/endocrine DCV exocytosis than for neuronal SV exocytosis. There are many important mechanistic similarities between DCV and SV exocytosis. However, \( \text{Ca}^{2+} \)-evoked DCV exocytosis is relatively slower than the neuronal synaptic vesicle exocytosis, although the basis for this difference is unclear (Ninomiya et al., 1997). The basis for this slower kinetics of DCV exocytosis in PC12 cells needs to be elucidated in order to extrapolate the findings in this model system to the mechanisms underlying faster modes of synaptic vesicle exocytosis (Martin and Grishanin, 2003). Indeed, inconsistent phenotypes of the same Munc18-1 mutations have been often observed between dense-core vesicle secretory pathway and synaptic vesicle secretory pathway. For example, DCV docking is severely perturbed in Munc18-1 deficient chromaffin cells or PC12 cells while synaptic vesicle docking is not affected in Munc18-1 null neurons (Verhage et al., 2000; Voets et al., 2001). Moreover, N-terminal binding has shown to be critical for synaptic transmission while dispensable for dense-core vesicle exocytosis (Han et al., 2009; Johnson et al., 2009; Malintan et al., 2009). However, the importance of N-peptide binding in synaptic transmission is challenged by the observation that
the F115E mutant that impairs the SNARE complex binding is capable of rescuing synaptic vesicle secretion (Meijer et al., 2012). Therefore, it is important to verify the findings from PC12 cells in neurons in order to better understand whether these two secretory pathways are regulated through the same mechanisms. Most importantly, although studies using cell lines can provide with very important information regarding the biological or biochemical mechanisms underlying phenomena of interest, in vitro analysis should always be supplemented with in vivo analysis to ensure the physiological relevance.

For the study in the thesis, shRNA-mediated Munc18-1/-2 double knockdown PC12 cells were used to study the phenotypes of various Munc18-1 mutants. Munc18-1/-2 DKD cells exhibit very severe phenotypes including dramatic reduction of syntaxin-1 expression level, severe localization of syntaxin-1, perturbed dense-core vesicle docking, and severe defect in dense-core vesicle secretion (Han et al., 2009). Fortunately, all the defective phenotypes could be rescued upon re-expression of wild-type Munc18-1. This is a great advantage for assessing the functional deficits of the mutations introduced in Munc18-1. However, protein knockdown 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 knockout method. Therefore, it is often considered that the effect of knockdown is transient and does not result in 100% deletion of the protein, raising a concern regarding the possible functional contribution of residual wild-type protein when assessing the phenotype of the mutant. However, our Munc18-1/-2 DKD cells were engineered in such way that shRNA targeting both Munc18-1 and Munc18-2 are stably expressed through continuous drug selection using puromycin and G418, respectively. Initial selection of shRNA expressing cells using these drugs followed by continuous culturing in presence of both drugs ensures the stable expression of shRNA for the target mRNAs in surviving cells. Although it is arguable that there may be residual wild-type Munc18-1 in cells, we always compare the
phenotypes of the mutants to that of the negative control (Munc18-1/-2 DKD cells infected with empty Em-GFP vector). Moreover, it is important to acknowledge that the severe phenotypes observed in the absence of Munc18-1 is comparable to Munc18-1 knockout neurons which suggests that such a minimum trace of Munc18-1 is insufficient to fully perform the functional role of Munc18-1 in mammalian cells.

Another limitation associated with this system involves inability to control the level of exogenously expressed protein. For my study, Munc18-1/-2 DKD PC12 cells were further stably rescued with various domain-specific Munc18-1 mutants through lentiviral infection system. The mutant gene was carried into the cells by pLVX CMV Em-GFP IRES blast vector. An IRES (internal ribosomal entry site) is a nucleotide sequence that allows for translation in the middle of a mRNA sequence during protein synthesis (Pelletier and Sonenberg, 1988). The presence of an IRES (internal ribosomal entry site) 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 Munc18-1 variants seem to be often over-expressed compared to the endogenous wild-type Munc18-1. Although comparable level of expression of exogenously introduced wild-type Munc18-1 and the mutants have allowed reasonable comparison of the phenotypes, whether there is any artifactual effect exerted by the over-expressed Munc18-1 variants on the general phenotypes of the cells need to be carefully considered during the interpretation of the results before generating conclusions.
6.9.2 Strengths/Limitations in the experimental approaches

6.9.2.1 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 Munc18-1 variants and monomeric syntaxin-1. Studying protein interaction using this 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; Miller and Stagljar, 2004). 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. Since it is well established that Munc18-1 and syntaxin-1 interacts with high affinity, this system was a great approach to study whether the mutations within Munc18-1 affects its interaction with syntaxin-1. 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. In our case, we transform empty vector for a negative control to make sure that there is no unspecific interaction between Munc18-1 and syntaxin-1. 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 may be over-estimated. Therefore, it is essential to consider these limitation of the technique when interpreting the data and drawing the conclusion from the data.

One puzzling observation has been made during the study of the binary interaction between syntaxin-1 and Munc18-1 variants, owing to the lack of sensitivity of this technique. In Chapter 3, the K46E/E59K mutant has been shown to completely lose its interaction with syntaxin-1. Theoretically, however, this result is quite surprising as it is believed that Munc18-1 can also interact with N-terminal peptide of syntaxin-1 through its outer surface of the hydrophobic pocket in domain-1. This means that even if the central cavity of Munc18-1 was mutated to disrupt its binary interaction with syntaxin-1, the residues that are thought to be important for N-terminal interaction are still intact in this mutant. Therefore, we should expect to be able to detect the residual signal that reflects intact N-terminal interaction between the Munc18-1 mutant and syntaxin-1. However, we did not observe such residual signal from interaction between the Munc18-1 K46E/E59K and syntaxin-1. This could be due to the possibility that N-terminal interaction is too weak to be detected in this experimental setting. However, yeast two-hybrid assay should be sensitive enough to detect even a very weak interaction. Another possibility would be that pVP16-syntaxin-1A construct has been designed in a way that the transcriptional activation domain of VP16 resides at the N-terminus of syntaxin-1. This may have somehow interfered with the interaction between the N-peptide of syntaxin-1 and Munc18-1 variants thus the signal detected from this assay solely reflects the binary interaction between the central cavity of Munc18-1 and four helical bundles of closed syntaxin-1.

Nonetheless, it is always important to further confirm the interactions detected using this method by employing other biochemical techniques. In this thesis, the binary interaction between
Munc18-1 and syntaxin-1 assessed by yeast two-hybrid assay were further confirmed by isothermal titration calorimetric (ITC) measurement (Chapter 3) or pull-down assay using GST-syntaxin-1 (Chapter 4). However, it should be noted that in all cases only the cytosolic domain of syntaxin-1 and/or synaptobrevin-2 were used to study both binary interaction and SNARE complex interaction. Considering that syntaxin-1 and snaptobrevin-2 both exist as membrane proteins with transmembrane regions in natural condition, it would be important to confirm whether Munc18-1 exhibit consistent interactive phenotype with full-length SNARE proteins. This will make important contribution for clarifying whether interactions observed by in vitro assays fairly reflect physiological scenario.

6.9.2.2 Assaying priming function of Munc18-1

In Chapter 4 of the thesis, it has been proposed that the KE/5I mutant represents the true “priming” mutant as it selectively impairs secretory function while retaining syntaxin-1 chaperone function. The KE/5I mutant has been designated as a priming mutant based on the observation that the mutation interferes with Munc18-1 function beyond vesicle docking assuming that Munc18-1 does not directly contribute to the fusion pore formation. However, it is arguable that this conclusion is largely justified as all that can be drawn from the phenotypes of the mutant is that it impairs the overall extent of exocytosis without affecting vesicle docking since the priming role of Munc18-1 has not been directly assessed.

Priming is generally understood to occur after the docking of vesicles but prior to the actual fusion, but what exactly is happening at this stage of exocytosis is unknown. Therefore, it is very difficult to directly assess the effect of mutations on this stage. However, it is believed that the priming stage could be defined based on specific assays that are usually carried out in neuronal preparations. During the priming stage, morphologically docked vesicles are thought to undergo
maturation to become fusion–competent so that they can be rapidly released upon Ca^{2+} trigger. This pool of vesicles that are readily releasable upon appropriate stimulation are thought to represent the pool of “primed” vesicles (Liu and Tsien, 1995; Schneggenburger et al., 1999; Stevens and Tsujimoto, 1995; von Gersdorff and Matthews, 1997; Wang and Kaczmarek, 1998; Wu and Borst, 1999). Therefore, the size of readily releasable pool of vesicles is often used as an indicative of intact or impaired priming stage in cells. Hypertonic sucrose stimulation is often used as a measure of priming as this method is known to stimulate only the secretion of already primed vesicles (readily releasable vesicles) by an unknown, Ca^{2+}-independent mechanism (Fatt and Katz, 1952). However, whether this method directly measures the priming activity of the cells is unclear as the molecular nature of the readily releasable pool is unknown. Considering that trans-SNARE complex formation is one of the events that should occur during priming of vesicles, it could be postulated that readily releasable vesicles represent a pool of vesicles that are tethered to the plasma membrane upon partial assembly of trans-SNARE complex (Lonart and Südhof, 2000).

Priming step could also be assessed by performing intracellular Ca^{2+}-uncaging followed by patch clamp capacitance measurement in which primed vesicles are rapidly released by direct stimulation with intracellular Ca^{2+}. It is believed that the different states of vesicles can be identified by the rate at which they fuse with the plasma membrane. For example, in chromaffin cells, a stepwise increase in [Ca^{2+}], has been suggested to lead to two kinetic components of secretion, a burst phase and a sustained phase as measured by membrane capacitance increase (Liu et al., 2010). Vesicles that fuse during the burst phase are presumably primed vesicles. Docked vesicles which are primed while [Ca^{2+}] remains high would be released during the sustained component (Haller et al., 1998; Neher and Marty, 1982). However, estimating priming and docking of vesicles with this method is indirect as membrane capacitance recording measures only
the increase in surface area upon fusion of vesicles with the plasma membrane. Alternatively, priming can be assessed through real-time imaging by total internal microscopic (TIRFM) analysis which allows to visualize the movement of vesicles within 200nm from the plasma membrane in live cells (Allersma et al., 2004; Ambrose, 1956; Axelrod, 1981; Tsuboi and Rutter, 2003). In the absence of stimulation, fluorescently labeled vesicles that are in proximity to the plasma membrane will be visualized. Then upon stimulation, the fusion kinetics of the docked vesicles can be observed by the rate at which the fluorescence signal disappears. This will allow assessing the fusion competence of the docked vesicles. However, as TIRFM only enables selective visualization of vesicles at the membrane close to the glass/cell interface, whether secretion observed at the footprint of the cell can represent the global exocytotic events over the entire cell surface is debatable. Together, assessing the role of Munc18-1 in the priming stage using additional assays would help to further address the importance of domain-3a of Munc18-1 in the later stage of exocytosis. Nonetheless, the strongly perturbed secretory ability of the KE/5I and KE/39I mutants without affecting the previous steps of exocytosis in addition to its largely reduced SNARE complex binding which is thought to be an underlying mechanism of the priming function of Munc18-1 clearly support the important role of Munc18-1 in the late stage of exocytosis which is highly likely to be at the priming stage.

6.10 Future directions

Current work has been focused on identifying the critical domains of Munc18-1 that are involved in different interaction modes of Munc18-1 and consequently in its pleiotropic functions. The results from the thesis clearly deliver two important messages regarding the mechanisms underlying Munc18-1 function in exocytosis. Firstly, both domain-1 and -3a of Munc18-1 plays a critical role in high affinity binary interaction with monomeric syntaxin-1 which is critical for its
synatxin-1 chaperone activity and consequently in dense-core vesicle secretion. Moreover, domain-3a of Munc18-1 plays a crucial role in the late stage of exocytosis, perhaps at the priming stage, through its interaction with assembled SNARE complex. Despite the clear messages delivered in the study, much more work needs to be done in order to completely understand the functions of Munc18-1 in exocytosis. This will require the molecular, cellular and physiological aspects of its roles to be converged. The first step towards this relies on filling in the gaps of the current studies.

In the thesis, it has been suggested that syntaxin-1 expression depends on the high affinity binary interaction between Munc18-1 and synatxin-1 as mutations in Munc18-1 that disrupt this binding results in reduction in cellular level of syntaxin-1. However, the mechanism of this decreased syntaxin-1 level is unclear although it is highly likely caused by instability of syntaxin-1 that leads to rapid degradation. Therefore, this hypothesis needs to be verified. Firstly, any change in mRNA level of syntaxin-1 in the absence of Munc18-1 should be verified, perhaps by using RT-PCR. If no difference is observed, whether syntaxin-1 is more prone to degradation in the absence of Munc18-1 can be assessed by investigating for any upregulation in ubiquitylated syntaxin-1. This could be done by immunoprecipitating syntaxin-1 and further probing with antibody against ubiquitin. Alternatively, proteasome-specific inhibitors such as lactacystin (Corey and Li, 1999; Fenteany et al., 1994; Omura et al., 1991) or epoxomicin (Meng et al., 1999; Sin et al., 1999) that interfere with the ubiquitin/proteasome pathway could be used to see if this alleviates the degradation of syntaxin-1 in the absence of Munc18-1. 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; Sharma et al., 2012). This will allow comparing the stability of syntaxin-1 in presence or absence of functional Munc18-1. Clarifying the mechanism underlying Munc18-1-dependent syntaxin-1 expression/degradation
would provide important information regarding whether Munc18-1 affects syntaxin-1 synthesis or stability.

The results from the thesis has also demonstrated that the ability of Munc18-1 to restore defective docking in Munc18-1/-2 double knockdown PC12 cells relies on its ability to rescue syntaxin-1 chaperone activity, thus suggesting that Munc18-1 indirectly mediates vesicle docking through syntaxin-1 regulation. Nonetheless, it has previously been suggested that Munc18-1 may affect docking phenotype by regulating the density or fenestration of the subplasmalemmal actin cytomatrix (Toonen et al., 2006a). Therefore, it would be very interesting to investigate whether there is any difference in the density or organization of filamentous actin network in the cells expressing either Munc18-1 mutants that cannot rescue vesicle docking or wild-type Munc18-1. The cortical subplasmalemmal network of actin filaments can be visualized by probing cells for filamentous actin followed by fluorescence microscopy (Falzone et al., 2012). This will further clarify the mechanism underlying Munc18-1 mediated vesicle docking.

Discovery of domain-3a mutants that selectively impairs the priming function has clearly demonstrated the importance of this domain of Munc18-1 in the later stage of exocytosis beyond syntaxin-1 chaperoning and dense-core vesicle docking stage. However, this does not exclude the possible contribution of this domain in the earlier stages of the secretory pathway. Indeed, the results from Chapter 5 suggest that domain-3a also contributes to the chaperone activity of Munc18-1. We have shown that K332E/K333E or Q336A/Y337L double mutations do not exhibit defective phenotype. However, when K332, K333, P335, Q336, and Y337 residues were mutated simultaneously (quintuple K332E/K333E/P335A/Q336A/Y337L), the ability of Munc18-1 to interact with monomeric syntaxin-1 was abolished and resulted in severe defect in the ability to restore cellular syntaxin-1 expression level and consequently resulted in impaired dense-core vesicle secretion. This is not surprising as all of these residues were highlighted in the previous
crystal structure to make direct contact with syntaxin-1 in closed conformation. In contrast, when K332, K333, Q336, and Y337 residues were mutated without manipulating P335 residue, the ability of the quadruple mutant to interact with monomeric syntaxin-1 was not impaired. The detrimental phenotype observed upon additional mutation in P335 residue implies that this residue has an important functional contribution. This is intriguing as P335 residue has previously been designated as a hinge point that allows domain-3a of Munc18-1 to undergo conformational change from bent to extended which allows it to adopt different binding mode (Hu et al., 2010). Therefore, it would be interesting to investigate the functional importance of P335 residue by analyzing the phenotype of Munc18-1 upon mutating the P335 residue alone in addition to mutations that target the flanking residues in addition to P335 residue. Furthermore, studying the phenotype of K332E/K333E/Q336A/Y337L quadruple mutant in the absence of P335A mutant will allow to further determine the functional contribution of P335 residue in the quintuple mutation that result in chaperoning defect. Narrowing down the residues important for this additional chaperone activity mediated by the domain-3a will further clarify the functional importance of domain-3a in exocytosis.

The current thesis has focused on integrating how the structural specificity and interactive property of Munc18-1 contribute to its essential roles in exocytosis. Understanding how Munc18-1 regulates exocytosis at molecular level is critical as this protein is an indispensable part of the fusion machinery. With the knowledge obtained from the studies discussed in this thesis, the next big step would be to understand how insufficiency in functional Munc18-1 is related to diseases in humans. De novo mutations in Munc18-1 have been associated with a wide spectrum of early infantile epileptic encephalopathy (EIEE), such as Ohtahara Syndrome, West Syndrome, and mental retardation with or without epilepsy in humans (Hamdan et al., 2011; Hamdan et al., 2009; Milh et al., 2011; Otsuka et al., 2010; Saitsu et al., 2008; Saitsu et al., 2010). Initially, V84D,
C180Y, M443R, and G544D mutations in Munc18-1 were screened in unrelated individuals with Ohtahara syndrome, which is one of the most severe and earliest forms of epilepsy that is characterized by early onset of tonic spasms, intractable seizure, and severe psychomotor retardation (Saitsu et al., 2008; Saitsu et al., 2010). A missense mutation, C180Y, showed structural instability and impaired binding to syntaxin-1A. However, their biochemical approaches did not analyze how the expression of these proteins would affect cellular phenotype. More recent studies have identified novel missense mutations in Munc18-1 that are implicated in Ohtahara syndrome and non-syndromic mental retardation. These mutations include L183R, R406H, P480L, C552R, and T574P (Hamdan et al., 2009; Mignot et al., 2011; Milh et al., 2011; Otsuka et al., 2010; Saitsu et al., 2010). Nonetheless, biological aspects of these mutants have never been assessed. Given the critical role of Munc18-1 in neuronal exocytosis, it could be hypothesized that these Munc18-1 mutants are functionally impaired leading to disrupted synaptic function in the human brain. However, detailed analysis on how these mutants affect neurosecretion is lacking. Therefore, it would be very interesting to characterize the nature of these mutations by investigating the mechanisms underlying the effect of these EIEE-associated mutants in neurosecretion. This can be done by thoroughly assessing their ability to interact with syntaxin-1, to chaperone syntaxin-1 and to restore vesicle docking and secretion. One of the following outcomes could be anticipated: 1) the mutation lead to structural instability leading to inefficient expression; 2) the mutation impairs binding to syntaxin-1 and consequently its chaperone function; 3) the mutation disrupts SNARE complex binding thus impairs priming function of the protein.

Preliminary findings regarding the binding ability of EIEE-associated Munc18-1 mutants, L183R, R406H, P480L, and C552R, revealed that L183R and C552R retain intact binary interaction with syntaxin-1 while R406H and P480L loses its ability to bind to monomeric syntaxin-1 (Figure 6.1). This clearly indicates that these mutants behave differently although they are all implicated in
detrimental phenotypes observed in affected individuals. Therefore, the results from this study will elucidate whether a common pathological mechanism exist among various forms of epilepsy associated with different Munc18-1 mutations. Understanding the molecular mechanisms underlying disease phenotypes will provide new insight into intractable spasms in infancy.
Figure 6.1 | Interactive properties of Munc18-1 mutants implicated in early infantile epileptic encephalopathy (EIEE). Binding between Munc18-1 mutants, L183R, R406H, P480L, or C552R, and syntaxin-1 was analyzed by yeast-two-hybrid assays. 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 Munc18-1 was set to 100%. Error bars indicate SEM (n=9-14).
6.11 Concluding Remarks

In summary, the results from the study presented in the thesis have demonstrated that the pleiotropic functions of Munc18-1 are mediated through distinctive binding modes with SNARE proteins which involve specific regions of the Munc18-1. Using Munc18-1/-2 double knockdown PC12 cells as a model system, it was found that high affinity interaction between domain-1 or domain-3a of Munc18-1 and syntaxin-1 is critical for syntaxin-1 chaperone activity of Munc18-1 which stabilizes and properly localizes syntaxin-1 to the plasma membrane (Chapter 3 and 5). Furthermore, interaction between domain-3a and pre-assembled SNARE complex has been demonstrated to underlie an important role of Munc18-1 in the later stage of exocytosis, perhaps at the priming stage (Chapter 4). These findings have not only greatly contributed to clarifying previous discrepancy revolving the functional importance of Munc18-1 but also revealed novel information that the chaperone activity and priming activity of Munc18-1 represent the sequential function of Munc18-1 that is mediated through independent mechanism. Overall, the findings from this study have provided thorough mechanistic insight into the structural/functional relationship of Munc18-1 in regulation of dense-core vesicle exocytosis. Dense-core vesicle secretion is critical for release of diverse signaling molecules such as hormones, growth factors, neuropeptides, and catecholamines which serve a wide range of physiological functions that is essential for maintaining the homeostasis of an organism. Exocytosis is a fundamental physiological 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 including neurons, neuroendocrine cells and endocrine 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


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