Roles of Sec5 in the Regulation of Dense-Core Vesicle Secretion in PC12 Cells

by

Tiandan Jiang

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Abstract

Thesis Title: Roles of Sec5 in the Regulation of Dense-Core Vesicle Secretion in PC12 Cells

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Student Name: Tiandan Jiang

Department of Physiology, University of Toronto

The exocyst is thought to tether secretory vesicles to specific sites on the plasma membrane. As a member of the exocyst, Sec5 is implicated in cell survival and membrane growth in *Drosophila*. Little is known of the exocyst function in mammals, with previous work suggesting involvement of exocyst in GTP-dependent exocytosis. Using RNA interference, we stably down-regulated Sec5 in PC12 cells. We found that these knockdown cells exhibit decreased GTP- and Ca^{2+}-dependent exocytosis of dense-core vesicles (DCVs), and contain less proportion of docked vesicles. Expression of Sec6/8 is also slightly reduced in Sec5 knockdown cells. Our results suggest that Sec5 is involved in both GTP- and Ca^{2+}-dependent exocytosis, possibly through the regulation of DCV docking. We also established doxycycline-inducible knockdown system for Sec5 in PC12 cells which may be more appropriate to study development-related proteins. Efforts were also made to re-introduce Sec5 into the Sec5 knockdown cells for rescue purposes.
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Abbreviations

α-SNAP       α-Soluble NSF-acceptor Proteins
BAPTA        1,2-bis(o-aminophenoxy)ethane-N,N,N’,N’-tetraacetic Acid
BoNT/E-LC    Light Chain of Botulinum Neurotoxin Type E
BSA          Bovine Serum Albumin
CAPS1        Calcium-dependent Activator Protein for Secretion 1
Cdc42         Cell Division Cycle 42
DCVs          Dense Core Vesicles
DG            Diacylglycerol
DMEM         Dulbecco’s Modified Eagle Medium
DOX          Doxycycline
dsRNA        Double Stranded RNA
EDTA         Ethylenediaminetetraacetic Acid
EGTA        Ethylene Glycol Tetraacetic Acid
EM           Electron Microscopy
ER           Endoplasmic Reticulum
FCS          Fetal Calf Serum
GABA         γ-Aminobutyric Acid
GFP          Green Fluorescent Protein
GTP          Guanosine-5’-triphosphate
GMP-PNP     Nonhydrolyzable GTP Analogue Guanosine 5’-(β,γ-imido)triphosphate
GppNHp       β,γ-imidoguanosine 5’-triphosphate
GTPγS        Guanosine 5’-[γ-thio]triphosphate
GDPβS        Guanosine 5’-[β-thio]diphosphate
GST          Glutathione S-transferase
HEK293FT     Modified Human Embryonic Kidney 293 Fibroblast
HEPES        4-(2-hydroxyethyl)-1-piperazineethanesulfonic Acid
HIV          Human Immunodeficiency Virus
IP3          Inositol Triphosphate
IRES         Internal Ribosome Entry Site
KD           Knock Down
KO           Knock Out
KRAB         Kruppel-associated Box
MDCK         Madin-Darby Canine Kidney
Munc18       Mammalian unc18
[3H] NE      Tritium Labeled Norepinephrine
NGF          Nerve Growth Factor
NSF          N-ethylmaleimide-sensitive Factor
PIP5K1       Type I Phosphatidylinositol 4-phosphate-5-kinase
PITP         Phosphatidylinositol Transfer Protein
PBS          Phosphate Buffered Saline
PC12         Pheochromocytoma 12
PEI          Polyethylenimine
PFA          Para Formaldehyde
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>PIP</td>
<td>Phosphorylate phosphatidylinositol 4-phosphate</td>
</tr>
<tr>
<td>PIP2</td>
<td>Phosphorylate phosphatidylinositol 4,5-bisphosphate</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein Kinase C</td>
</tr>
<tr>
<td>PPI</td>
<td>Polyphosphoinositide</td>
</tr>
<tr>
<td>PSS</td>
<td>Physiological Saline Solution</td>
</tr>
<tr>
<td>Ral GTPase</td>
<td>Ras-like GTPase</td>
</tr>
<tr>
<td>RhoGDI</td>
<td>Rho GDP dissociation inhibitor</td>
</tr>
<tr>
<td>RNAi</td>
<td>RNA Interference</td>
</tr>
<tr>
<td>RRP</td>
<td>Readily Releasable Pool</td>
</tr>
<tr>
<td>RT</td>
<td>Room Temperature</td>
</tr>
<tr>
<td>SE</td>
<td>Standard Error</td>
</tr>
<tr>
<td>shRNA</td>
<td>Short Hairpin RNA</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small Interfering RNA</td>
</tr>
<tr>
<td>SNAP-25</td>
<td>Synaptosome-Associated Protein of 25 kDa</td>
</tr>
<tr>
<td>SNARE</td>
<td>Soluble N-ethylmaleimide-sensitive Factor Attachment Protein Receptor</td>
</tr>
<tr>
<td>SNM</td>
<td>Silent Nucleotide Mutation</td>
</tr>
<tr>
<td>SVs</td>
<td>Synaptic Vesicles</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris Buffered Saline</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission Electron Microscopy</td>
</tr>
<tr>
<td>tetO</td>
<td><em>tet</em> operator</td>
</tr>
<tr>
<td>TetR</td>
<td>Tetracycline Repressor</td>
</tr>
<tr>
<td>TGN</td>
<td>trans Golgi Network</td>
</tr>
<tr>
<td>VAMP</td>
<td>Vesicle Associated Membrane Protein</td>
</tr>
<tr>
<td>VCP</td>
<td>Valosin-Containing Protein</td>
</tr>
<tr>
<td>VSV-G</td>
<td>Vesicular Stomatitis Virus Glycoprotein</td>
</tr>
<tr>
<td>WT</td>
<td>Wild Type</td>
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1. Introduction

In eukaryotic cells, selected cargoes are transported to the plasma membrane for growth and secretion via vesicular traffic. Upon formation in the Golgi apparatus, the secretory vesicles are typically transported along cytoskeletal elements to reach the plasma membrane where fusion of the vesicular/plasma membrane occurs and the vesicular contents are ultimately released. This phenomenon, known as exocytosis, is indispensable to numerous physiological processes. For instance, secretion of membrane proteins and lipids from Golgi-derived vesicles provides new components for plasma membrane growth; release of catecholamines from adrenal chromaffin cells and sympathetic ganglion neurons are important for regulating blood pressures and heart rate; insulin secretion from pancreatic β-cells is crucial for glucose homeostasis; and inflammatory substances secreted by mast cells play critical roles in allergy and defense against pathogens. In the brain, neurotransmitter release across the synaptic cleft is responsible for neuronal communication, and abnormal neurotransmitter release has been implicated in mental disorders. To try to understand the mechanisms underlying exocytosis has therefore become an imperative duty of researchers. It is generally accepted that secretion from neurons or neuroendocrine cells involves two major types of secretory vesicles: the synaptic vesicles (SVs) and the dense-core vesicles (DCVs). SVs are small and clear vesicles that store and release neurotransmitters such as glutamate, glycine, and γ-aminobutyric acid (GABA); while DCVs are characterised by their electron-dense cores and are responsible for storing and excreting neuromodulators such as biogenic amines, peptides and neurotrophins. These secreted neurotransmitters /
neuromodulators then go on to regulate various physiological processes as previously mentioned. The mechanisms by which SVs and DCVs exert exocytosis are quite similar, with some notable differences. My thesis mainly focuses on the later.

1.1. Stages in DCV secretion

Upon leaving the trans Golgi network (TGN), DCVs are transported along the cytoskeletal elements and undergo several stages before secretion of the vesicular contents takes place (Figure 2.1.1) (Sudhof, 1995; Sugita, 2008). For the vesicles to be secreted, it is important for them to be in apposition to the plasma membrane (PM). The first stage in DCV secretion is therefore morphologically described as **docking** or **tethering**, where the vesicles are linked to the PM at a distance within about 50 nanometers from the PM, but are not yet in contact with the PM. The stage after docking/tethering is known as **priming** during which the attachment sites between DCVs and PM mature and become competent for exocytosis (Robinson and Martin, 1998). The primed DCVs are then ready for **fusion** with the PM upon Ca^{2+} influx or GTP signaling **triggering**. What happens to the DCVs following fusion/exocytosis, however, is still controversial. The SVs, which go through similar stages as the DCVs, are believed to be endocytosed and recycled via three alternative pathways. SVs in the presynaptic nerve terminals may be re-acidified and refilled with neurotransmitters after undocking (“kiss and run”) or without undocking (“kiss and stay”). Alternatively, the SVs could be recycled through the endosome. Similarly, it is possible that DCVs also go through endocytosis and recycling. Peptide-containing DCVs may be recycled via the Golgi complex (Gerber and Sudhof, 2002). Each stage of the DCV cycle is mediated by the
complex interactions of a number of proteins in the exocytosis machinery, which will be further discussed in later sections (Figure 1.1.1).

![Diagram showing stages of dense-core vesicle (DCV) secretion]

**Figure 1.1.1. Stages of the dense-core vesicle (DCV) secretion.** The stages are indicated by italicized, bold letters. The proteins involved in each stage are shown below each stage. Modified from Sugita (2008).

### 1.2. \( \text{Ca}^{2+} \)-dependent exocytosis

Regulated exocytosis of DCVs can be triggered by elevated intracellular \( \text{Ca}^{2+} \) level or GTP signaling, with the former being more prevalent (Sugita, 2008). As mentioned, only the primed (i.e. exocytosis-competent) DCVs are ready for fusion upon \( \text{Ca}^{2+} \) influx. The **current model** for \( \text{Ca}^{2+} \)-dependent secretion suggests that priming of the DCVs requires the formation of the SNARE (Soluble N-ethylmaleimide sensitive factor attachment protein receptor) complexes, as well as ATP (Holz et al., 1989; Sollner et al., 1993; Xu et al., 1998; Chen and Scheller, 2001; Jahn et al., 2003). The neuronal...
SNARE proteins are syntaxin1, SNAP-25 (synaptosomal-associated protein) and synaptobrevin2 (also known as vesicle associated membrane protein 2 or VAMP2) (Sollner et al., 1993). Among the SNARE proteins, syntaxin1 and SNAP-25 are associated with the target plasma membrane (thus target-SNAREs or t-SNAREs), whereas synaptobrevin2 locates at the vesicle membrane (thus vesicular-SNARE or v-SNARE) (Rothman and Warren, 1994). All three SNARE proteins possess SNARE motifs which are specific heptad repeat sequence, with SNAP-25 containing two such motifs and the other two members of the SNARE complex containing one each (Poirier et al., 1998; Burgoyne and Barclay, 2002). Tetramerization of the SNARE proteins through the alignment of the four SNARE motifs forms the trans-SNARE complex and brings together the DCVs and the plasma membrane, getting ready for Ca^{2+} -triggered membrane fusion. Formation of the trans-SNARE complex is thought to be able to overcome the energy barrier that prevents fusion of the two membranes (McNew et al., 2000; Parlati et al., 2000).

Meanwhile, ATP is also believed to be crucial to DCV priming. Evidence for this conclusion includes: 1). ATP could possibly be utilized by type I phosphaticylinositol 4-phosphate-5-kinase (PIP5KI) to phosphorylate phosphaticylinositol 4-phosphate (PIP) and generate phosphatidylinositol 4,5-bisphosphate (PIP_2). PIP_2 in turn binds to proteins involved in Ca^{2+} -triggered membrane fusion, including synaptotagmin and calcium-dependent activator protein for secretion 1 (CAPS1). 2). The ATPase N-ethylmaleimide sensitive factor (NSF), together with \( \alpha_\gamma \)-soluble NSF-acceptor proteins (\( \alpha_\gamma \)-SNAPs), bind to the \( \text{cis} \)-SNARE complex that is
inactive for exocytosis in an ATP-dependent manner (Malhotra et al., 1988). The cis- but not the trans-SNARE complex is disrupted by the hydrolysis of ATP by NSF, favouring the formation of the active trans-SNARE complex (Weber et al., 2000). When cell membrane is depolarized, Ca\(^{2+}\) channels on the plasma membrane open and Ca\(^{2+}\) flows down its concentration gradient into the cytoplasm. Increased intracellular Ca\(^{2+}\) concentration is then sensed by synaptotagmin, a membrane protein associated with DCVs/SVs and a plausible candidate for Ca\(^{2+}\) sensor, resulting in membrane fusion and extrusion of vesicle’s contents. The optimal concentrations of Ca\(^{2+}\) for triggering exocytosis are around 1-10 micromolar (Sugita, 2008).

1.3. GTP-dependent exocytosis

A wide variety of secretory cell types employ Ca\(^{2+}\)-independent exocytosis. Examples include stimulation of platelets with collagen, and the GTP-dependent exocytosis in which secretory processes are activated by non-hydrolysable GTP analogues β,γ-imidoguanosine 5’-triphosphate (GppNHp) or guanosine 5’[γ-thio]triphosphate (GTP\(\gamma\)S) (Fernandez et al., 1984; Barrowman et al., 1986). GTP-dependent exocytosis has been observed in mast cells (Gomperts et al., 1983; Fernandez et al., 1984), chromaffin cells (Bittner et al., 1986; Ahnert-Hilger et al., 1992; Burgoyne and Handel, 1994), pituitary gonadotrophs (Tse and Tse, 2000), melanotrophs (Okano et al., 1993), insulin-secreting β-cells (Regazzi et al., 1989), and platelets (Padfield et al., 1996; Chen et al., 2000). In 1984, Fernandez and colleagues demonstrated that when rat mast cells are dialysed with patch pipette filled with solution containing 5 μM of GTP\(\gamma\)S and virtually no Ca\(^{2+}\), rapid degranulation occurred as shown by increased membrane
capacitance (Fernandez et al., 1984). The underlying mechanisms for GTP-dependent exocytosis, however, remain unresolved (Pinxteren et al., 2000).

Studies using permeabilization techniques took the effort to examine the possible sites and modes of action by the GTP analogues but resulted in limited progress, and different effect has been observed in different secretory cell types. In permeabilized platelets, it was found that guanine nucleotides enhanced sensitivity of secretion to Ca$^{2+}$ (Haslam and Davidson, 1984), whereas effects of Ca$^{2+}$ and the GTP$\gamma$S were shown to be independent of each other in Sendai virus-permeabilized rabbit neutrophils in the presence of Ca$^{2+}$ chelators (EGTA and BAPTA) (Barrowman et al., 1986). The Barrowman group further postulated that the GTP analogue-induced secretion may be due to its activation of polyphosphoinositite (PPI) phosphodiesterase and subsequent liberation of diacylglycerol (DG) which could potentially activate protein kinase C (PKC) in a transient manner. In addition, since GDP and GDP$\beta$S inhibit both GTP- and Ca$^{2+}$-dependent secretion, it is also reasonable to assume that the GTP-dependent pathway must interact with the Ca$^{2+}$-dependent pathway at some level common to both, such as membrane fusion or microtubule assembly (Barrowman et al., 1986). Unlike in mast cells or neutrophils, Bittner et al. showed that GppNHp did not stimulate production of inositol triphosphate (IP$_3$) or DG in permeabilized bovine chromaffin cells; the GTP-dependent secretion was independent of Ca$^{2+}$-induced secretion, and could not be attributed to possible effects of GTP on cAMP levels or microtubule assembly (Bittner et al., 1986). In permeabilized PC12 cells, GTP-dependent exocytosis does not require cytosolic proteins, ATP or Ca$^{2+}$ (Klenchin et al., 1998), but is nonetheless blocked by clostridial neurotoxins which cleaves the SNARE proteins essential for Ca$^{2+}$-dependent exocytosis.
(Banerjee et al., 1996; Glenn and Burgoyne, 1996; Wang et al., 2004). Thus, despite the obscurity in our understanding of the GTP-dependent exocytotic pathway, two things are becoming clear: 1) the action is through GTPases (i.e. sensors for GTP signals), and 2) the \( \text{Ca}^{2+} \)- and GTP-dependent exocytosis pathways may interact at some common SNARE protein-dependent step(s) (Sugita, 2008).

The Ral (Ras-like) GTPases (RalA and RalB) have recently been found to be the major GTP sensors in mammalian cells (Wang et al., 2004; Li et al., 2007). The Ral proteins interact with an octameric protein complex termed the exocyst complex and physically bind to two of its members, Sec5 and Exo84, in a GTP-dependent manner (Moskalenko et al., 2002; Sugihara et al., 2002). Ral proteins have been shown to associate with secretory granules and synaptic vesicles (Bielinski et al., 1993; Volknandt et al., 1993; de Leeuw et al., 1999). In permeabilized PC12 cells, GTP-dependent exocytosis is dominant-negatively inhibited by bacterially expressed soluble RalA protein which lacks prenylation for proper membrane localization. Same effect is seen with a fragment of Sec5 that retains binding to RalA (Wang et al., 2004). Furthermore, GTP- but not \( \text{Ca}^{2+} \)-dependent exocytosis was reduced by RNA interference-mediated silencing of RalA and/or RalB (Li et al., 2007). How the Ral-exocyst interaction functions to trigger GTP-dependent exocytosis, however, is still a mystery.

1.4. **PC12 cells as a tool to study DCV secretion**

The PC12 (pheochromocytoma 12) cell line is a single cell clonal line derived from a transplantable rat pheochromocytoma, a tumor of the rat adrenal gland (Greene
and Tischler, 1976). PC12 cells are relatively easy to grow, and have been intensively used in the study of cell development and regulation of gene expression. More importantly, these cells serve as a good model system for primary neuronal cells due to their ability to reversibly differentiate into neuron-like cells in response to nerve growth factor (NGF) (Greene and Tischler, 1976). Undifferentiated PC12 cells are similar to those of the immature rat adrenal chromaffin cells in many ways, including their ability to secrete neurotransmitters. When treated with NGF, PC12 cells stop proliferation, extend neurites, become electrically excitable, increase in the number of calcium channels and the synthesis of several neurotransmitters. Removal of NGF results in degradation of the protruding processes in most differentiated PC12 cells, whereas the cell bodies remain unaffected, with replication resumed within several days (Greene and Tischler, 1976; Greene et al., 1982).

The PC12 cells employed in my study were derived from the PC12 cell line which was developed and characterized by Thomas Martin (Klenchin et al., 1998; Martin and Grishanin, 2003). Undifferentiated PC12 cells of 10-15 μm in diameter contain mainly DCVs (40-350 nm in diameter), whereas both DCVs and clear SVs (20-70 nm in diameter) have been observed in the differentiated cells (Greene and Tischler, 1976; Martin and Grishanin, 2003). The major neurotransmitters produced within PC12 cells are catecholamines (dopamine and to a lesser extent norepinephrine) and neuropeptides which are typically stored in the DCVs, as well as acetylcholine which is stored in the SVs (Greene and Tischler, 1976; Schubert et al., 1980; Kobayashi et al., 1998; Schubert et al., 1980; Travis and Wightman, 1998). Neurotransmitter release from DCVs in PC12 cells can be regulated by Ca$^{2+}$- and GTP-dependent exocytic/exocytotic pathways (Sugita,
PC12 cells thus make an excellent model for studying regulated neurotransmitter release in mammalian cells.

There are several reasons for choosing PC12 cell line over primary neurons in my study. Firstly, PC12 cells are relatively easy to maintain, and their robustness allows for a wide range of genetic manipulations. Moreover, DCV secretion can be assayed in both intact and permeabilized PC12 cells (Li et al., 2007). DCVs of PC12 cells are able to stay exocytosis-competent even after permeabilization of the plasma membrane as long as cytosolic proteins and MgATP are provided. Several types of secretion assays that measure the release of radioactive norepinephrine (\[^{3}\text{H}\]-NE) from prelabeled cells have already been well established (Hay and Martin, 1992; Klenchin et al., 1998); and have previously been successfully performed in our laboratory (Wang et al., 2004; Li et al., 2007).

1.5. Tethering/docking of DCVs to the plasma membrane

A simple model of vesicle targeting and membrane fusion suggests that the SNARE complex, together with the vesicle and target membrane, is sufficient to determine their compatibility for fusion (McNew et al., 2000; Parlati et al., 2000). However, several lines of evidence indicate that the interactions between v-SNARE and t-SNAREs may not be able to account for all the specificity of membrane transport events in vivo (Pelham, 2001). In yeast cells and neurons, the t-SNAREs (Sso1p and Sso2p in yeast; syntaxin1 and SNAP-25 in neurons) show ubiquitous distribution along the plasma membrane, whereas vesicles only fuse with certain subdomains of the plasma membrane.
(Brennwald et al., 1994; Garcia et al., 1994). Disruption of the SNAREs in the squid giant synapse and *Drosophila* caused reduced secretion without blocking vesicle docking (Hunt et al., 1994; Broadie et al., 1995). In chromaffin cells, docking is strongly reduced when syntaxin1A/1B/2/3 are cleaved by botulinum neurotoxin C1 (de Wit et al., 2006). However, knockout of the other two members of the SNARE complex, SNAP-25 and synaptobrevin, do not seem to affect docking although DCV secretion is significantly reduced (Sorensen et al., 2003; Borisovska et al., 2005). These results suggest that SNARE complex formation is insufficient for the precise pairing of the vesicle membrane and target site, and some sort of physical linkage between the two membranes have already been established before the trans-SNARE complex is formed, with the vesicles still at considerable distance from the target sites.

The formation of the physical links between the vesicle membrane and the target site is thought to take place during the tethering/docking stage of the vesicle cycle, representing probably the earliest stage that confers targeting specificity (Guo et al., 2000; Lowe, 2000; Waters and Hughson, 2000; Whyte and Munro, 2001, 2002). A number of “tethering factors” have been proposed to be involved in the tethering processes, including a group of long coiled-coil proteins and several multi-subunit protein complexes such as the exocyst complex, the conserved oligomeric Golgi (COG) complex, the Golgi-associated retrograde protein (GARP) complex, the transport protein particle (TRAPP) complex, the Class C Vps complex, and the Dsl1p complex (Whyte and Munro, 2002). The coiled-coil proteins are thought to passively hold the vesicles to the target membranes, whereas the large protein complexes actively promote the interactions between the two membranes, increasing probability of SNARE-mediated fusion (Whyte
and Munro, 2002). The exocyst is one of the best-characterised tethering complexes. In contrast to the ubiquitous distribution of t-SNAREs along the plasma membrane, components of the exocyst tend to be located to regions of active exocytosis and membrane growth in yeast and mammalian cells (TerBush and Novick, 1995; Finger et al., 1998; Grindstaff et al., 1998; Guo et al., 1999a; Guo et al., 1999b; Hazuka et al., 1999). Sec3p in the yeast exocyst locates to the plasma membrane independently of the other components and potentially serves as a landmark for site-specific vesicle fusion (Finger et al., 1998). All of these give rise to the possibility that the exocyst could be responsible for targeting secretory vesicles to the appropriate sites on the plasma membrane.

1.6. Structure/function of the exocyst

The exocyst, also known as Sec6/8 complex in the mammalian system, is a protein complex considered to be required for exocytosis by possibly tethering vesicles to specific sites on the plasma membrane. The complex was originally identified in the budding yeast and comprises eight protein subunits: Sec3p, Sec5p, Sec6p, Sec8p, Sec10p, Sec15p, Exo70p and Exo84p. The six Sec subunits were discovered by Novick and colleagues through genetic screening, and were so named because mutants of these proteins inhibit secretion. The subunits were found to interact with each other, and were later purified together with two additional subunits, Exo70p and Exo84p, by the same group (TerBush and Novick, 1995; TerBush et al., 1996; Kee et al., 1997). It has been shown that loss of function of any of the exocyst members resulted in blockade of secretion and accumulation of secretory vesicles in yeast (TerBush et al., 1996; Guo et al.,
The exocyst proteins are conserved from yeast to mammals, with the mammalian homologues termed Sec3, Sec5, Sec6, Sec8, Sec10, Sec15, Exo70 and Exo84 with homology ranging from 20% to 25% (Hsu et al., 1996). Despite the relatively low sequence homology, the mammalian exocyst is strikingly similar to its yeast counterpart in terms of protein sizes, complex stability, and cellular distribution. The complex has been found in soluble and membrane-bound forms in both yeast and mammal. In both organisms, staining of the exocyst complex was enhanced in areas of active exocytosis: budding sites in yeast, regions of basolateral membrane addition in polarized epithelial cells and areas near the plasma membrane of the nerve terminals (Hsu et al., 1996), making it a better candidate than the SNAREs in conferring the specificity required for vesicle targeting. In addition, studies of the yeast secretory mutants revealed genetic interactions between Sec8/Sec15 and the yeast t-SNAREs Sso1/Sec9; and immunoprecipitation experiments in rat demonstrated in vivo interactions between rat Sec8 and syntaxin (Aalto et al., 1993; Brennwald et al., 1994; Hsu et al., 1996). The yeast exocyst is known to be required prior to the assembly of the SNARE complex and membrane fusion (Grote et al., 2000). These results together suggest a role for the exocyst complex in vesicle exocytosis upstream of the SNARE complex.

The yeast exocyst functions as an effector complex of the Sec4p, a member of the Rab GTPase family, which is believed to be important for a post-Golgi vesicular traffic event in yeast (Guo et al., 1999b). The Sec15p subunit specifically associates with Sec4p in its active GTP-bound form. Both Sec15p and Sec4p are associated with secretory vesicles, and the Sec15p/Sec4p interaction is thought to trigger further interactions with the other members of the exocyst complex, ultimately leading to
docking and fusion of the vesicles with appropriate areas of the plasma membrane (Lipschutz and Mostov, 2002). Boyd and colleagues showed that six out of the eight yeast exocyst components arrive at the exocytic sites with the secretory vesicles, whereas Exo70p gets to the destination using both vesicular and non-vesicular transport (Boyd et al., 2004). Sec3p, on the other hand, localizes to the secretion sites independent of the other components (Finger et al., 1998). Thus, Sec3p and to some extent Exo70p may serve as spatial landmarks, tethering the vesicles to precise sites on the plasma membrane through assembling with the other exocyst members associated with the vesicles.

Several additional GTPases have been implicated in the regulation of exocyst function, including Rho1p, Rho3p, and Cdc42p of the Rho family (members of the Ras superfamily). Rho1p and Cdc42p interact with Sec3p and are important for its localization (Guo et al., 2001; Zhang et al., 2001). Rho3p interacts with Exo70p, an interaction required for cell growth and efficient secretion of cell-wall hydrolases (Adamo et al., 1999; Robinson et al., 1999). The mammalian GTPase with the highest sequence homology to Sec4p is Rab3, which does not seem to interact with any of the exocyst members (Wang et al., 2004; Munson and Novick, 2006). Like in the yeast, the mammalian Exo70 also interacts with a member of the Rho family: TC10; and the interaction has been implicated in the translocation of GLUT4, an insulin-regulated glucose transporter in adipocytes, upon insulin signaling (Inoue et al., 2003). Two other GTPases, RalA/B (the Ras-like proteins) and Rab11 (also a member of the Ras family), have recently been suggested to possibly function in similar ways as the yeast Sec4p (Guo et al., 1999b; Brymora et al., 2001; Moskalenko et al., 2002; Sugihara et al., 2002; Beronja et al., 2005; Satoh et al., 2005). RalA and RalB GTPases physically interact with
Sec5 and Exo84 in a GTP-dependent manner (Figure 1.6.1a) (Fukai et al., 2003; Moskalenko et al., 2003; Jin et al., 2005). These interactions are thought to be crucial for regulated exocytosis and neurite branching (Wang et al., 2004; Lalli and Hall, 2005). Mammalian Rab11, on the other hand, has recently been shown to directly bind to Sec15 in a GTP-dependent manner (Zhang et al., 2004). Since the interaction of Sec15p and Sec4p Rab GTPase is thought to be critical for the exocyst assembly in yeast, the mammalian Rab11 may also take the place of the yeast Sec4p as transport vesicle-associated small GTPase that recruits the exocyst (Guo et al., 1999b). The involvement of various GTPases suggests that the exocyst may be integrating multiple signals from different signalling pathways before choosing the precise spots for vesicle fusion (Munson and Novick, 2006).
Figure 1.6.1. Structures of the exocyst subunits. (a) RalA-binding domain of Sec5 binding to RalA (left) (Fukai et al. 2003); RalA-binding domain of Exo84 binding to RalA (right) (Jin et al. 2005, Fukai et al. 2003). (b) Structures of Exo70p (Dong et al. 2005, Hamburger et al. 2006), Exo84p C-terminal domain (Dong et al. 2005), Sec15 C-terminal domain (Wu et al. 2005), Sec6p C-terminal domain (Sivaram et al. 2006). Courtesy of Munson and Novick (2006).
The protein-protein interactions between the GTPases and exocyst subunits and those among the exocyst members were detected through yeast two-hybrid assays or binding assays using either in vitro-translated or purified recombinant proteins (Table 1.6.1) (Matern et al., 2001; Vega and Hsu, 2001; Inoue et al., 2003; Moskalenko et al., 2003; Prigent et al., 2003; Zhang et al., 2004; Jin et al., 2005). Further understanding of the molecular mechanisms underlying the actions of the exocyst requires elucidation of the structures of the individual subunits as well as the complex in its assembled form, both of which are difficult due to the large size and low solubility of the subunits and the tendency of the complex to disassemble during the process of purification (Munson and Novick, 2006). Structural studies in Drosophila and yeast have identified a motif of a tandem repeat of helical-bundle units in all of Sec15, Exo70p, Exo84p and Sec6p despite their low sequence similarity (Figure 1.6.1b) (Dong et al., 2005; Wu et al., 2005; Hamburger et al., 2006; Sivaram et al., 2006). Secondary structure predictions of the other four exocyst members suggest that they may also have similar helical bundles, giving rise to the hypothesis that the subunits pack on to one another via these rod-like helical structures (Munson and Novick, 2006). Further support of this hypothesis comes from images of the exocyst complexes generated using quick-freeze/deep-etch EM (Figure 1.6.2a,b). The unfixed exocyst complex exhibits radially symmetric “flower-like” conformations with petals of different lengths. When fixed with glutaraldehyde, the complex appears as a wider structure with fewer and smaller arms and is thought to represent the native exocyst structure more closely (Hsu et al., 1998). Based on these results, Munson and Novick came up with a speculative schematic representation of the
assembled yeast exocyst in association with related GTPases and the vesicle/plasma membranes (Figure 1.6.2c) (Munson and Novick, 2006).

The precise roles of the exocyst complex are not well understood in higher eukaryotic cells. This is largely due to the lack of good models for loss-of-function studies in these organisms, as will be discussed in more details in the following section.

**Figure 1.6.2. Model for the exocyst in its assembled form.** (a) Quick-freeze/deep-etch EM of the unfixed purified mammalian brain exocyst complex. (b) Quick-freeze/deep-etch EM of the purified mammalian brain exocyst complex fixed with glutaraldehyde (Hsu et al. 1998). (c) Speculative schematic of the assembled yeast exocyst and a network of molecular interactions that links secretory vesicles to exocytotic patch on the plasma membrane. Courtesy of Munson and Novick (2006).
Table 1.6.1. Protein-protein interactions between the exocyst subunits and with small GTPases.

<table>
<thead>
<tr>
<th>Exocyst subunit</th>
<th>Interactions</th>
<th>Small GTPase interactions</th>
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<tbody>
<tr>
<td></td>
<td>(Yeast)</td>
<td>(Animal)</td>
</tr>
<tr>
<td>Sec3</td>
<td>Sec5 (ref. 31)(^a,b)</td>
<td>Sec5 (ref. 45)(^a)</td>
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<tr>
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<td>Sec6 (ref. 31)(^b)</td>
<td>Sec8 (ref. 45)(^a)</td>
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<td>Sec5</td>
<td>Sec3 (ref. 31)(^a,b)</td>
<td>Sec3 (ref. 45)(^a)</td>
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<td></td>
<td>Sec6 (ref. 31)(^b)</td>
<td>Sec6 (ref. 45)(^a)</td>
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<td></td>
<td>Sec10p (ref. 31)(^a)</td>
<td>Exo84 (ref. 40)(^a)</td>
</tr>
<tr>
<td></td>
<td>Exo70p (ref. 31)(^b)</td>
<td>Exo84 (ref. 40)(^a)</td>
</tr>
<tr>
<td>Sec6</td>
<td>Sec5 (ref. 31)(^b)</td>
<td>Sec5 (ref. 45)(^a)</td>
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<tr>
<td></td>
<td>Sec6 (ref. 17)(^c)</td>
<td>Sec8 (ref. 45,49)(^a,c)</td>
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<td></td>
<td>Sec8p (ref. 9,31)(^b,c)</td>
<td>Sec10 (ref. 49)(^c)</td>
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<td></td>
<td>Sec10p (ref. 9)(^c)</td>
<td>Exo70 (ref. 45)(^p)</td>
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<td>Exo70p (ref. 6)(^c)</td>
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<tr>
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<td>Sec6p (ref. 9,31)(^b,c)</td>
<td>Sec3 (ref. 45)(^a)</td>
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<td>Exo70 (ref. 49)(^c)</td>
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<td>Sec6 (ref. 49)(^c)</td>
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<td>Sec8 (ref. 45,49)(^a,c)</td>
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<td>Sec15p (ref. 31)(^a,b)</td>
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<td>Exo70p (ref. 6)(^c)</td>
<td>Exo70 (ref. 49)(^c)</td>
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<td>Exo84p (ref. 46)(^a)</td>
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<td>Sec10p (ref. 31)(^a,b)</td>
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<td>Sec15 (ref. 45)(^a)</td>
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<td></td>
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<td>Exo70 (ref. 45)(^a)</td>
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</table>

\(^a\): Protein-protein interactions identified through yeast two-hybrid assays.  
\(^b\): Protein-protein interactions identified by binding assays using either *in vitro*-translated proteins.  
Sec5 is a central member of the exocyst complex. The yeast Sec5p has a molecular weight of 107 kDa, whereas its homolog in rat is predicted to be 104 kDa. The protein sequence of mammalian Sec5 is 21% identical to its yeast counterpart (32% similarity) (Kee et al., 1997). A higher sequence homology exists between Sec5 of *C. elegans* and mammals (Sugihara et al., 2002). A 21-amino acid region of the protein has a high probability of forming coiled-coil domain which may allow its interaction with similar domains in the other exocyst subunits (Kee et al., 1997).

As mentioned, functions of the exocyst proteins (including Sec5) are poorly understood in organisms other than yeast due to the absence of a functional genetic analysis. The limited knowledge that we currently have are mostly obtained from studies in *Drosophila*. Knocking out of the genes of the exocyst members often results in embryonic lethality. Homozygous Sec5 mutants *Drosophila* were born but died as growth-arrested larvae once maternal gene products were depleted. Neuromuscular junctions did not expand in these Sec5 mutant larvae, and neurite outgrowth also failed (Murthy et al., 2003). Functions of the Sec5 and Sec6 are very similar in many ways. Sec6 mutations also caused cell lethality and disrupted plasma membrane growth in *Drosophila* (Beronja et al., 2005). Sec5 and Sec6 mutant larvae of *Drosophila* exhibited significant defects in the insertion of membrane proteins into the plasma membrane, whereas Ca$^{2+}$-dependent neurotransmitter release was unaffected at certain muscular junctions, at least in Sec5 mutants (Murthy et al., 2003; Beronja et al., 2005; Murthy et al., 2005). These results suggest that the function of Sec5 may differentiate between the trafficking for cell/membrane growth and that for transmitter release. Photoreceptor cells
with reduced Sec6 function accumulated secretory vesicles and failed to transport proteins to the rhabdomere, a microvillar specialization on the apical surface (Beronja et al., 2005). Together, these results suggest that in *Drosophila*, Sec5 and Sec6 are involved in the insertion of a broad range of proteins into the plasma membrane. Furthermore, Murthy et al. (2003) demonstrated in *Drosophila* using trafficking assays that newly synthesized proteins are transported down the axon properly in Sec5 null mutants, and Sec5 may therefore be required at the docking/tethering step downstream of the earlier transport steps.

Little research regarding Sec5 and its regulatory GTPases has been conducted in mammals. As previously mentioned, the N-terminus of Sec5 physically binds the Ral GTPases in a GTP-dependent manner (Moskalenko et al., 2002; Jin et al., 2005). Moskalenko et al. (2002) demonstrated that inhibition of Ral function in PC12 cells blocked the release of human growth hormone (hGH) from DCV. In mouse pancreatic islets and insulinoma cell lines, RalA depletion has been shown to inhibit biphasic release of insulin, possibly due to a reduction of the readily releasable pool (RRP) of insulin granules and subsequent recruitment of granules from the reserve pool (Lopez et al., 2008). Collectively, these results suggest possible involvement of Sec5 in DCV secretion. Using permeabilized PC12 cells, Dr. Sugita’s lab has previously shown that bacterially-expressed soluble RalA GTPase (but not RhoA or Rab3A) which lacks prenylation for proper membrane localization dominant-negatively inhibits GTP-dependent exocytosis (Wang et al., 2004). Similarly, a fragment of Sec5 that binds to Ral is shown to inhibit GTP-dependent exocytosis by preventing endogenous Ral from interacting with the exocyst complex. This inhibition is, however, reverted when the RalA-Sec5 fragment
interaction was disrupted by point mutations in either RalA (E38R) or the Sec5 fragment (T11A) (Wang et al., 2004). Furthermore, silencing of RalA and RalB by RNA interference also inhibited GTP-dependent exocytosis without affecting Ca\(^{2+}\)-dependent exocytosis (Li et al., 2007; Kawato et al., 2008). These results have recently been confirmed in GTP-dependent dense granule secretion in platelets, except that the Ca\(^{2+}\)-sensitivity was enhanced by GppNHp (Kawato et al., 2008). Together, these data suggest that the RalA-exocyst interaction is involved in GTP-dependent exocytosis, and RalA/B are the major sensors for GTP in the GTP-dependent triggering of DCV exocytosis. Nonetheless, based on the data in yeast and Drosophila, the function of the exocyst complex including Sec5 seems not to be limited to GTP-dependent exocytosis, but also includes processes involved in development such as neurite branching. In order to reveal some of the possible roles that Sec5 may play in the regulation of exocytosis, I employed several strategies of RNA interference (RNAi) to suppress the expression of sec5 in PC12 cells and tested the effect of loss-of-function of sec5 on the tethering/docking and secretion of DCVs.

1.8. Mechanisms of lentivirus-mediated RNAi

RNA interference (RNAi) is an approach used to silence specific genes by the use of short interfering RNA (siRNA) or short hairpin RNA (shRNA). It was first identified in C. elegans, where injection of double-stranded RNAs (dsRNAs) into the worm was found to silence the genes with complementary sequences (Fire et al., 1998). The injected dsRNAs are processed into siRNAs of 19-22 nucleotides in length by the RNase enzyme Dicer (Ketting et al., 2001). These siRNAs are then uptaken into a RNA-
induced silencing complex (RISC) which recognizes and destroys the complementary messenger RNAs (mRNAs) (Dykxhoorn et al., 2003). This strategy, however, has not been successful in mammalian species because long dsRNAs induce the interferon responses (non-specific responses to foreign dsRNAs) as a part of the cell’s antiviral mechanism, resulting in a global decrease in mRNA translation (Stark et al., 1998; Elbashir et al., 2001). To overcome this hurdle, transfection of siRNAs have been used to direct sequence-specific RNAi pathway (Elbashir et al., 2001). Alternatively, expression of shRNA encoded within an expression vector could mimic endogenous trigger of the RNAi mechanism in mammalian cells (Paddison et al., 2004). The encoded shRNA is first transcribed from the DNA template into a single-stranded RNA molecule. The two complementary sequences that flank a loop then pair with each other and cause the RNA to fold back onto itself, forming a loop-like structure. The RNAi machinery then recognizes and converts the shRNA into siRNA (McIntyre and Fanning, 2006). In my study, the shRNA strategy was adopted for the purpose of down-regulating Sec5 due to its advantages in silencing longevity and better delivery options. Selection of hairpin sequence was done through an algorithm designed to increase the chance of good knockdown and decrease the chance of off-target effects (http://www.broad.mit.edu/genome_bio/trc/rnai.html).

I employed the pLKO-puro lentiviral vector to express our designed shRNA expression cassette for Sec5 target sequence. The pLKO-puro vector is a third-generation self-inactivating lentiviral vector generated using a three-plasmid packaging system in which the gag, pol and rev, and the gene encoding the vesicular stomatitis virus glycoprotein (VSV-G) coat are expressed through separate vectors. This design is to
minimize the chance for reconstitution of the host genome through recombination to create replication competent viruses (Dull et al., 1998; Zufferey et al., 1998; Follenzi et al., 2000). Expression of the shRNA is driven by the human U6 promoter (hU6). The mammalian selection marker puromycin resistance gene (PAC, encoding a puromycin N-acetyl-transferase) controlled by the PGK promoter allows for selection of transduced cells. The vector also contains the ampicillin resistance gene (AmpR) which allows the vector to be amplified in bacteria for DNA purification purposes (Root et al., 2006).

1.9. Mechanisms of drug-inducible gene suppression

As mentioned, Sec5 has been implicated in the cells’ ability to survive. Therefore, it could be difficult to select cells with strongly down-regulated Sec5 since those cells may not be able to survive to the point of selection by puromycin. To overcome this difficulty, I tried also to establish the lentivirus-mediated drug-inducible knockdown system that allows for conditional suppression of Sec5 after cell development has been finished. I adopted a lentivirus vector-based system that took the advantage of the repression activity of TetR-KRAB, a tetracycline-controlled fusion protein between the tetracycline repressor (TetR) from Escherichia coli T10 and the Kruppel-associated box (KRAB) domain of human Kox1 (Gossen and Bujard, 1992; Deuschle et al., 1995). KRAB is able to modulate transcription from an integrated promoter which lays beside the tet operator (tetO) sequences (Deuschle et al., 1995). In the absence of the drug doxycycline (a derivative of tetracycline), TetR-KRAB specifically binds to tetO and suppresses the activity of the juxtaposed promoter and thus the transcription of siRNA, resulting in normal expression of the cellular gene. In the presence of doxycycline, TetR-
KRAB is bound by doxycycline particles and is thus prevented from binding with \textit{tetO}, leading to transcription of the siRNA and subsequent down-regulation of the target gene (Figure 1.9.1, modified from Wiznerowicz and Trono 2003) (Deuschle et al., 1995; Wiznerowicz and Trono, 2003). I chose to use the lentivirus-mediated knockdown plasmid – the pLKO-puro vector for expressing \textit{tetO}-linked transcriptional units so that they can be easily integrated into the genome of PC12 cells for TetR-KRAB-mediated repression to take effect. TetR-KRAB was expressed using an HIV-1-based lentiviral expression vector, the pLVX-IRES-hygro vector. This vector utilizes the constitutively active human cytomegalovirus promoter (P_{CMV}) to drive the expression of the protein of interest, in this case the TetR-KRAB hybrid protein. The IRES (internal ribosome entry site) is a nucleotide sequence that allows for translation to be initiated in the middle of an mRNA sequence. The use of IRES rather than a traditional promoter allows the hygromycin-resistant gene and the gene encoding TetR-KRAB to be expressed in a more co-ordinated manner.
Figure 1.9.1. Mode of action of the doxycycline-controllable transrepressor. (A) In the absence of doxycycline, TetR-KRAB binds to tetO and suppresses siRNA transcription, resulting in normal expression of target gene. (B) In the presence of doxycycline, TetR-KRAB no longer binds to tetO. siRNA is therefore produced, leading to suppression of target gene. Modified from Wiznerowicz and Trono (2003).
2.10. Rationale of study

The secretory pathway has been well conserved from yeast to mammals. A lot of mammalian proteins that are involved in exocytosis have homologues in yeast and other organisms. For example, the homologues of Munc18, NSF, α-SNAP and SNAP25 are Sec1, Sec18, Sec17 and Sec9, respectively (Novick et al., 1980). As a central component of the exocyst, Sec5 has been implicated in the protein trafficking in Drosophila and possibly GTP-dependent exocytosis of DCVs in PC12 cells (Murthy et al., 2003; Wang et al., 2004). In order to investigate the roles played by Sec5 in the regulation of DCV secretion in intact mammalian neurosecretory cells, I knocked down Sec5 in PC12 cells and tested its effects on DCV docking and regulated catecholamine secretion.
2. Hypothesis

Sec5, a member of the exocyst complex, plays critical roles in both Ca^{2+}-dependent and GTP-dependent secretions through the regulation of tethering/docking of DCVs in PC12 cells.

Specific Aims:

Specific Aim 1: Establish PC12 cell line in which Sec5 is stably down-regulated.

Specific Aim 2: Analyze secretion in Sec5 KD cells through GTP-dependent and Ca^{2+}-dependent secretion assays

Specific Aim 3: Analyze the proportion of docked vesicles in Sec5 KD cells in single cell electron micrographs

Specific Aim 4: Establish the doxycycline-inducible KD system for Sec5 in PC12 cells

Specific Aim 5: Re-introduce Sec5 in Sec5 KD cells.
3. Materials and Methods

3.1. Maintenance of PC12 and HEK293-FT cells

The wild-type PC12 cells used in my study were first isolated by Thomas Martin (University of Wisconsin, Madison, WI) and Erik Schweitzer (University of California at Los Angeles, Los Angeles, CA). Cells were cultured on regular 10cm uncoated plastic culture dishes using Dulbecco’s Modified Eagle Medium (DMEM) (Invitrogen, Carlsbad, CA) containing 5% calf serum, 5% horse serum (both from HyClone, Logan, UT), and penicillin/streptomycin (Sigma) (Schweitzer, 1993; Sugita et al., 2001; Sugita et al., 2002; Wang et al., 2004; Li et al., 2005; Wang et al., 2005). I will refer to culture medium with the above mentioned components as complete medium. Cells were allowed to grow in standard incubators with 37°C and 9.5% of CO₂, and were split upon confluence. For splitting, one dish of PC12 cells is usually incubated with 1ml of Hank’s Buffer containing 1 mM EDTA for 3 minutes, followed by addition of 7 ml of complete media and 5-10 times of trituration to prevent cell clusters. Cells were typically split at a 1:8 ratio on a weekly basis.

Human embryonic kidney-fibroblast (HEK293-FT) cells were cultured in DMEM containing 110 mg/L sodium pyruvate, 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, plus 0.1% Amphotericin B (A/B). The HEK293-FT cells were incubated with 5% of CO₂ at 37°C. Splitting of HEK293-FT cells is the same as that of PC12 cells, except that 1ml of trypsin EDTA is used instead of Hank’s Buffer for the initial detaching purpose.
3.2. Stable downregulation of Sec5 in PC12 cells using lentivirus mediated RNAi

3.2.1. pLKO-rSec5 KD construct

Targeted rat sec5 sequence used for the generating the short hairpin RNA (shRNA) was ligated into pLKO-puro vector (purchased from Sigma) containing puromycin-resistant gene, resulting in the construct pLKO-rSec5-puro (Figure 3.2.1). KD sequence was sub-cloned between AgeI and EcoRI sites.

**Target sequence in rat sec5:**

5’-GTGTTATTCCATGGCATGAGT-3’

**Forward primer:**

5’-CCGG**GTGTTATTCCATGGCATGAGT**CTCGAG**ACTCATGCCATGGAATAACAC**TTTTTG-3’

**Reverse primer:**

5’-AATTCAAAAA**GTGTTATTCCATGGCATGAGT**CTCGAG**ACTCATGCCATGGAATAACAC**-3’

![Figure 3.2.1. Designed shRNA sequence for pLKO-rSec5 KD construct.](image.png)

The 21-base bolded/underlined segments represent the sense and anti-sense strands which were inserted into the pLKO-puro vector for the generation of pLKO-rSec5-puro construct.

3.2.2. Transfection of HEK293-FT cells for virus production

The viral particles containing shRNA for Sec5 KD were generated in HEK293-FT cells. To transfect HEK293-FT cells with pLKO-puro (control) or pLKO-rSec5-puro (KD) constructs, two separate mixtures were prepared. One mixture contains 9µg of either control or KD plasmid, 3 µg of MD2G plasmid, and 4.8 µg of pCMV-dR8.74
plasmid in 500 µl of 0.15 M NaCl. The other mixture contains 48 µg of the transfection agent polyethylenimine (PEI) in 500 µl of 0.15 M NaCl. The two mixtures were then combined into one tube, vortexed vigorously for 10-20 seconds, and left at room temperature (RT) for 10 minutes. The final mixture was then applied to HEK293-FT cells that have reached a confluence of about 70%. Cells were incubated overnight and were replenished with fresh HEK293-FT cell media the next day, allowing the plasmids that were not taken up to be washed away. The cells were further incubated for another two days during which time the viral particles were being produced and secreted into the culture media. After this, media containing viral particles were collected and stored at 4°C for future infection use.

3.3.3. Lentivirus infection of PC12 cells

WT PC12 cells were infected with either control or KD viral particles. Typically, cells with 70% confluence are infected with 2.5 ml of virus-containing media plus 0.5ml of complete media (with 0.1% A/B) for 2-3 days. The low total volume (3 ml) allows for closer contact between virus and the cells, giving higher chance of infection. Cells were then replenished with fresh complete media (with 0.1% A/B), and were further incubated for 2-4 days before selection of transduced cells. Selection was conducted using 2.5 µg/ml of puromycin (for puromycin-resistant cells containing the PAC gene) and/or 0.70 mg/ml of G418 (for G418-resistant cells containing neo gene). Transduced cells were then subjected to immunoblot analyses for protein expression.

3.3. Immunoblot analyses of Sec5 KD cells
Upon reaching about 70% confluence, selected cells were harvested and lysed in phosphate buffered saline (PBS) supplemented with 1ug/ml PMSF (in isopropanol) and 1 mM EDTA to limit endogenous protease activity. The buffer-cell mixture was passed through a 23G1 syringe for at least 10 times to fully disrupt the cell membranes. Protein concentration was measured using Bradford assay (Bio-Rad) to facilitate loading of equal amount of proteins from each cell culture. Lysed cells were then mixed with equal volumes of 2X sample buffer containing β-Mercaptoethanol. Samples were sonicated briefly to break down nucleic acid strands, and were boiled in 100°C for 5 minutes to help denature the proteins better. Equal amounts of each cell lysate, typically 20 µg, were then loaded to SDS-PAGE gels for protein separation, followed by transfer of the proteins from gels to nitrocellulose membranes (15V, overnight). The next morning, membranes were first stained by Ponceau to check even loading across lanes. Membranes were then blocked with 5% skim milk (in TBS-T) prior to being probed with a primary antibody (1° Ab) specific to the protein of interest. Following this, the membranes were again probed by a 2° Ab conjugated with horseradish peroxidase. Western Lightning®, a chemiluminescent reagent (Perkin Elmer Life Sciences, Inc. Boston, MA, USA), was added to the blotted membrane, and signals produced by the enzymatic reaction were detected by exposing a sensitive sheet of photographic film. Quantification of the signal was performed using ImageJ.

Monoclonal 1° Abs for Sec5 and Exo84, and polyclonal 1° Ab for Exo70 were kind gifts from Dr. Richard H. Sheller (Genentech). Monoclonal 1° Abs for Sec6 and Sec8 were purchased from Stressgen Biotechnologies (Victoria, BC, Canada). Polyclonal
1° Ab for Tet repressor (anti-TetR Ab) was obtained from Bioscience Inc. (San Diego, CA, USA). Monoclonal 1° Ab for valosin-containing protein (VCP, loading control) was kindly given to us by Dr. Thomas Sudhof (University of Texas Southwestern Medical Center, Dallas, TX, USA).

### 3.4. Secretion assays

#### 3.4.1. GTP-dependent secretion assay

PC12 cells in 10cm dishes were labeled with 4 μCi [³H]-NE (Amersham Biosciences) in the presence of 0.5 mM ascorbic acid, and were left in the incubator overnight so that radioactive NE could be incorporated into DCVs. The next day, cells were washed with K-Glu buffer (20 mM HEPES, pH 7.2; 120 mM potassium glutamate; 20 mM potassium acetate; and 2 mM EGTA) with 0.1% bovine serum albumin (BSA), permeabilized with freeze-and-thaw as described in Klenchin et al. (1998), and were left on ice for 1-3 hours in the presence of 10 mM EGTA to extract the cytosolic proteins (Wang et al., 2004). Permeabilized PC12 cells were washed three times with K-Glu buffer containing 0.1% BSA; and each of control or KD cells were resuspended in a total volume of 1.2 to 1.4 ml. Even volumes (90 μl) of each of control or KD cells were distributed into 12 eppendorf tubes (6 wells x 2 for duplicates). GTP-dependent NE secretion was induced by addition of K-Glu buffer (120mM K-Glu, 20mM Hepes of pH7.2, 20mM KAc, and 2mM EGTA) with 0.09% BSA containing a series of increasing concentrations of GppNHp (0 μM, 0.1 μM, 1 μM, 10 μM, 100 μM, and 1000 μM; 10 μl of each) (trisodium salt, Sigma). Cells were incubated for 25 minutes at 30°C. Secretion
was terminated by chilling on ice. Samples were centrifuged for 3 minutes (14000 rpm, 4°C). Supernatants which represent the extracellular solution (i.e. secreted NE) were collected and mixed well with 2 ml scintillation liquid for counting of secreted radioactivity. Meanwhile, 100 µl of 0.1% Triton X-100 was added to each tube to solubilise the cell pellets. The resulting mixtures represented the cytosolic solutions (i.e. retained NE), and were then mixed well with 2 ml of scintillation liquid for counting of retained radioactivity. Secretion is presented as a percentage of secreted [3H]-NE over the total amount of incorporated [3H]-NE:

\[
\frac{\text{Count in Supernatant}}{\text{Count in supernatant + count in cytosol}} \times 100\%
\]

3.4.2. Ca\textsuperscript{2+}-dependent secretion assay

On day 0, cells in 10 cm dishes were split into six 1 cm wells within a 24-well plate. On day 3, cells in each well were labeled with 300 µl of complete media containing 0.15 µCi [3H]-NE and were incubated overnight. On the morning of day 4, the radioactive (“hot”) media was removed and replaced with non-radioactive (“cold”) complete media; and the cells were incubated for at least another 2 hours prior to the secretion assay. After this, cells were washed with 300 µl of physiological saline solution (PSS) (145 mM NaCl, 5.6 mM KCl, 2.2 mM CaCl\textsubscript{2}, 0.5 mM MgCl\textsubscript{2}, 5.6 mM glucose, 15 mM HEPES, pH adjusted to 7.4), stimulated with 200 µl of PSS or High K\textsuperscript{+}-PSS (81 mM NaCl, 70 mM KCl, 2.2 mM CaCl\textsubscript{2}, 0.5 mM MgCl\textsubscript{2}, 5.6 mM glucose, 15 mM HEPES, pH adjusted to 7.4), and were incubated for 20 minutes. Secretion was terminated by chilling to 0°C. Similar to GTP-dependent secretion assay, extracellular solution was collected into
Eppendorf tubes, centrifuged for 3 minutes (14000 rpm, 4°C), and the supernatant was mixed well with 2 ml scintillation liquid for counting of secreted radioactivity. 200 µl of 0.1% Triton X-100 was added to each well in the 24-well plate to lyse the cells. These were later combined with corresponding pellet part in the centrifuged tubes from the previous step, and again mixed well with 2 ml of scintillation liquid for counting of retained radioactivity. Secretion is calculated in the same way as in GTP-dependent secretion. The same assay was repeated on day 5 and day 6.

3.5. Electron microscopy analysis

PC12 cells grown in 10 cm dishes were fixed upon reaching a confluence of ~70%. For primary fixation, cells were washed 3 times with PBS, and were fixed for 1 hour at RT using modified Kamovsky’s fixative (2.5% glutaraldehyde, 3.2% paraformaldehyde, 0.1 M cacodylate buffer, pH 7.6). Cells were then scraped off the Petri dish, collected into microcentrifuge tubes, and centrifuged for 4 minutes (4000 rpm, 4°C). Supernatant fixative was removed with caution, and the remaining loose pellets were centrifuged again under the same condition mentioned. Any residual fixative in the pellets was then carefully removed, and 1.5 ml of new fixative was added to the pelleted cells. Cells were left at 4°C overnight. The following day, cells were centrifuged again. Fixative was removed and the pellets were washed three times, 10 minutes each time with 1 ml of 0.1 M cacodylate buffer. The pellets were then subjected to post fixation with 1% osmium tetroxide (in 0.1 M cacodylate buffer, pH 7.6) for 1 hour at RT in the dark. After this, the pellets were first washed again with 1 ml of 0.1 M cacodylate buffer (5 min), then with double distilled water (5 min x 2) to remove any residual cacodylate buffer. To enhance contrast, samples then subjected to En bloc staining with 1% uranyl
acetate (aqueous solution) for 1 hour at RT in the dark. After En bloc staining, pellets were washed twice with double distilled water, and were dehydrated through increasing graded ethanol series (1.5 ml of 50%, 70%, 80% and 90% ethanol, 10 min incubation each; 1.5 ml of 100% ethanol, 10 min incubation x 3). The samples were further subjected to infiltration with ethanol-modified Spurr’s resin using increasing resin-to-ethanol ratios (25%, 50%, and 75% of Spurr’s resin, 20 min incubation each; 100% Spurr’s resin, 20 min incubation x 3), and was left in 100% resin overnight on tissue rotator. The following morning, samples were supplemented with fresh 100% resin which was repeated in the afternoon of the same day before embedding samples in BEEM™ capsules. Capsules were incubated at 65°C overnight for polymerization. Polymerized cells were cut into semi-thin sections (0.5-1.0 mm) and stained with Toluidine bluemethylene blue stain for 60-90 seconds for locating regions of interest by light microscopy. Ultrathin sections (60-90 nm) were then cut, mounted onto copper grids and stained with 3% uranyl acetate in 50% methanol for 45 min, followed by post stain by Reynold’s lead citrate for 13-15 min. The grids were viewed using Hitachi (Tokyo, Japan) H7000 Transmission Electron Microscope (TEM) at 75 kV. Electron micrographs of cross-sections of individual cells from control and Sec5 KD cell types were taken (n=30-31 for each group), which were used to analyze DCV docking. As described in Diaz et al. (2009), two types of DCVs have been identified in the micrographs: 1) stand-alone DCVs of radii between 60-120 nm with a dark appearance (i.e. granules); 2) DCVs with halos (i.e. white vacuoles around granules). The distance from each vesicle to the PM was measured using ImageJ, based on which I calculated the percentages of DCVs within 25
nm, 25-50 nm, 50-100 nm, 100-150 nm, 150-200 nm, or >200 nm of the PM from control or KD groups.

3.6. TetR-tetO drug-inducible suppression of Sec5 in PC12 cells

To establish the lentivirus-mediated drug-inducible knockdown system for Sec5, wild type PC12 cells were first infected with virus containing pLVX-IRES-hygro-TetR-KRAB. Transduced cells were selected with 250 µg/ml hygromycin, and expression of the fusion protein was checked using anti-TetR polyclonal 1° Ab. After protein expression was confirmed, the same cells were infected again using pLKO-tetO-rSec5-puro-containing viral particles and were later selected with 250 µg/ml hygromycin together with 2.5 µg/ml puromycin. Wild type PC12 cells infected with pLVX-IRES-hygro-TetR-KRAB and pLKO-tetO-puro served as control for this experiment. To induce Sec5 downregulation, both control and KD cells were incubated in media containing (1 µg/ml) doxycycline hyclate (DOX) for 7 days before expression levels of Sec5 protein were examined by western blot.

3.7. Reintroduction of Sec5 expression in Sec5 KD cells

To reintroduce Sec5 protein in pLKO-puro control and pLKO-rSec5-puro KD cells, I infected the cells with viral particles containing pLVX-IRES-blast-mSec5(SNM)-EmdGFP which expresses mouse Sec5 fused with emerald GFP. To prevent the mSec5 from being degraded by the pre-existing shRNA in the cells, we included silent nucleotide mutation (SNM) in its sequence. The original sequence GTGTTATTCCATGGCATGAGT was changed to GTTCTTTTCAACGTATGTCC.
without changing the encoded amino acids. Transduced cells were selected with 5 µg/ml blastocidin.

3.8. Statistical data analysis

Data in GTP-/Ca^{2+}-dependent secretion assays and EM analysis were represented by mean ± SE (standard error). Two-way ANOVA (Origin 7.0) followed by paired t-test was used for statistical analysis of the GTP-dependent secretion assays. Two sample t-test was used for determining statistically difference between control and KD cells in Ca^{2+}-dependent secretion assays as well as for statistical analysis of DCV docking (Minitab version 14, Minitab™, Inc.).
4. Results

4.1. Sec5 expression is reduced by lentivirus-mediated RNAi

To test the effectiveness of our Sec5-puro KD construct, PC12 WT cells infected with pLKO-puro (control) or pLKO-rSec5-puro (KD) were harvested and subjected to western blot analyses (Figure 5.1.1). Figure 5.1.2 summarizes the results from western blot quantification of relevant proteins. The protein expression levels were tested by probing membranes with anti-Sec5/anti-Sec6/anti-Sec8/anti-Exo70/anti-Exo84 antibodies, and the expression levels of the exocyst proteins were quantified using ImageJ. Sec5 expression level in the KD cells was reduced by 58.5 ± 1.7 % compared to pLKO-puro control cells. Interestingly, expression of Sec6 was also reduced (by 21.1 ± 9.9 %) in these Sec5 KD cells, whereas Sec8/Exo70/Exo84 expressions were not significantly altered (Figure 5.1.2). Valosin-containing protein (VCP) served as a loading control (Figure 5.1.1).

In order to further down-regulate Sec5, I generated 2X KD cells by infecting pLKO-rSec5-puro KD cells with pLKO-rSec5-neo-containing lenti viruses. The pLKO-rSec5-neo and pLKO-rSec5-puro vectors target the same KD sequence for Sec5. The doubling of Sec5 KD sequence copies could potentially result in greater KD of Sec5. Transduced cells were selected with puromycin plus G418 and will be referred to as pLKO-rSec5-puro/neo KD cells. Corresponding control (pLKO-puro/neo) cells were generated by infecting pLKO-puro control cells with pLKO-neo-containing lenti virus. Figure 5.1.4 summarizes the results from western blot quantification of relevant proteins.
Different from our expectation, Sec5 expression level was not further reduced in these 2X KD cells (57.2 ± 7.5 % reduction), suggesting that KD of Sec5 might be already saturated in the 1X KD cells. Expression of Sec6 was again found to be reduced by 17.8 ± 4.5 % in the 2X KD cells, and that of Sec8 was also slightly decreased by 13.0 ± 1.8 %. Other exocyst proteins tested were unaffected by Sec5 down-regulation (Figure 5.1.4).
Figure 5.1.1. Immunoblot analyses of pLKO-rSec5-puro KD PC12 cells. The same membrane was probed by anti-Sec5/Sec6/Sec8/Exo70/Exo84 antibodies. VCP was used as loading control. A total of 30 µg protein was loaded into each well.
**Figure 5.1.2. Normalized expression levels of the exocyst proteins in pLKO-puro and pLKO-rSec5-puro cells.** Quantification of signal intensity using ImageJ shows that Sec5 expression was reduced by about 58.5 ± 1.7 % (p<0.0001) in the KD cells. Sec6 level was also decreased by 21.1 ± 9.9 % but failed to achieve statistical significance (p=0.0997). Expression levels of other exocyst members were not significantly affected by Sec5 KD. Unpaired t-test was performed for statistical analysis (*** p<0.0001).
Figure 5.1.3. Immunoblot analysis of pLKO-rSec5-puro/neo 2X KD PC12 cells. The membrane was probed by anti-Sec5/Sec6/Sec8/Exo84 antibodies. VCP was used as loading control. A total of 30 µg protein was loaded into each well.
Figure 5.1.4. Normalized expression levels of the exocyst proteins in pLKO-puro/neo and pLKO-rSec5-puro/neo cells. Quantification of signal intensity using ImageJ shows that Sec5 expression was reduced by 57.2 ± 7.5 % (p<0.0001) in the KD cells. Sec6 level was down-regulated by 17.8 ± 4.5 % (p=0.0012), and Sec8 expression was reduced by 13.0 ± 1.8 % (p<0.0001). Expression levels of other exocyst members were not significantly affected by Sec5 2X KD. Unpaired t-test was performed for statistical analysis (*p<0.05, *** p<0.0001).
4.2. GTP-dependent secretion is reduced in Sec5 KD cells

GTP-dependent secretion assays were performed for pLKO-rSec5-puro KD and its paired control cells. Figure 4.2.1 summarizes the average of 10 secretion assays, with duplicates within each assay (n=19 for control; n=20 for control). The left y-axis of Figure 4.2.1 represents the mean actual percentage of secreted radioactivity, which was normalized as indicated by the y-axis on the right where the basal secretion (i.e. at 0 mM GppNHp) was set to 0 and the maximal secretion achieved by the control cells was considered 100%. Percentage of secretion was calculated using the previously mentioned formula, and error bars represent standard error. Compared to control cells, Sec5-puro KD cells exhibited a 41.1 ± 10.0 % reduction in GTP-dependent exocytosis. Two-way ANOVA was used to determine the statistical difference between secretion of control and KD cells. The effects of both factors (i.e. cell types and concentration of GTP analogue) are statistically significant (control vs. KD: F₁=14.0, p=2.4x10⁻⁴; and the different concentrations of GppNHp: F₅=15.2, p=7.0x10⁻¹³), with no significant interaction between the two factors (F=0.91, p=0.47). Paired t-test was then performed to determine the statistical difference between control and KD cells at each concentration of GppNHp, with significant differences found at 0.1 μM (p<0.05), 10 μM (p<0.05), 100 μM (p<0.001), and 1000 μM (p<0.001) of GppNHp (Figure 4.2.1).
Figure 4.2.1. GTP-dependent secretion of [3H]-NE from DCVs is partially reduced in pLKO-rSec5-puro KD cells. Secretion was induced by incubating pre-labeled cells with a series of increasing concentrations of GppNHp for 25 min at 30°C. Percentage values of secretion were normalized by setting the basal level secretion (i.e. at 0 µM of GppNHp) to 0% and the maximal secretion achieved by pLKO-puro control cells (at 100 µM of GppNHp) to 100%. Error bars represent SE (n=19 for control cells, n=20 for KD cells). Analysis using two-way ANOVA indicates that both cell types (control vs. KD) and concentrations of GppNHp are statistically significant (control vs. KD: F₁=14.0, p=2.4x10⁻⁴; concentrations of GppNHp: F₅=15.2, p=7.0x10⁻¹³), with no significant interaction between the two factors (F=0.91, p=0.47). Asterisk(s) indicates statistically significant difference between control and KD cells at specific concentrations of GppNHp (paired t-test, * p<0.05; ** p=0.001).
5.3. *Ca\textsuperscript{2+}-dependent secretion is reduced in Sec5 KD cells*

*Ca\textsuperscript{2+}-dependent secretion assays were performed for pLKO-rSec5-puro KD as well as pLKO-rSec5-puro/neo 2X KD cells. As described in the previous section, each set of cells were assayed for 3 consecutive days (day 4, 5 and 6 after plating on day 0), with triplicates for each treatment (PSS or high K\textsuperscript{+}) within each assay. Figure 4.3.1A shows the mean actual percentage of secreted radioactivity for pLKO-rSec5-puro KD cells. All secretion % values were normalized to that of the average secretion of pLKO-puro control cells under high K\textsuperscript{+} and were presented in Figure 4.3.1B. Secretion was usually lowest on day 4, higher on day 5 and highest on day 6. Nevertheless, the percentage of decreased secretion in KD cells compared to control cells stayed unchanged. A 30% decrease (29.8% for day 4, 32.1% for day 5, and 32.6 % for day 6) was consistently observed in Sec5-puro KD cells stimulated with high K\textsuperscript{+}. Similar analyses were performed for pLKO-rSec5-puro/neo 2X KD cells, and were presented in Figures 3.3.2A and B. Secretions on days 5 and 6 were higher than that on day 4, but normalized percentage secretion reduction in KD cells was similar for all 3 days (16.1% for day 4, 22.9% for day 5, and 23.8% for day 6). Interestingly however, on days 4 and 5, the basal level secretion under PSS for the 2X KD cells was significantly higher than that of the control cells. Similar result was not observed on day 6. Percent of secretion was calculated using the previously mentioned formula, and error bars represent standard error. Paired t-test was used to determine statistical difference between paired control and KD cells.*
A

Day 4

Day 5

Day 6

% of total radioactivity secreted

pLKO-puro
pLKO-rSec5-puro

PSS
High K

Normalized % of secreted radioactivity

pLKO-puro
pLKO-rSec5-puro

PSS
High K

B

Day 4

Day 5

Day 5

% of total radioactivity secreted

pLKO-puro
pLKO-rSec5-puro

PSS
High K

Normalized % of secreted radioactivity

pLKO-puro
pLKO-rSec5-puro

PSS
High K
Figure 4.3.1. High K⁺-stimulated Ca²⁺-dependent exocytosis was reduced pLKO-rSec5-puro KD cells. Secretion was induced by 70 mM K⁺ for 20 min at 37°C. (A) Mean actual percentage of secreted radioactivity. Average secretion was lowest on day 4, higher on day 5 and highest on day 6 in both control and KD cells. (B) Normalized percentage secretion in control and KD cells. Percentage secretion values were normalized to the mean percentage secretion of pLKO-puro control cells under high K⁺. Normalized secretion provides better visualization of the consistent decrease (29.8% for day 4, 32.1% for day 5, 32.6 % for day 6) in KD cells despite the increased actual percentage secretion on days 5 and 6. Error bars indicate SE (n=24 for day 1 and day 2, n=12 for day 3). Asterisks indicate statistically significant difference between control and KD cells (paired t-test, *** p<0.001)
Figure 4.3.2. High K$^+$-stimulated Ca$^{2+}$-dependent exocytosis was reduced pLKO-rSec5-puro/neo 2X KD cells. Secretion was induced by 70 mM K$^+$ for 20 min at 37°C. (A) Mean actual percentage of secreted radioactivity in control and KD cells. Average secretion was lower on day 4 and higher on days 5 and 6 in both control and KD cells. (B) Normalized percentage of secretion in control and KD cells. Percentage secretion values were normalized to the mean percentage secretion of pLKO-puro/neo control cells under high K$^+$. Normalized secretion provides better visualization of the consistent decrease (16.1% for day 4, 22.9% for day 5, and 23.8% for day 6) in KD cells despite the increased actual percentage of secretion on days 5 and 6. Error bars indicate SEM (n=12 for day 1 and day 2, n=6 for day 3). Asterisks indicate statistically significant difference between control and KD cells (paired t-test, ** p=0.003; *** p<0.001).
4.4. DCV docking is affected in Sec5 KD cells

The exocyst has been postulated to be critical for the tethering of secretory vesicles to the plasma membrane (Tsuboi et al., 2005). Since Sec5 is a central component of the exocyst complex, I tested whether down-regulation of Sec5 could result in perturbation of DCV docking by electron microscopy. Representative electron micrographs for pLKO-puro control, pLKO-rSec5-puro KD, pLKO-puro/neo control, pLKO-rSec5-puro/neo 2X KD cells are shown in Figures 4.4.1 to 4.4.10, with arrows indicating exemplary DCVs. Figure 4.4.11 summarizes the distribution of DCVs within single cells for each cell type, where the average proportion of vesicles localized within particular distances from the PM displayed in panel A and the mean number of vesicles for each cell type in panel B. Error bars indicate SE. Compared to pLKO-puro control cells, pLKO-rSec5-puro KD cells exhibited a 36.4% reduction in the proportion of DCVs located within 25 nm from the PM (or a 22.4% reduction within 50 nm). There was no difference between the mean numbers of DCVs in control or KD cells, as was also supported by a 41.6% increase in the proportion of DCVs located farther than 200 nm from the PM. Similar results were obtained from pLKO-rSec5-puro/neo 2X KD cells (Figure 4.4.12). Whereas the mean numbers of DCVs were similar for control and 2X KD cells, a 31.3% decrease was found for the percentage of “docked” DCVs (within 50 nm from the PM), together with a 31.5% increase in the percentage of DCVs located farther than 200 nm from the PM. Together, these results suggest that Sec5 KD results in deficiencies in DCV docking.
Figure 4.4.1. Electron micrograph of a single pLKO-puro control cell. Examples of DCVs are indicated by arrows.
Figure 4.4.2. Electron micrograph of a second single pLKO-puro control cell. Examples of DCVs are indicated by arrows.
Figure 4.4.3. Electron micrograph of a single pLKO-rSec5-puro KD cell. Examples of DCVs are indicated by arrows.
Figure 4.4.4. Electron micrograph of a second single pLKO-rSec5-puro KD cell. Examples of DCVs are indicated by arrows.
Figure 4.4.5. Electron micrograph of a third single pLKO-rSec5-puro KD cell. Examples of DCVs are indicated by arrows.
Figure 4.4.6. Electron micrograph of a single pLKO-puro.neo control cell. Examples of DCVs are indicated by arrows.
Figure 4.4.7. Electron micrograph of a second single pLKO-puro/neo control cell. Examples of DCVs are indicated by arrows.
Figure 4.4.8. Electron micrograph of a single pLKO-rSec5-puro/neo 2X KD cell. Examples of DCVs are indicated by arrows.
Figure 4.4.9. Electron micrograph of a second single pLKO-rSec5-puro/neo 2X KD cell. Examples of DCVs are indicated by arrows.
Figure 4.4.10. Electron micrograph of a third single pLKO-rSec5-puro/neo 2X KD cell. Examples of DCVs are indicated by arrows.
Figure 4.4.11. DCV docking is reduced in pLKO-rSec5-puro KD cells. (A) Percentages of DCVs within 0-25 nm, 25-50 nm, 50-100 nm, 100-150 nm, 150-200 nm and beyond 200 nm from the plasma membrane in pLKO-puro (control) and pLKO-rSec5-puro (KD) cells. (B) Mean number of DCVs in control and KD cells. Error bars represent SE (n=31 for each of control and KD). Asterisks indicate statistically significant difference between control and KD cells (two sample t-test, ** p<0.01; *** p<0.001).
**Figure 4.4.12. DCV docking is reduced in pLKO-rSec5-puro/neo 2X KD cells.** (A) Percentages of DCVs within 0-25 nm, 25-50 nm, 50-100 nm, 100-150 nm, 150-200 nm and beyond 200 nm from the plasma membrane in pLKO-puro/neo (control) and pLKO-rSec5-puro/neo (2X KD) cells. (B) Mean number of DCVs in control and KD cells (two sample t-test, p=0.05). Error bars represent SE (control: n=30; KD: n=31). Asterisks indicate statistically significant difference between control and KD cells (two sample t-test, ** p<0.01; *** p<0.001)
4.5. Sec5 downregulation by TetR-tetO drug-inducible system

Results from secretion assays and EM analysis show that Sec5 1X and 2X KD cells exhibited reduced GTP-/Ca\(^{2+}\)-dependent secretion as well as DCV docking, but only to certain extent. This might be a result of the incomplete suppression of Sec5 in these KD cells. In order to achieve better KD and more prominent phenotypes, multiple single colonies were isolated in the hope of establishing stable cell lines in which Sec5 is more strongly down-regulated. Unfortunately, this method was not able to improve the level of Sec5 KD (data not shown). This is reasonable since Sec5 has been implicated in cell survival and development, and the cells in which Sec5 is strongly knocked down may have already died before reaching the stage of selection. To overcome this hurdle, I established the lentivirus-mediated TetR-tetO drug-inducible KD system in PC12 cells which allows Sec5 to be down-regulated in fully developed cells. Preliminary data show that inducible Sec5 KD has been achieved using this conditional KD system (Figure 5.5.1). When doxycycline was added, Sec5 was significantly reduced in the KD cells. However, Sec5 expression also slightly decreased in the KD cells even in the absence of doxycycline, possibly because of the incomplete suppression of siRNA expression by TetR. Secretion and docking experiments have not been performed on these cells due to time limit. Analyses of the secretory phenotypes of the drug-induced KD cells may be able to provide further mechanistic insights regarding the functions of Sec5 and the exocyst in exocytosis of mammalian cells.
Figure 4.5.1. Immunoblot analysis of doxycycline-induced Sec5 KD in PC12 cells.
Inducible KD of rSec5 was achieved using a lentivirus vector-based system for conditional gene suppression. pLVX-IRES-hygro-TetR-KRAB-tetO-puro (control) and pLVX-IH-TetR-KRAB-tetO-rSec5-puro (KD) cells were incubated with or without (1 µg/ml) doxycycline hyclate for 7 days before they were harvested for western blot analysis.
4.6 Over-expression of mouse Sec5-GFP in wild type PC12 cells

To examine whether the reductions in GTP/Ca\(^{2+}\)-dependent exocytosis and DCV docking were truly caused by Sec5 KD, I reintroduced Sec5 protein in pLKO-puro control and pLKO-rSec5-puro KD cells through infection of the cells with viral particles containing pLVX-IRES-blast-mSec5(SNM)-EmdGFP which expresses mouse Sec5 fused with emerald GFP. To prevent the mSec5 from being degraded by the pre-existing shRNA in the cells, we included silent nucleotide mutation (SNM) in its sequence. Emerald GFP was introduced for two purposes: 1) to serve as a marker of successful infection; and 2) to distinguish between endogenous rSec5 and over-expressed mSec5 by size difference. Transduced cells were selected with 5 µg/ml blastocidin. Figure 5.6.1 shows a western blot of over-expressed mSec5-EmdGFP as well as endogenous rSec5 in the control and KD cells. mSec5-EmdGFP was expressed at similar levels in both control and KD cells, although the endogenous rSec5 was significantly reduced in the KD cells. Again, secretory phenotypes of these cells have not yet been examined due to time limit; and would be our crucial next step in the analysis of Sec5 function in mammalian cell exocytosis.
Figure 4.6.1. Immunoblot analysis of pLKO-puro control and pLKO-rSec5-puro KD cells in which mSec5-EmdGFP was over-expressed. mSec5-EmdGFP expression was achieved by infecting the cells with pLVX-IRES-blast-mSec5(SNM)-EmdGFP. Transduced cells were selected using 5 µg/ml blastocidin. mSec5-EmdGFP was expressed at similar levels in both control and KD cells despite significant reduction of endogenous rSec5 levels in the KD cells. Equal amount of protein (25 µg) was loaded into each well. VCP served as loading control.
5. Discussion

Despite the well-established roles played by Sec5 and other interacting exocyst proteins in the vesicular trafficking for cell growth and the insertion of many proteins into the plasma membrane, their function in vesicle exocytosis and the potential underlying mechanisms remain unclear, particularly in mammalian cells. Current knowledge regarding exocyst functions mainly comes from studies in yeast and *Drosophila*. The uncertainty of mammalian exocyst function is at least in part due to a lack of good loss-of-function models for direct comparison. PC12 cells have long been used as excellent models of mammalian neurosecretory cells in their native undifferentiated form, as well as neurons in their differentiated form upon stimulation by nerve growth factor (NGF). Our lab has successfully generated stable knockdown (KD) of a number of exocytosis-related proteins, including Munc18-1, syntaxin1A/1B, CAPS1, and RalA/B within PC12 cells using the pSuper KD system (Brummelkamp et al., 2002). In my study, I used the undifferentiated form of PC12 cells for the ease of analysing various secretory phenotypes and a broader application of the results in mammalian secretory cell types such as pancreatic β-cells, platelets and mast cells. Using the latest lentivirus-mediated RNA interference (RNAi) technology (Root et al., 2006), I generated PC12 cells in which Sec5 is down-regulated, and focused on analysing the various secretory phenotypes including GTP- and Ca\(^{2+}\)-dependent dense core vesicle (DCV) exocytosis and DCV docking in these cells. Furthermore, in the search for a more appropriate KD system for Sec5 which is thought to be heavily involved in cell development, I established a lentivirus-mediated TetR-*tetO* drug-inducible KD system allowing for conditional
suppression of Sec5. Efforts were also made to re-introduce Sec5 with silent nucleotide mutations (SNM) to the Sec5 KD cells for rescue purposes.

5.1. Expression of Sec5 upon lentivirus-mediated RNAi in PC12 cells

The establishment of stable Sec5 KD cell line is crucial for our subsequent analysis of Sec5 function in DCV exocytosis. As mentioned, there are few good KD models for Sec5 in mammals due to its heavy involvement in cell growth. In *Drosophila*, homozygous Sec5 or Sec6 mutant animals die as growth-arrested larvae once the maternal gene products are depleted (Murthy et al., 2003; Beronja et al., 2005; Murthy et al., 2005). Sec5 KD by siRNA has been shown in normal rat kidney (NRK) cells but only to a moderate extent (Rosse et al., 2006). Studies of the other exocyst subunits mostly depend on over-expression of wild type of mutant proteins in the presence of endogenous wild type proteins or the introduction of antibodies against specific subunits (Vega and Hsu, 2001; Yeaman et al., 2001; Lipschutz et al., 2003; Prigent et al., 2003; Sans et al., 2003).

As mentioned, our lab has previously used the pSuper plasmid system to generate stable RalA/RalB KD PC12 clones. However, the success rate of isolating strong KD cells was relatively low as a result of the low frequency of incorporation of transfected DNA into the chromosome of the host cells. For the purpose of down-regulating Sec5, I adopted the lentivirus-mediated infection system which has been shown to transport and integrate the KD plasmid into host chromosome with a higher efficiency (Root et al., 2006). The stable Sec5 1X and 2X KD cells in this study showed a
~60% reduction in the expression of Sec5 and indicated no problem in cell survival. Morphology of the KD cells was largely normal. Growth of these KD cells was originally slightly slower but eventually caught up with that of the corresponding control cells. Two times KD of Sec5 using pLKO-rSec5-puro and pLKO-rSec5-neo did not result in greater reduction in Sec5 level, indicating KD at the specific target site has reached saturation, suggesting the possibility that the cells in which Sec5 was strongly down-regulated might indeed have problem surviving/growing, and were not present in our heterogeneous pool of selected cells. Nonetheless, our Sec5 KD cells exhibited defects in DCV secretion and DCV docking, as will be discussed in later sections.

5.2. Co-dependent expressions of Sec5 and Sec6 in PC12 cells

Interestingly, expression of Sec6 was reduced by ~20% in both of the 1X and 2X KD cells, whereas expression of the other exocyst proteins were unaltered (Figure 5.1.3). These results are consistent with previous findings in Drosophila where mutants of Sec5 and Sec6 share similar phenotypes indicative of shared functions of the two proteins. In Drosophila, homozygous mutants of either sec5 or sec6 were born but died around 96 hours after egg laying. Larvae from both mutants displayed similar defects in membrane protein trafficking, a phenotype also observed in germ line clones mutant for either sec5 or sec6 (Murthy et al., 2003; Murthy and Schwarz, 2004; Murthy et al., 2005). Sec5 is mislocalized in sec6 mutants (Murthy et al., 2005). In addition, it has been shown that Sec5 and Sec6 could be co-immunoprecipitated from embryonic lysates using Sec5 antibody, and their sedimentation profiles from membrane fractionation experiment largely overlap, suggesting that they associate with a relatively common membrane
population (Beronja et al., 2005). In fact, immunoblot analysis has shown that Sec5 is down-regulated almost as much as Sec6 in our recently generated Sec6 KD cells (data not shown). These results together suggest that Sec5 and Sec6 may exist in the same subcomplex and function together in multiple secretory events. Alternatively, reduced expression of Sec6 in our Sec5 KD cells could be explained by off-target effect of our KD construct. To rule out this possibility, we could use other target sites to knock down Sec5. Murthy et al. (2005) also found that Sec8 is mislocalized in both sec5 and sec6 germ line clones, although the patterns of mislocalization were different. We did observe a slight reduction in Sec8 expression levels in our Sec5 2X KD cells. However, in our Sec5 1X KD cells, the expression levels of Sec8/Exo70/Exo84 were comparable to those of the control cells.

5.3. Common and distinct roles of the exocyst subunits

The similar phenotypes in sec5 and sec6 mutant Drosophila suggest that the components of the exocyst may act in a common pathway essential for cell viability and membrane trafficking. Nonetheless, emerging evidence indicates that the exocyst subunits may not always function as a complex. Firstly, distribution patterns of the exocyst subunits are not identical. In Drosophila photoreceptor cells (PRCs), for example, Sec6 appears to have a broader distribution than Sec5 and Sec8 suggesting its possible association with additional membrane populations (Beronja et al., 2005). In the PRCs, Sec5 and Sec8 highly co-localize with Sec15 in the lamina, while similar expression pattern is not seen for Sec6. Secondly, Sec5 and Sec6 are broadly required for exocytosis and cell survival, Sec10 seems to be involved in only a few secretory events (Andrews et
al., 2002; Murthy et al., 2003; Beronja et al., 2005; Murthy et al., 2005). Thirdly, in PRCs, the function of Sec15 is very much different from that of Sec5 or Sec6. Unlike PRCs with homozygous mutations in sec5 or sec6 which die as growth-arrested larvae, PRCs homozygous for loss-of-function in sec15 survive and differentiate normally, with defects only in their selection of specific synaptic targets as a result of disrupted trafficking of specific cell adhesion and signalling molecules (Mehta et al., 2005). These results suggest that each member of the exocyst complex may play common and distinct roles in vesicular trafficking and regulated fusion of secretory vesicles. Whether the phenotypes observed in our Sec5 KD cells are attributable to the loss of function of the complex as a whole or only to the disruption of some kind of sub-complex containing Sec5 requires further investigation. Since Sec5 binds in the center of the assembled exocyst (Guo et al., 1999b), it is reasonable for us to assume that the function of the complex is at least disrupted to some extent in our Sec5 KD cells.

5.4. Functional significance of Sec5 in regulated DCV exocytosis

Murthy et al. (2003) found that Sec5 is necessary for many aspects of the membrane traffic within neuronal tissues of Drosophila, including neurite outgrowth and neuromuscular junction (NMJ) expansion which could be explained by its requirement in membrane protein insertion. However, by measuring the size of the nerve-evoked responses and the size of the individual minis between 48 and 96 hours AEL, they also found that Sec5 is not required for the regulation of synaptic vesicle exocytosis, at least in specific NMJs. The trace amount of maternal Sec5 present during this time of larval development could not account for the unaltered transmitter release since this level of
maternal contribution could no longer support other forms of membrane traffic. Sec5 is therefore thought to differentiate between the membrane trafficking for membrane growth and that for synaptic transmission (Murthy et al., 2003). More specifically, it is likely that Sec5 regulates the transport of vesicles derived from the trans-Golgi network (TGN); whereas the local cycling of vesicles primarily from the plasma membrane or through the endosomal pathway may be exocyst-independent, as in the case of SV exocytosis.

It remains unclear, however, whether the DCVs which are TGN-derived but which exocytose in a regulated manner could fuse with the plasma membrane without Sec5 or the exocyst complex. This has also been the main focus of my study. As mentioned, the exocyst is found to be regulated by small GTPases such as Sec4p in yeast, Rab11 in multi-cellular organisms, and RalA/B in mammals. The interaction between RalA and the exocyst complex was first identified in neuronal cells using a pull-down assay in which recombinant GST-RalA was used to isolate RalA-binding proteins (Brymora et al., 2001). The Ral-exocyst interaction was confirmed by several other groups as well, with Sec5 identified as a direct target of Ral in its active GTP-bound form (Moskalenko et al., 2002; Sugihara et al., 2002). Expression of a dominant-negative form of Ral in the brain of transgenic mice was shown to influence the readily releasable pool (RRP) of SV (Polzin et al., 2002), and inhibition of Ral function blocks the K⁺-stimulated release of human growth hormone from PC12 cells. Also, as previously discussed, findings from our lab also indicate the GTP-dependent interaction between RalA/B and the exocyst complex mediates the GTP-dependent exocytosis (Wang et al., 2004; Li et al.,
These results may suggest a role for Sec5 or the exocyst in the regulation of DCV exocytosis, and I therefore tested the functional importance of Sec5 in PC12 cells.

GTP-dependent secretion of norepinephrine from DCVs in our Sec5-puro KD cells was reduced by 40% compared to that of the control cells, which was significant considering the fact that Sec5 was only knocked down by 60%. The basal levels of GTP-dependent secretion in both control and Sec5-puro KD cells were around 16% before normalization (Figure 5.2.1A), which was relatively high. One explanation could be that our cells have been damaged to some extent and became leaky due to the freeze-thaw method used for permeabilization of the cell membrane. This may also help explain the relatively low level of GppNHp-stimulated secretion later observed. In addition, results from some of the GTP-dependent secretion assays were not as good as the others, which may be due to the delicate balance of the rates of freezing and thawing required for efficient permeabilization (Klenchin et al., 1998). Alternatives of the freeze-thaw method include some bacterial or peptide toxins which make small pores on the plasma membrane which allow passage of GTP analogues.

Our lab has previously shown that silencing of RalA and/or RalB in PC12 cells had no effect on high [K⁺]-stimulated Ca²⁺-dependent exocytosis (Li et al., 2007). Our Sec5 KD cells, however, did exhibit partially reduced Ca²⁺-dependent secretion of norepinephrine, indicating Sec5 may be involved in additional steps in DCV cycle that are required for both GTP- and Ca²⁺-dependent exocytosis. Ca²⁺-dependent neurotransmitter release was only moderately reduced by 30% in the 1X KD and 20% in
the 2X KD cells (Figures 5.3.1B and 5.3.2B), likely due to the incomplete suppression of Sec5. One point I would like to note about the Ca\(^{2+}\)-dependent assay is that secretions of both control and KD cells were generally lower on the first day (day 4) and increased on the subsequent two days (Figures 5.3.1A and 5.3.2A), possibly due to better expression of certain types of the L-type Ca\(^{2+}\) channels and other unknown reasons (Elhamdani et al., 1998). Another interesting observation is that the basal level secretion under PSS for days 4 and 5 was significantly higher in the pLKO-rSec5-puro/neo KD cells compared to pLKO-puro/neo control cells. Moreover, the ratio of stimulated (high K\(^+\)) : basal (PSS) secretion was poorer on day 4, better on day 5 and best on day 6. Further investigations are necessary to explain these phenomena.

Stronger and more explicit phenotypes would require the establishment of a model system in which Sec5 could be more strongly down-regulated. But before that, I tried to identify the stage at which Sec5 is exerting its effect. Studies in yeast have shown that the exocyst complex is required before the assembly of the SNARE complex and therefore the final steps in vesicle fusion. And since the exocyst complex is heavily involved in protein trafficking, it is reasonable to hypothesize that Sec5 KD would be involved in the tethering/docking stage of the DCV cycle. This was indeed the case seen from our EM analysis as will be discussed in the next section.

5.5. DCV docking defects in the Sec5 KD PC12 cells

Spatial distribution of DCVs in the electron micrographs of PC12 cells were quantified by means of the distance from the vesicles to the plasma membrane that have
been previously adopted by many others (Gutierrez et al., 1997; Plattner et al., 1997; Koval et al., 2000, 2001; Pothos et al., 2002; Gong et al., 2003). Anatomically, DCVs located within 50 nm from the plasma membrane are generally considered as being in the docking stage (Sugita, 2008). According to this standard, DCV docking was reduced by ~20% and ~30% in our Sec5-puro and Sec5-puro/neo KD cells, respectively. Since the mean numbers of DCVs in control and KD cells did not differ significantly, this decrease in the percentage of docked vesicles was accompanied by an increase in the percentage of vesicles located beyond 200 nm from the plasma membrane (Figures 5.4.11 and 5.4.12). Thus our results are supportive of a model in which Sec5 acts upstream of the RalA/B GTPases and the SNARE complex in DCV secretion by regulating the tethering/docking of the DCVs to the plasma membrane.

### 5.6. Significance of the establishment of drug-inducible Sec5 KD system in PC12 cells

It is almost impossible to generate a Sec5 KO system due to the many roles played by Sec5 in cell survival and development. As discussed, one major disadvantage of our method in selecting stable Sec5 KD cell lines is that the cells in which Sec5 expression was strongly suppressed might have suffered from growth defects and could not survive to the stage of drug selection. The cells we were able to select thus represented a heterogeneous population of cells with various degrees of partial Sec5 down-regulation. To avoid this problem, we took the effort to establish a drug-controllable KD system for Sec5 in PC12 cells. Despite the slight leakiness of TetR (Figure 5.5.1), this system will be a valuable tool in analysing the functions of the developmentally essential Sec5.
5.7. Limitations of the study

There are advantages and limitations associated with any system, and our model of study is no exception. One major problem of our stable Sec5 KD model is the incomplete suppression of Sec5 expression which makes it hard to determine the possible underlying causes of the relatively moderate phenotypes observed. Also, the expression of some exocyst subunits tend to be affected by each other, making it difficult to pinpoint the functions associated with each individual subunit. Another problem is that since the exocyst complex is involved in cell growth, and trafficking/insertion of various membrane proteins (Murthy et al., 2003; Beronja et al., 2005; Murthy et al., 2005), it is possible that disruption of the exocyst function will affect the expression of the voltage-gated K⁺ and Ca²⁺ channels which could not be examined in our model. Furthermore, for EM analysis, we combined the distances obtained from different cells within a cell group and later compared between different groups. This method has recently been criticized by Diaz et al. because it does not take into consideration the interdependence among the distances observed within single cells which might be related to the interactions among the vesicles (Diaz et al., 2009). Other limitations of our model of study have been discussed previously.

5.8. Future directions

The secretory phenotypes of the drug-inducible Sec5 KD cells will be analysed in the next step. In addition to GTP- and Ca²⁺-dependent secretion assays and EM analysis, the roles for Sec5 in directional trafficking could be examined by visualizing DCVs and SVs labeled with fluorescent protein markers in living PC12 cells.
differentiated by NGF. To further investigate the inter-dependence between Sec5 and Sec6, we are currently working to KD Sec6 using the same RNAi strategies (both stable and drug-inducible). Initial data show that Sec5 is suppressed almost as much as Sec6 in the stable Sec6 KD cells. Also, I over-expressed wild-type mouse Sec5 (SNM) fused with Emerald GFP in the Sec5-puro KD cells to rescue the docking/secretion defects in the stable Sec5 KD cells. Functional analysis of these rescued cells may answer if the observed defects are really caused by Sec5 down-regulation and may reveal possible roles played by Sec6. Upon confirmation of the rescue ability of WT Sec5, abilities of various mutants in rescuing the phenotypes will also be tested to elucidate the structural determinants of Sec5.
6. References


