Identification and characterization of the interaction between VPS33B and SNAREs

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science

Department of Biochemistry
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Abstract

VPS33B is a Sec1/Munc18 (SM) protein known to be required for the biogenesis of α-granules in megakaryocytes, large polyploid cells found primarily in the bone marrow, which give rise to platelets. Mutations in VPS33B cause arthrogryposis, renal dysfunction and cholestasis (ARC) syndrome, a multisystem disorder characterized by a failure to thrive and death within the first year of life. Platelets from ARC patients completely lack α-granules, causing a bleeding disorder. As a member of the SM protein family, VPS33B is thought to play a role in vesicular fusion events through its interaction with the SNARE proteins, though these interactions have yet to be identified. Here, it is shown that VPS33B interacts with STX6, a member of the syntaxin subfamily of SNAREs. The introduction of ARC missense and nonsense mutations into VPS33B, as well as VPS33A missense mutations, completely abrogated binding to STX6. Confocal microscopy studies revealed STX6 also co-localizes well with markers of the α-granule biogenesis pathway. Taken together, this implies a role for the interaction of VPS33B with STX6 in α-granule biogenesis. Co-immunoprecipitation, sucrose gradient centrifugation, and immunofluorescence data suggested that the interaction between VPS33B and STX6 is transient, and the majority of the cellular pools of each protein exist in distinct cellular compartments. This data, along with the known structure of STX6 and that predicted of VPS33B, suggests a novel and unique mode of binding between VPS33B and STX6 compared to other identified SM-STX pairs.
Acknowledgments

I would like to extend my deepest thanks to my supervisor, Dr. Walter Kahr, whose eternal optimism helped me through the most frustrating days; to my supervisory committee Dr. William Trimble and Dr. David Williams for their wisdom and guidance; to all members of the Kahr lab, Anson Chen, Ling Li, Denisa Urban, and Dr. Fred Pluthero for helpful advice, support, and entertainment; and to Moshe Kim for technical assistance with the yeast two-hybrid experiments, and all members of the Trimble lab for helpful advice and allowing the occasional borrowing of reagents.
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List of Abbreviations

AMEM: Minimal Essential Medium Alpha
ARC: arthrogryposis, renal dysfunction, and cholestasis
CFTR: cystic fibrosis transmembrane conductance regulator
CIP: calf-intestinal phosphatase
CORVET: class C core vacuole/endosome tethering
DMEM: Dulbecco’s modification Eagle’s medium
DMSO: dimethyl sulfoxide
DSP: Dithiobis[succinimidyl propionate]
DTT: dithiothreitol
ECL: enhanced chemiluminescence
EDTA: Ethylenediaminetetraacetic acid
EMEM: Eagle’s Minimal Essential Medium
FAK: focal adhesion kinase
GEF: guanosine exchange factor
GFP: green fluorescent protein
HA: haemagglutinin
HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HOPS: homotypic fusion and protein sorting
HPS: Hermansky-Pudlak syndrome
HRP: horseradish peroxidase
IRAP: insulin-responsive amino-peptidase
ISG: immature secretory granule
LBPA: lysobisphosphatidic acid
LB: Lysogeny broth
MVB: multivesicular body
MWCO: molecular weight cut-off
NEM: N-ethylmaleimide
NSF: N-ethylmaleimide sensitive factor
PBS: phosphate-buffered saline
PCR: polymerase chain reaction
PEG: polyethylene glycol
PMA: phorbol-12-myristate-13-acetate
RPMI: Roswell Park Memorial Institute
SBP: streptavidin-binding peptide
SDS-PAGE: sodium dodecyl sulphate polyacrylamide gel electrophoresis
SM: Sec1/Munc18
α-SNAP: soluble N-ethylmaleimide-sensitive factor attachment protein, alpha
SNAP: synaptosome-associated protein
SNARE: soluble N-ethylmaleimide-sensitive factor attachment protein receptor
STX: syntaxin
TBS: Tris-buffered saline
TPO: thrombopoietin
VAMP: vesicle-associated membrane protein
VIPAR: VPS33B-interacting protein involved in polarity and apical protein restriction
VPS: Vacuole protein sorting
VWF: von Willebrand Factor
YNB: yeast nitrogen base
YPD: yeast extract, peptone, dextrose
Chapter 1

Introduction

1.1 – Platelets and megakaryocytes

Platelets are small (~3 by 0.5 μm), disc-like, anucleate blood cells that circulate in the vasculature for 7-10 days and play a significant role in hemostasis and other functions. They are derived from megakaryocytes; large, polyploid cells found primarily in the bone marrow.

1.1.1 – General platelet structure and function

Platelets are characterized by the presence of four types of granules, the secretory α-granules and dense granules, which are responsible for secreting proteins and other small molecules at the sites of platelet function (Italiano and Hartwig, 2007), as well as lysosomes and peroxisomes. The presence of the secretory granules can be seen in Figure 1.

![Platelet structure](image)

**Figure 1.** Platelet structure. Platelets are anucleate blood cells characterized by the presence of four types of secretory granules responsible for storing proteins and small molecules essential for platelet function. Adapted from George, 2000.

Perhaps most widely established is the role of platelets in establishing hemostasis at sites of vascular injury. It is known in humans that patients with too few or dysfunctional platelets can suffer moderate to severe bleeding problems (Richardson et al., 2005; Nurden and Nurden,
Platelets adhere and aggregate at sites of vascular injury, which is followed by subsequent platelet activation, characterized by cytoskeletal rearrangements and the secretion of specialized granules containing proteins and small molecules important for platelet plug formation. This leads to fibrin clot formation to prevent blood loss. Platelets have also been shown to contribute to wound healing and organ regeneration, as serotonin present in platelet dense granules is required for liver regeneration (Clavien, 2008). Platelets play a role in angiogenesis, containing both pro- and anti-angiogenic factors (Coppinger et al., 2004; Reed, 2004), and they defend against infection and play a role in the innate and adaptive immune response, through the interaction with endothelium and leukocytes and the release of inflammatory and immune modulating factors contained in α- and dense granules (Elzey et al., 2003; Weyrich and Zimmerman, 2004; May et al., 2008). Clearly, these cells play an important role in the human body, and the understanding of proper platelet function is a key and expanding area of research.

1.1.2 – Megakaryocyte maturation and platelet formation

Platelets are formed from larger progenitor cells called megakaryocytes, with platelet formation representing the final stage of megakaryocyte development. Megakaryocytes are highly specialized cells found predominantly in the bone marrow, and function solely to produce platelets and release them into the circulation (Italiano and Hartwig, 2007). Megakaryocytes arise from pluripotent stem cells, and undergo a series of DNA replications without cell division through a process known as endomitosis. Upon completion of this process, polyploid (up to 128N) megakaryocytes begin rapid cytoplasmic expansion and the accumulation of cytoplasmic proteins and granules that are essential for platelet function. The final stage of megakaryocytic development involves cytoplasmic reorganization, allowing for the formation of long, thin, beaded extensions known as proplatelets (Italiano and Hartwig, 2007). It is thought that the megakaryocyte protrudes these proplatelet extensions through the endothelium and into the vasculature. Subsequently, sheer flow is thought to provide the force required to allow the proplatelet extension to break away from the megakaryocyte mother cell and result in the release of fully-formed platelets into the bloodstream, as shown in Figure 2 (Junt et al, 2007; Geddis and Kaushansky, 2007).
Figure 2. Generation of platelets in the bloodstream. Platelets are formed from megakaryocytes, large, polypoid cells that extend finger-like pro-platelet extensions across endothelial cells into the bloodstream. It is thought the shear flow of the blood vessel allows the pro-platelet extensions to break down and release platelets into the bloodstream. Adapted from Geddis and Kaushansky, 2007.

1.1.3 – Platelet α-granules and biogenesis of α-granules in megakaryocytes

The α-granule is the more abundant of the two secretory granules produced in the megakaryocyte and subsequently transported into platelets. Each platelet contains roughly 50-80 α-granules ranging from 200-500 nm in size. They comprise approximately 10% of the platelet volume, and the extra membrane provided by the secretion of these granules, along with that of the open canalicular system (a system of invaginations of the cell membrane unique to the platelet) (White and Clawson, 1980), allows the platelet to increase its surface area by 2-4-fold upon platelet stimulation (Blair and Flaumenhaft, 2009). α-granules contain over 300 different secretory proteins, including adhesion molecules, chemokines, cytokines, fibrinolytic regulators, immunologic modulators and an assortment of coagulation, complement, growth, and pro- and anti-angiogenic factors (Harrison and Cramer, 1993; Coppinger et al., 2004; Reed, 2004). These play important roles in clotting, angiogenesis, inflammation, wound healing, bone remodelling, and the development of atherosclerotic lesions. It has also been suggested that different α-granule cargo proteins are packaged into different α-granules, such that not all α-granules contain identical cargo proteins (Sehgal and Storrie, 2007; Italiano et al, 2008; Kamykowski et al., 2011).

Biogenesis of α-granules occurs in the megakaryocyte, deriving in part from the budding of small vesicles from the trans-Golgi network that contain α-granule cargo (Cramer et al., 1990; Hegyi et al., 1990). However, vesicles budding off from the trans-Golgi network can also be
sorted to the multivesicular bodies (MVBs), endosomal structures found in most cells that form from the limiting membrane of the endosome (Piper and Katzmann, 2007; Woodman and Futter, 2008). MVBs are involved in sorting vesicles containing endocytosed and newly-synthesized proteins. In megakaryocytes, MVBs serve as an intermediate phase in granule production, and both α-granules and dense granules are sorted via MVBs (Heijnen et al., 1998; Youssefian and Cramer, 2000). It is not clear whether all vesicle trafficking to α-granules proceeds through MVBs, as some trafficking may occur directly from the trans-Golgi network. In addition, clathrin-mediated endocytosis mediates the delivery of plasma membrane and extracellular cargo such as fibrinogen to α-granules, and these endocytic vesicles may also be sorted through the MVBs (Heijnen et al., 1998; Blair and Flaumenhaft, 2009) (Figure 3).

**Figure 3.** α-granule biogenesis in megakaryocytes. α-granules are formed from vesicles that bud off either directly from the trans-Golgi network, or from the trans-Golgi network and subsequent sorting through the multi-vesicular bodies (MVBs). Endocytosis at the plasma membrane also delivers cargo to α-granules, and these endocytic vesicles may also be sorted by MVBs. Adapted from Blair and Flaumenhaft, 2009.

α-granules formed in megakaryocytes are transported to platelets during the formation of proplatelet extensions that occur in the final stages of megakaryocyte development. The α-granules, and other organelles, are transported along microtubule tracks from the megakaryocyte cell body into the proplatelet extensions (Richardson et al, 2005). Movement occurs at a rate of 0.1-2 μm/min in an apparent random direction. α-granules travel along microtubules both in a kinesin-dependent manner, and also “piggyback” on the surface of sliding microtubules, the force for which is provided by cytoplasmic dynein. α-granules are captured in the developing platelet via microtubule coils present in platelets (Richardson et al, 2005). It is thought that
morphologically distinct sub-types of α-granules exist, and that high spatial segregation of cargo exists within individual α-granules, as seen in Figure 4 (van Nispen tot Pannerden et al., 2010).

![Figure 4](image_url)

**Figure 4.** Trafficking of α-granules during platelet formation. α-granules and other organelles are transported along microtubules from the the megakaryocyte cell body to the proplatelet extensions. Limited colocalization between α-granule proteins fibrinogen (green) and von Willebrand Factor (red) demonstrates the existence of distinct sub-types of α-granules. Adapted from Italiano et al., 2008 and Blair and Flaumenhaft, 2009.

1.2 – The role of the Sec1/Munc18 and SNARE protein families in vesicular trafficking

Trafficking of proteins and lipids through membrane-enclosed vesicles is required for a wide range of functions fundamental to eukaryotic cells. These include organelle formation and maintenance, neurotransmitter secretion, protein targeting and cell growth. Several conserved families of cytoplasmic and membrane-bound proteins participate in these trafficking events, and these proteins interact with one another to allow for the formation of vesicle-attachment and membrane-fusion complexes necessary for the fusion of vesicles with the proper target membrane. Two such protein families include the soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs), and the Sec1/Munc18 (SM) proteins.

1.2.1 – Characterization of the SNAREs

The SNARE proteins have been shown to function in the final event of docking of vesicles with target membranes and catalyzing the fusion of the opposing membranes of the transport vesicle and target compartment (Sollner et al., 1993b; McNew et al., 2000; Hu et al., 2003). In humans, 36 SNAREs have been identified, with their major features summarized in Table 1 (adapted from Hong, 2005).
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<td>111</td>
<td>25-77</td>
<td>87-106</td>
<td>Q_c</td>
</tr>
<tr>
<td>GS27</td>
<td>212</td>
<td>130-182</td>
<td>192-212</td>
<td>Q_b</td>
</tr>
<tr>
<td>GS28</td>
<td>250</td>
<td>170-222</td>
<td>231-250</td>
<td>Q_b</td>
</tr>
<tr>
<td>Vti1a</td>
<td>217</td>
<td>132-184</td>
<td>193-214</td>
<td>Q_b</td>
</tr>
<tr>
<td>Vti1b</td>
<td>232</td>
<td>146-198</td>
<td>107-229</td>
<td>Q_b</td>
</tr>
<tr>
<td>Slt1</td>
<td>259</td>
<td>173-225</td>
<td>232-252</td>
<td>Q</td>
</tr>
<tr>
<td>Sec20</td>
<td>228</td>
<td>132-184</td>
<td>203-220</td>
<td>?</td>
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</tbody>
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The SNAREs are generally small proteins of approximately 100-300 amino acids, and all contain an evolutionally-conserved, α-helical SNARE motif of about 60 residues in length (Weimbs et al., 1997). All identified SNAREs contain one SNARE motif, with the exception of SNAP-23, SNAP-25, and SNAP-29, which contain two tandem SNARE motifs separated by a linker region. These three proteins, along with syntaxin 11 and Ykt6, are also the only SNAREs to lack a C-terminal transmembrane domain. These SNAREs instead become membrane-attached via prenylation, palmitoylation of cysteine residues, or through interaction with other membrane-
attached SNAREs (Veit et al., 1996; Prekeris et al., 2000; Vogel et al., 2000; Fukasawa et al., 2004).

The SNAREs can be further divided into subfamilies based on structure similarity, the most prominent of which are the syntaxins (STXs), synaptosome-associated proteins (SNAP), and the vesicle-associated membrane proteins (VAMPs). The STXs contain both the C-terminal transmembrane domain (with the exception of STX11) and one SNARE motif common to most SNARE proteins, but they are also characterized by the presence of an N-terminal, three-helix bundle known as the H_{abc} domain, as shown in Figure 5. The H_{abc} domain of some STXs is able to fold back onto the SNARE motif, and in this case the STX is said to be in a “closed” conformation, unable to interact with other SNARE proteins. When the H_{abc} domain is not folded back on the SNARE motif, the STX is said to be in an “open” conformation, and able to interact with other SNAREs.

![Figure 5](image)

**Figure 5.** Syntaxins can adopt two functionally-distinct conformations. In (A) the H_{abc}-domain of STX1A (red) folds back on the SNARE motif, rendering the syntaxin in a “closed” conformation, unable to interact with other SNAREs. In (B) the H_{abc}-domain is released from the SNARE motif, rendering the syntaxin in the “open” conformation required for interaction with other SNAREs, such as SNAP-25, shown in green. Adapted from Toonen and Verhage, 2003.

The VAMP subfamily is found predominantly on vesicular membranes and contains a C-terminal transmembrane domain, one SNARE motif, but no H_{abc} domain. The synaptosome-associated proteins (SNAP), as described above, contain no transmembrane domain and two tandem SNARE motifs separated by a linker region.
1.2.2 – The SNARE hypothesis for membrane fusion

After a vesicle is brought into close contact with its target membrane, SNARE proteins mediate vesicle fusion through the formation of a SNARE complex, in which SNARE proteins on the vesicular membrane (v-SNAREs) interact with those of the target membrane (t-SNAREs) (Sollner et al., 1993b). A SNARE complex is composed of four α-helical SNARE motifs which form a twisted, parallel four-helical bundle. When this complex is formed by SNAREs on opposing membranes, it is referred to as a trans-SNARE complex, as seen in Figure 6. After membrane fusion, all SNAREs are present on the same membrane in a cis-SNARE complex. cis-SNARE complexes are broken apart and recycled by the action of the AAA ATPase N-ethylmaleimide sensitive factor (NSF) and soluble NSF attachment protein, alpha (α-SNAP) (Sollner et al., 1993a). The first crystal structure of a trans-SNARE complex was solved for the synaptic SNARE complex consisting of STX1A, SNAP-25, and VAMP2 (Sutton et al., 1998).

In a typical trans-SNARE complex, one v-SNARE contributes one SNARE motif to the complex, while either two or three t-SNAREs contribute the other three SNARE motifs. The interaction of the SNAREs and an N- to C-terminal “zippering” of the SNARE motifs into the four-helix bundle is thought to provide the energy necessary to overcome the energy barrier created by the negative charges of the phospholipid headgroups of the lipid bilayers to allow for the fusion of the vesicular membrane with its target (Fasshauer, 2003).

Figure 6. The SNARE hypothesis for membrane fusion. t-SNAREs and v-SNARE interact via their SNARE motifs to form a four-helix bundle known as a SNARE complex. Zippering of this bundle from N- to C-termini provides the energy required for fusion of the vesicular membrane to its target. Adapted from Toonen and Verhage, 2003.
Interaction between the four helices is maintained by the 16 residues present in each helix which form 16 areas of interaction (termed “layers”) between all four helices, usually consisting of hydrophobic residues and arranged perpendicular to the axis of the four-helix bundle. The middle of the four-helix bundle is characterized by a layer of interaction mediated by hydrophilic residues termed the “zero layer”. The 7 layers N-terminal to the zero layer are defined as -7 to -1, and the eight layers C-terminal to the zero layer are termed 1 to 8. The zero layer always consists of three glutamine (Q) residues and one arginine (R) residue (Fasshauer et al., 1998).

Thus, SNARE proteins which contribute a Q residue are termed Q-SNAREs, and those which contribute an R residue are termed R-SNAREs. The SNARE motifs of Q-SNAREs can be further subdivided into three groups. Those Q-SNAREs with motifs most similar to the SNARE motif of STX1A are termed Qa-SNAREs. Those that are most similar to the N-terminal SNARE motif of SNAP-25 are termed Qb-SNAREs, and those that are most similar to the C-terminal SNARE motif of SNAP-25 are termed Qc-SNAREs (Bock et al., 2001). It is believed that all SNARE complexes are composed of one each of the Qa-, Qb-, Qc- and R-SNAREs, and this is the case in the first identified SNARE complex of STX1A (Qa), SNAP-25 (Qb and Qc), and VAMP2 (R). This is further supported by the crystal structure of an endosomal SNARE complex consisting of STX7 (Qa), Vti1b (Qb), STX8 (Qc), and VAMP8 (R) (Antonin et al., 2002). All STXs are Qa-SNAREs with the exception of STX6, 8, and 10, which are Qc-SNAREs, while all VAMPs are R-SNAREs and the three SNAPs contain Qb and Qc motifs. At this time, the significance of the zero-layer is unclear. It was hypothesized that the zero-layer residues are critical for SNARE complex disassembly by N-ethylmaleimide-sensitive Factor (NSF) (Sutton et al, 1998; Scales et al., 2001), though this was shown not to be the case (Lauer et al., 2006). However, the zero-layer may be important for the formation of a SNARE complex in the correct orientation and establishing the desired four-helix oligomeric state, or it may serve as a check point of sorts, providing an intermediate “stop point” at which SNARE complexes are half-way zippered (Lauer et al., 2006).

1.2.3 – Characterization of the Sec1/Munc18 protein family

Members of the SM protein family were first identified in *Saccharomyces cerevisiae*, when *SEC1* was identified as one of ten genes identified as secretion mutants required for the final
stage of protein secretion, the fusion of secretory vesicle to the cell membrane, and subsequent release of vesicular contents to the cell exterior (Novick et al., 1980). The existence of a distinct family of Sec1-like proteins was realized when it was noted that three functionally-related *S. cerevisiae* proteins, Sec1, Sly1, and Slp1 (later renamed Vps33) shared a low (~23%) but significant sequence identity (Aalto et al., 1992). The Sec1-like proteins were shown to be conserved in metazoans with the identification of Sec1 homologues in *Caenorhabditis elegans* (Unc-18) (Hosono et al., 1992; Gengyo-Ando et al., 1993) and *Drosophila melanogaster* (Rop) (Salzberg et al., 1993). A mammalian homologue to Sec1 was first identified in rat brain homogenate, and was termed Munc18 (later, Munc18-1) (Hata et al., 1993). In all, seven mammalian SM proteins have been identified: Munc18-1, Munc18-2, Munc18-3, SLY1, VPS45, VPS33A, and VPS33B (Toonen and Verhage, 2003). One of these seven proteins is required at each of the numerous vesicular trafficking events that occur within cells, and some have been shown to be required in certain cell types for cell-specific fusion events. For example, Munc18-1 is required for release of synaptic vesicles in both the central and peripheral nervous systems (Verhage et al., 2000), and also plays a role in the release of large dense-core vesicles in adrenal chromaffin cells (Voets et al., 2001), while VPS33B has been shown to be required for α-granule biogenesis in megakaryocytes and is essential for trafficking in multiple cells from various tissues (Gissen et al., 2004; Lo et al., 2005).

SM proteins are hydrophilic, each of approximately 600 amino acids, though their primary amino-acid sequences yield no recognizable domains or motifs which might suggest how SM proteins could promote vesicle trafficking or fusion (Carr and Rizo, 2010). However, structural analysis of several SM proteins has revealed that the overall fold of these proteins is highly conserved both between different family members in the same organism, and between homologues of different organisms. Each SM protein is composed of three domains (domains 1-3) that form an arched, horseshoe-like shape. This results in the presence of a deep cavity on one side of the protein, and in some SM proteins, a large groove on the opposite side, both of which have been implicated in SNARE protein binding (see Section 1.2.4) (Misura et al., 2000; Bracher and Weissenhorn, 2001; Bracher and Wissenhorn, 2002). SM binding of SNAREs via the deep cavity and large groove is shown in Figure 7.
1.2.4 – SM-SNARE interactions

The SM proteins have been implicated in SNARE binding and the formation and assembly of SNARE complexes. However, the exact role of SM proteins in vesicle fusion remains unclear. Different SM proteins may in fact play different roles in this process, as evidenced by the fact that different SM proteins bind to their cognate SNAREs in different ways. The first connection between SM proteins and SNAREs came after the identification of a tight interaction between Munc18-1 and STX1A, both of which are required for neurotransmitter release from synaptic vesicles (Hata et al., 1993). The co-crystal structure of this complex revealed that STX1A is held in its “closed” conformation in the deep cavity of Munc18-1 (Misura et al., 2000). This seemed to indicate an inhibitory role for Munc18-1 in vesicle trafficking, though this was at odds with its essential role in neurotransmitter release (Verhage et al., 2000). Instead, it was suggested that this structure represented an intermediate state where Munc18-1 was instead responsible in assisting the transition of STX1A from the “closed” conformation to an “open” one (Dulubova et al., 1999). To date, this mode of binding has only been identified for STXs involved in exocytosis (Sudhof and Rothman, 2009).

Figure 7. Structure of Munc18-1 (green/blue) bound to STX1A (pink). In the first crystal structure acquired for an SM-STX pair (Misura et al., 2000), Munc18-1 binds to the “closed” conformation of STX1A in its deep cavity. A large groove exists on the opposite side of Munc18-1 which can interact with the N-peptide of STX1A (red). Adapted from Carr and Rizo, 2010.
Some SM proteins contain a large groove, found on the opposite side of the deep cavity, which has been implicated in SNARE-binding. This groove is likely involved in an interaction at the N-terminus of STXs with a conserved N-peptide motif (shown in red in Figure 6) (Munson and Bryant, 2009). The large groove is composed of a pocket of hydrophobic residues, a small acidic groove, and a basic patch (Hu et al., 2007). All SM proteins that contain this groove bind STXs containing the N-peptide motif, while those SM proteins that lack this groove interact with STXs lacking the motif (Hu et al., 2007), though this may not be in a one-to-one manner. For example, yeast Sec1p, which lacks the groove, is known to interact with the syntaxin Sso1p only when Sso1p is part of its SNARE complex (Carr et al., 1999; Togneri et al., 2006). The significance of the N-peptide interaction is unclear; some findings support its functional importance, as disruption of this interaction in different SM-STX pairs can cause disruption of Golgi structure (Yamaguchi et al., 2002) or neurotransmitter release (Khvotchev et al., 2007; Johnson et al., 2009), while other studies report milder or no functional effects upon disruption of this interaction (Peng and Gallwitz, 2004; Carpp et al., 2006; Han et al., 2009). It may be that the SM protein’s interaction with the N-peptide may allow the SM protein to remain bound to the STX once the STX enters its “open” conformation and binds to other SNAREs (Sudhof and Rothman, 2009). The yeast SM proteins Sly1p and Vps45p (and their mammalian homologues) were the first to be shown to bind their cognate STXs in a one-to-one manner via the STX N-terminus (Dulubova et al., 2002; Yamaguchi et al., 2002), and Munc18-1 has also been shown to bind STX1A in this manner (Shen et al., 2007; Dulubova et al., 2007).

SM proteins are also thought to bind to, and assist in the zippering of, trans-SNARE complexes in their deep cavity. Binding to intact SNARE complexes was initially observed for two yeast SM proteins, Sly1p (Sogaard et al., 1994; Peng and Gallwitz, 2004) and Sec1p (Carr et al., 1999), and more recently, this direct interaction with SNARE complexes has been observed for Munc18-1 (Shen et al., 2007; Dulubova et al., 2007), Munc18-3 (Latham et al., 2006) and VPS45 (Carpp et al., 2006; Burkhardt et al., 2008; Furgason et al., 2009).

The interaction of SM proteins with “closed” STXs, STX N-peptides, and SNARE complexes, and specifically the ability of Munc18-1 to participate in each manner of binding, had led to
speculation of a general, three-phase model for SM-SNARE interactions involved in exocytosis fusion events, as shown in Figure 8. First, the SM protein binds its cognate STX in the “closed” conformation and assists in its transition to an “open” conformation, while remaining bound to the STX via the N-peptide motif throughout the transition. After the remaining SNAREs join the STX to form a SNARE complex, the SM protein folds back on the complex, expediting the SNARE motif zippering process in a manner that remains undetermined. As stated above, binding of SM proteins to “closed” STXs has only been identified for STXs involved in exocytosis. SM proteins involved in non-exocytosis fusion events may bind their “open” STXs followed by binding to the trans-SNARE complex, or the SM protein may only be involved in one of these binding motifs.

Figure 8. Three modes of binding to SNAREs by SM proteins. Some SM proteins bind the “closed” conformation of STX (left), with the STX N-peptide bound to a large pocket on the opposite side of the SM protein (inset). SM proteins may transition STXs from a “closed” to “open” conformation, while remaining in contact with the STX via its N-peptide motif (middle). Subsequent SNARE complex formation allows for the SM protein to fold back on the assembling trans-SNARE complex and assist in the zippering required for membrane fusion to occur. Adapted from Sudhof and Rothman, 2009.

However, as stated above, not all SM proteins possess the groove to bind the STX N-peptide, and these SM proteins are thought to bind STXs that lack the N-peptide anyway. Also, some SM proteins do not bind to their cognate STXs in a one-to-one manner. It is this variability in the SM protein family that has made deducing their exact role in vesicle fusion so difficult to determine.

It has also been hypothesized that SM proteins may act as molecular chaperones for their cognate syntaxins, allowing for the proper targeting and expression of the syntaxin, and there is a growing body of evidence that suggests Munc18-1 acts as a chaperone of syntaxin-1. It has been
shown in rat kidney fibroblast cells and other non-neuronal cells that ectopically expressed syntaxin-1 becomes trapped in the Golgi and endoplasmic reticulum, but when co-transfected with Munc18-1, syntaxin-1 correctly localizes to the plasma membrane (Rowe et al., 1999, 2001; Medine et al., 2007; Rickman et al., 2007). Furthermore, in Munc18-1 knockdown PC12 cells, syntaxin-1, but not SNAP-25, is mislocalized, accumulating in the perinuclear region. This mislocalization is rescued upon re-expression of Munc18-1 (Arunachalam et al., 2008). In addition to mislocalization, it has also been shown that expression levels of syntaxin-1 are tremendously decreased in Munc18-1 deficient neurons and neuroendocrine cells, as well as in Munc18-1/-2 double knockdown PC12 cells (Verhage et al., 2000; Voets et al., 2001; Han et al., 2009). Taken together, these studies strongly support the notion that Munc18-1 acts as a molecular chaperone of syntaxin-1, allowing for its proper expression and localization. Whether other SM proteins provide a similar function to their cognate syntaxins remains unanswered.

1.3 – VPS33B

One of the least-characterized mammalian SM proteins is VPS33B. VPS33B is 617 residues in length, has a molecular weight of approximately 71.6 kDa, and, like all SM proteins, is predominantly hydrophilic. Based on sequence alignment with Munc18-1, it contains three putative syntaxin-binding domains, from residues 35-60, 260-275, 310-340 (Gissen et al., 2005). It also contains a stretch of 31 amino acids (residues 450-480) not present in VPS33A, with which VPS33B shares 32% sequence identity (Figure 9).

Figure 9. Schematic representation of VPS33B. Boxes A, B, and C represent the putative syntaxin-binding regions of VPS33B, while the black box represents a stretch of 31 residues present in VPS33B but not in VPS33A.

VPS33B is a homolog of the yeast protein Vacuolar protein-sorting (Vps) 33. Vps33p was initially identified in S. cerevisiae through a genetic screen where mutations in the class C vacuole protein sorting genes resulted in absent vacuoles (Raymond et al., 1992). Vps33p is known to associate with Vps11p, Vps16p, and Vps18p to form the Class C-Vps core complex. This core complex interacts with Vps39p and Vps41p to form the homotypic fusion and protein sorting (HOPS) complex (Sato et al., 2000; Seals et al., 2000; Wurmser et al., 2000; Peterson and
Emr, 2001; Subramanian et al., 2004; Collins et al., 2005). Vps39p is a guanosine exchange factor (GEF) that activates Ypt7p, the yeast Rab 7 homolog (Wurmser et al., 2000), while Vps41p serves as a Ypt7p effector (Brett et al., 2008). A second class C-Vps complex known as class C core vacuole/endosome tethering (CORVET) has also been identified. In this complex, Vps39p and Vps41p are, in effect, replaced by Vps3p and Vps8p. The exact roles of Vps3p and Vps8p are unclear, but it is known that CORVET interacts with Vps21p, the yeast Rab5 homolog (Peplowska et al., 2007). HOPS is present at vacuoles and is required for vacuolar trafficking, CORVET is present at endosomes and is required for endosomal trafficking. By exchanging the accessory proteins (Vps39p and 41p for Vps3p and 8p), these complexes can interconvert to provide organelle specificity during trafficking (Peterson and Emr, 2001; Subramanian et al., 2004; Peplowska et al., 2007), as shown in Figure 10.

![Figure 10](image_url)

**Figure 10.** Class C-Vps complexes formed in yeast. (A) The class C-Vps core complex is joined by either Vps39 and Vps41 (HOPS) or Vps3 and Vps8 (CORVET). Interconversion between these two complexes is thought to provide organelle specificity during trafficking. (B) Trafficking events mediated by HOPS are shown in red arrows, while those mediated by CORVET are shown in purple arrows. Adapted from Nickerson et al., 2009.

In metazoans, there exist two homologues to Vps33p, VPS33A and VPS33B. It is thought that, like Vps33p, each of these proteins exists in a high-molecular weight, multi-subunit complex. In support of this, it has been shown in our lab, through non-denaturing Blue Native gel electrophoresis, that VPS33B exists in complexes of approximately 480 and 720 kDa (unpublished data). Other studies suggest that mVPS11, mVPS16, mVPS18, mVPS39, and mVPS41 participate in endosomal fusion (Richardson et al., 2004), and that human VPS11,
VPS16A, VPS18 and VPS33A form a hetero-oligomeric complex that is predominantly associated with late endosome and lysosomes (Kim et al., 2001). To date, VPS33B in metazoans has only been shown to interact with one protein, VPS16B, a homologue of yeast Vps16. The interaction of VPS33B with this protein has been identified and characterized in *D. melanogaster* (dVPS16B) (Pulipparacharuvil et al., 2005), *C. elegans* (Spe-39) (Zhu et al., 2009), and humans (VIPAR) (Cullinane et al., 2010). Studies in our lab also identified a protein that interacts with VPS33B, which, based on sequence analysis, was shown to be hVPS16B (same protein as VIPAR) (unpublished data).

To this point, no interactions of VPS33B with SNARE proteins have been identified. In mammals, the complex formed by VPS11, VPS16A, VPS18 and VPS33A has been shown to interact with STX7, although a direct interaction with STX7 was only shown for VPS11 and VPS18, but not VPS33A (Kim et al., 2001). In *Drosophila*, a direct interaction has been observed between dVPS33A and dSTX16 (Akbar et al., 2009). In yeast, the Class C-Vps core complex is known to interact with the syntaxin Vam3p (Sato et al., 2000; Seals et al., 2000) presumably through Vps33p, although to this point, no direct Vps33p/Vam3p interaction has been identified.

It is thought that the complexes in which VPS33A and VPS33B exist are distinct and non-redundant. In support of this theory, studies in *Drosophila* show that dVPS33A interacts specifically with dVPS16A, while dVPS33B interacts specifically with dVPS16B. Moreover, loss of dVPS33A function is not restored by expression of dVPS33B (Pulipparacharuvil et al., 2005). In mammals, it has been shown that in mice a homozygous Asp251Glu missense mutation in VPS33A results in the *buff* mouse phenotype, characterized by platelet dense granule deficiency (Suzuki et al., 2003). In contrast, mutations in VPS33B cause a deficiency in α-granules (Lo et al., 2005), while dense granule biogenesis remains unaffected.

**1.4 – ARC syndrome and other mutant VPS33B phenotypes**

In humans, mutations in VPS33B have been shown to cause arthrogryposis, cholestasis, and renal dysfunction (ARC) syndrome (Gissen et al., 2004). ARC syndrome is an autosomal
recessive condition associated with failure to thrive and death within the first year of life. It is a multi-system disorder with many clinical manifestations, including cerebral malformations, sensorineural deafness, congenital heart disease, nephrogenic diabetes insipidus, ichthyosis, dysmorphic features, recurrent infections, diarrhea, and a bleeding diathesis. ARC patients display hepatocyte accumulation of lipofuscin granules and abnormal localization of the membrane proteins in polarized epithelial cells of the liver and kidneys (Gissen et al., 2004). In keeping with the bleeding problems observed, ARC patients have unusually large platelets, characterized by a complete lack of α-granules, as observed through thin section electron microscopy, as seen in Figure 11, thus confirming the requirement for VPS33B in megakaryocyte and platelet α-granule biogenesis (Lo et al., 2005). More recently, mutations in VIPAR/VPS16B have also been identified in ARC patients (Cullinane et al., 2010).

Both VPS33B and VPS16B have recently been implicated in phagosomal maturation (Akbar et al., 2011). In a process similar to receptor-mediated endocytosis, cells defend against microbial invaders through phagocytosis. Microbes are engulfed into early phagosomes by actin-driven extensions of the plasma membrane and quickly fuse with endosomes to induce phagosomal maturation (Flannagan et al., 2009). Continued maturation depends on the fusion of phagosomes with early and late endosomal compartments, and finally with lysosomes, facilitating the formation of phagolysosomes, and allowing for the degradation of the phagolysosomal cargo (Kinchen and Ravichandran, 2008). It has been demonstrated that after infecting host cells, *Mycoplasma tuberculosis* secretes the tyrosine phosphatase PtpA, and VPS33B is a substrate of PtpA. PtpA secretion combined with active-phosphorylated VPS33B
inhibits phagosome-lysosome fusion (Bach et al., 2008). In *Drosophila*, it has recently been shown that dVPS16B is required for the formation of phagolysosomes through the fusion of phagosomes and lysosomes. Hemocytes of flies null for dVPS16B are able to engulf bacteria but fail to digest them, as seen in **Figure 12**. This phenotype is also observed in wildtype hemocytes treated with dVPS33B siRNA (Akbar et al., 2011).

**Figure 12.** dVPS16B and dVPS33B are required for phagosomal maturation. (A) Wildtype *Drosophila* hemocytes treated with labelled *E.coli* BioParticles are able to engulf and degrade the bacteria, while hemocytes lacking dVPS16B (B) cannot degrade the bacteria, due to a failure in the fusion of phagosomes with lysosomes. A similar phenotype was observed in wildtype hemocytes treated with siRNA against dVPS33B. Adapted from Akbar et al., 2011.

1.5 – Syntaxin 6

Most members of the syntaxin subfamily are classified as Qa-SNAREs. However, STX6 is an atypical syntaxin in that it is classified as a Qc-SNARE. The only other syntaxins that are Qc-SNAREs are STX8 and STX10, and indeed, there are some structural similarities shared between STX6, 8, and 10 which differ slightly from the Qa-SNARE syntaxins, based on the crystal structure of the STX6 N-terminal domain (Misura et al., 2002). Like the Qa-SNARE syntaxins, the Qc-SNARE syntaxins contain an N-terminal, three-helix H<sub>abc</sub>-domain. In the Qa-SNARE syntaxins, the first helix of the H<sub>abc</sub>-domain begins at approximately residue 30 of the protein. The first 30 residues are made up of the N-peptide motif implicated in SM protein-binding and a linker region connecting this motif to the H<sub>abc</sub>-domain. However, in the Qc-SNARE syntaxins the first helix of the H<sub>abc</sub> domain begins at the extreme N-terminus of the protein, and thus the Qc-SNARE syntaxins lack any N-peptide motif (Misura et al., 2002; Hong, 2005). In addition, it is predicted that the H<sub>abc</sub>-domain of STX6 is unable to fold back on its SNARE motif to adopt a “closed” conformation similar to that adopted by STX1A, based on the fact that the H<sub>abc</sub>-domain of STX6 lacks a hydrophobic, SNARE motif-binding groove that is present in the H<sub>abc</sub>-domain
Thus, since the Qc-SNARE syntaxins lack the N-peptide motif and are likely unable to adopt a “closed” conformation, any interaction of these syntaxins with SM proteins must occur in some manner that is distinct from the “normal” mode of syntaxin-binding by SM proteins described in Section 1.2.4. Alternatively, since each SNARE complex is thought to be comprised of a Qa-, Qb-, Qc-, and R-SNARE, each of the Qc-SNARE syntaxins should form a SNARE complex that also includes one Qa-SNARE syntaxin. Thus, it is possible that only the Qa-SNARE syntaxins interact in a one-to-one manner with SM proteins, and Qc-SNARE syntaxins, despite their nomenclature, act more like the non-syntaxin Qc-SNAREs Bet1, GS15, and Slt1, and do not interact with SM proteins at all. To this point, all STX-SM interactions identified to date involve Qa-SNARE syntaxins. However, STX6 has been shown to take part in exocytosis in neutrophils, and here STX6, together with SNAP-23, was shown to play a role in granule fusion with the plasma membrane. Since SNAP-23 contains both a Qb- and Qc-SNARE motif, this has led to speculation that in some instances STX6 can act as a “true” (ie. Qa-SNARE) syntaxin (Martin-Martin et al., 2000). Thus, it follows that STX6, as well as the other Qc-SNARE syntaxins, are also able to bind SM proteins, just as their “true” syntaxin counterparts do.

STX6 was originally identified through its homology to the yeast syntaxin Pep12p (Bock et al., 1996). However, Pep12p is a Qa-SNARE, and thus it seems the closest orthologue in the S. cerevisiae genome appears to be Tlg1p (Bock et al., 2001). Both Pep12p and Tlg1p have been implicated in post-Golgi trafficking events (Coe et al., 1999; Gerrard et al., 2000). Similarly, STX6 seems to localize to trans-Golgi and endosomal compartments. Studies in fibroblast cell lines reveal that STX6 localizes to trans-Golgi and endosomal structures (Bock et al, 1996; Bock et al., 1997; Klumperman et al., 1998). In addition, a tyrosine-based sorting motif in STX6 (140YGRL143) has been identified as a trans-Golgi retention signal and has been shown to be required for recycling of STX6 from the plasma membrane back to the trans-Golgi (Watson and Pessin, 2000). STX6 has been shown to form a SNARE complex with STX7, Vti1b, and one of VAMP7 or VAMP8, and these two complexes likely play a role in late endosomal regulation (Wade et al., 2001).
STX6 has also been shown to be required for homotypic fusion of immature secretory granules to form mature secretory granules in PC12 cells, a rat neuronal cell line (Wendler et al., 2001). STX6 is present on immature but not mature secretory granules, and thus not required for the final secretion event. It has been demonstrated that after homotypic fusion of immature secretory granule, STX6 localizes to clathrin-coated pits on the granules and is likely removed via clathrin-coated vesicles (Klumperman et al., 1998). As described above, STX6 is also involved in granule fusion at the plasma membrane of neutrophils (Martin-Martin et al., 2000). Other examples of known STX6-positive SNARE complexes include that of STX16, Vti1b, STX6 and VAMP4, which has been shown to be responsible for retrograde trafficking from early/recycling endosomes to the trans-Golgi (Mallard et al., 2002). STX6 has also been shown to be involved in a membrane-trafficking step that sequesters the glucose transporter GLUT4 away from the plasma membrane in adipocytes (Perera et al., 2003), and has been shown to be required for sorting of proteins from endosomes toward either the trans-Golgi or lysosomes in pancreatic beta-cells (Kuliawat et al., 2004). More recently, STX6 has been implicated in caveolar endocytosis (Choudhury et al., 2006), as well as the recycling of insulin-responsive amino-peptidase (IRAP) from the plasma membrane back to insulin-responsive compartments of muscle cells and adipocytes (Watson et al., 2008). STX6 also acts as an effector and regulator of the p53 family of transcription factors in the regulation of cell adhesion and survival. STX6 is required for the proper targeting of focal adhesion kinase (FAK) and integrin α5 to maintain cell adhesion, and FAK has been shown to interact with p53 and inhibit p53-dependent apoptosis (Zhang et al., 2008). Finally, STX6 has also been shown to play a role in mediating the degradation of the cystic fibrosis transmembrane conductance regulator (CFTR) that regulates Cl⁻, Na⁺, HCO⁻, and H₂O transport across the apical membrane of epithelial cells (Cheng et al., 2010).

1.6 – Rationale and Hypothesis

VPS33B remains one of the least characterized of the seven mammalian SM proteins. Only one binding partner, VPS16B, has been established. Despite the fact that SM proteins play an essential role in vesicle trafficking through their interactions with the SNARE proteins, no such VPS33B-SNARE interaction had been identified when I commenced this project. With the
exception of VPS33A and VPS33B, all mammalian SM proteins have been shown to interact with at least one syntaxin.

As such, the focus of my project centred around identifying and characterizing VPS33B-SNARE interactions, specifically focussing on the syntaxin sub-family of SNAREs, since other SM proteins are known to bind STXs in a one-to-one manner. Given VPS33B’s role in α-granule biogenesis in megakaryocytes, I hypothesize that any SNAREs identified to interact with VPS33B could also be specifically required for this process. In addition, I hypothesize that mutations in VPS33B that are known to cause ARC syndrome may abrogate binding to any SNARE proteins with which wildtype VPS33B normally interacts.
Chapter 2

Materials and Methods

2.1 – Antibodies and plasmids

Antibodies used for immunoprecipitation, immunoblotting and immunofluorescence experiments include: α-STX6 (rabbit polyclonal, Synaptic Systems; rabbit monoclonal C34B2, Cell Signalling Technology; and mouse monoclonal 30, BD Biosciences); α-VPS33B (rabbit polyclonal, produced in-house); α-VPS16B (mouse polyclonal, produced in-house); α-GFP (rabbit polyclonal, Invitrogen); α-HA (mouse monoclonal HA.11, Covance); α-myc (mouse monoclonal 9E10, Covance); α-SBP (mouse monoclonal sc-101595, Santa Cruz Biotechnology); α-LexA (rabbit polyclonal, Invitrogen); α-AP-1 (mouse monoclonal 100/3, Sigma-Aldrich); α-TGN46 (sheep monoclonal AHP500, MorphoSys); α-Lamp-1 (mouse monoclonal H4A3, University of Iowa); α-vWF (rabbit polyclonal, DakoCytomation; α-EEA1 (rabbit monoclonal C45B10, Cell Signalling Technology; and α-CD63 (mouse monoclonal H5C6, BD Biosciences).

Plasmids used for yeast two-hybrid experiments were pEG202 (Genebank Acc. #89960) and pJG4-5 (Genebank Acc. #U89961) (both acquired from Origene). Plasmids used for co-immunoprecipitation experiments were pCMV-myc (Clontech Laboratories), pEGFP-N1 (Clontech Laboratories), and pcDNA3-HA (acquired from Addgene plasmid repository). SBP-VPS33B and VPS33B-SBP inserts were cloned into pIRES2-DsRed-Express (Clontech).

2.2 – Cell culture and transfections

Both HeLa and HEK293 cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) (Wisent Bioproducts) supplemented with 10% v/v fetal bovine serum (HyClone). Dami cells were cultured in Iscove’s modified DMEM (Wisent Bioproducts) supplemented with 10% v/v horse serum (HyClone). For the co-localization experiments described in Section 3.7, Dami cells were also treated with 1μM phorbol-12-myristate-13-acetate (PMA) and 8.3 nM thrombopoietin (TPO). Control fibroblasts were maintained in Minimum Essential Medium Alpha (AMEM) (Wisent Bioproducts) supplemented with 10% v/v fetal bovine serum, and ARC fibroblasts were maintained in Eagle’s Minimum Essential Medium (EMEM) (Wisent Bioproducts).
supplemented with 10% v/v fetal bovine serum. RAW cells were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium (Wisent Bioproducts) supplemented with 10% v/v fetal bovine serum. All cells were maintained at 37°C in 5% CO₂.

All transient transfections were performed using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer’s instructions. For the immunoprecipitation experiments, HEK293 cells were grown to 90% confluency on a 10cm-dish, and transfected using 6 μg total DNA in the presence of 30 μL Lipofectamine 2000 in 3 mL serum-free media added to the cells growing in 15 mL DMEM. Cells were harvested and lysed 24h after transfection. For transfection of HeLa cells with siRNA against VPS33B, cells were plated at approximately 30% confluency in 6-well plates and each well was transfected using 100 pmol siRNA in the presence of 5 μL Lipofectamine 2000 in 500 μL serum-free media added to the cells growing in 2 mL DMEM. Media was replaced 4-6h after transfection. Cells were harvested and lysed 48h after transfection.

2.3 – Cloning

All cloning was performed using the same protocol, with different restriction enzymes used depending on the reaction. Briefly, all required inserts were acquired via polymerase chain reaction (PCR). The required primers were designed and ordered from IDT Integrated DNA Technologies and the amplification reaction was performed using Pfx DNA polymerase (Invitrogen). PCR products were purified using a PCR purification kit (BioBasic Inc.) according to the manufacturer’s instructions. PCR products and vectors were digested with the required restriction enzymes (New England BioLabs Inc.); calf intestinal phosphatase (CIP) (New England BioLabs Inc.) was included in all vector digestions. Digests were purified using a PCR purification kit (BioBasic Inc.), and vector-insert ligation was performed using T4 DNA Ligase (New England BioLabs Inc.). Ligation products were transformed into DH5α E.coli made competent by treatment with rubidium chloride and plated on Lysogeny Broth (LB) plates containing the required antibiotic for selection. Colonies were picked and grown in liquid LB containing antibiotics overnight, and DNA was extracted using a QIAprep Spin Miniprep Kit (Qiagen) according to the manufacturer’s instructions.
2.4 – Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting

All SDS-PAGE and Western blotting was performed using the same protocol, varying the antibodies used depending on the experiment. Briefly, samples were treated with SDS-loading buffer (20% v/v glycerol, 1% v/v β-mercaptoethanol, 2% w/v SDS, 65 mM Tris-HCl, pH 6.8, 0.001% Bromophenol Blue), boiled 5 min, and loaded onto acrylamide gels (4% stacking layer, 10% separating layer), and run at 200V for 45 mins, and subsequently transferred to nitrocellulose membranes at 100V for 2h or 30V overnight. Membranes were blocked in Tris-buffered saline-Tween (TBS-T) (20 mM Tris-HCl, pH 7.6, 137 mM NaCl, 0.1% v/v Tween 20) containing 5% skim milk powder for 1h, then incubated with primary antibody in blocking solution for 1h. This was followed by washing 3x for 10 min in TBS-T, incubation with horse radish peroxidise (HRP)-conjugated secondary antibody in blocking solution for 1h, and final washing 3x for 10 mins in TBS-T. Membranes were treated with enhanced chemiluminescence (ECL) reagent for 1 min and exposed to film.

2.5 – Yeast two-hybrid screen of syntaxins against SM proteins

cDNA for all syntaxins, as well as Munc18-1, VPS33A, and VPS33B were ordered from the Signalling Identification Network (SIDNET) at the Hospital for Sick Children. The “bait” cDNAs, Munc18-1, VPS33A, and VPS33B, were cloned into the vector pEG202, encoding for the LexA DNA-binding domain fused to the N-terminus of the bait protein, and containing the yeast selectable marker HIS3. The “prey” syntaxin cDNAs were cloned into the vector pJG4-5, encoding for a nuclear localization signal, B42 acid blob activation domain, and HA-tag fused to the N-terminus of the prey protein, and containing the yeast selectable marker TRP1, as well as the GAL1 promoter for galactose-inducible expression of the fusion protein. All syntaxins were cloned such that they were lacking the transmembrane domain. Subsequent screens were performed with truncations and point mutants of VPS33B. For the truncation mutants, primers were designed to PCR amplify DNA encoding for the N-terminal truncation of VPS33B, residues 70-617, as well the C-terminal truncations, residues 1-290, 1-350, and 1-438, 1-502, and 1-532. The L30P, S243F, G249V, and D252E point mutations were introduced using the
Stratagene Quik-Change Mutagenesis Kit (see Section 2.8). The E385G point mutant was generated serendipitously through an error made by the DNA polymerase enzyme during PCR amplification of VPS33B cDNA.

The fusion constructs were transformed into yeast following the protocol described in the DupLex-A™ Yeast Two-Hybrid System (Origene). The “bait” constructs were transformed, together with the vector pSH18-34 (containing the lacZ reporter gene and the yeast selectable marker URA3), into the yeast strain EGY48. The “prey” constructs were transformed into the yeast strain RFY206. Initially, the yeast strains were grown from glycerol stock onto plates containing the rich media YPD (20 g/L peptone, 10 g/L yeast extract, 20 g/L glucose, 0.1 g/L NaOH, 20 g/L agar in distilled water). Single colonies were picked and grown in 5 mL YPD overnight at 30°C. The next morning, the overnight culture was used to inoculate 300 mL YPD such that the OD600 = 0.1. The culture was shaken vigorously at 30°C until OD600 = 0.5-0.7. The yeast cells were harvested by centrifugation at 1500x g for 5 minutes and the supernatant was removed. The cells were resuspended in 30mL distilled water, centrifuged as previously, and the supernatant was again removed. The cells were then resuspended in 1.5 mL 1x TE/LiOAc buffer (0.01 M Tris, pH 7.5, 0.001 M EDTA, 0.1 M lithium acetate, in distilled water) and 50 μL aliquots were used for each transformation. Salmon sperm DNA was denatured by boiling for 5 minutes, and 10 μg was added, along with 1 μg of the “bait” or “prey” DNA, to the cells (in the case of the “bait” transformations, 1 μg of reporter plasmid pSH18-34 was also added). 300 μL of 1x TE/LiOAc/PEG buffer (0.01 M Tris, pH 7.5, 0.001 M EDTA, 0.1 M lithium acetate, in 50% PEG-3350) was added to each reaction and mixed by inversion. Cells were incubated at 30°C for 30 minutes. Next, 40 μL DMSO was added to each reaction, and the cells were heat shocked at 42°C for 20 minutes. 150 μL was removed from this mixture and plated onto YNB(glucose) selective growth media (6.7 g/L yeast nitrogen base with ammonium sulphate, w/o amino acids, 0.6 g/L –his –ura –leu –trp dropout mix, 20 g/L glucose, 20 g/L agar in distilled water). For EGY48 yeast transformed with the “bait” construct (containing selectable marker HIS3) and the reporter plasmid pSH18-34 (containing selectable marker URA3), cells were plated on “YNB –his –ura” media by adding back 0.04 mg/mL tryptophan and 0.06 mg/mL leucine to the YNB selective growth media. For RFY206 yeast transformed with the “prey”
construct (containing selectable marker TRP1), cells were plated on “YNB –trp” media by adding back 0.06 mg/mL leucine, 0.02 mg/mL uracil, and 0.02 mg/mL histidine to the YNB selective growth media. All plates were placed at 30ºC for 2-3 days. Single colonies were picked from each plate, resuspended in 200 μL distilled water, re-plated and placed at 30ºC until a lawn of yeast had grown.

Mating was then performed on YPD plates by transferring each “bait” or “prey” strand, using sterilized wooden tongue depressors, onto the plate in a grid pattern such that mating only occurs at the intersection of these strands. After allowing 24h for mating, the yeast were transferred using velvet cloth to “YNB –his –ura –trp” plates (0.06 mg/mL leucine added back to YNB selective media) and grown for a further 24h. All surviving cells, which contain the “bait” construct, “prey” construct, and reporter plasmid, were then transferred to “YNB –his –ura –trp” plates containing YNB (galactose) selective media with X-gal (6.7 g yeast nitrogen base with ammonium sulphate, w/o amino acids, 0.6 g –his –ura –leu –trp dropout mix, 20 g galactose, 10 g raffinose, and 20 g agar in 900 mL distilled water; 7 g sodium phosphate (dibasic) and 3 g sodium phosphate (monobasic) in 100 mL distilled water; 0.8 mL of 100 mg/mL X-gal in N,N-dimethyl formamide). Cells were grown for a further 24-48h and were screened for successful “bait”/“prey” interactions.

2.6 – Immunofluorescence microscopy

Cells grown on coverslips were fixed in 4% (v/v) paraformaldehyde in phosphate-buffered saline (PBS) (Wisent Bioproducts) for 20-30 minutes. Coverslips were washed with PBS three times for 10 minutes; cells were permeabilized with 0.5% (v/v) Triton X-100 in PBS for 5 minutes, and washed with PBS three times for 10 minutes. Cells were then incubated with blocking solution (3% (w/v) bovine serum albumin in PBS) for one hour, followed by incubation with primary antibody in blocking solution for one hour. The coverslips were then washed three times with PBS for 15 minutes, and incubated with Alexa fluor®-conjugated secondary antibody (Invitrogen) at a 1:1000 dilution in blocking solution for one hour. Coverslips were washed three times with PBS for 15 minutes, followed by incubation with Hoechst stain at 1:5000 dilution in PBS for 5 minutes. The coverslips were further washed three times in PBS for 5
minutes, and mounted onto microscope slides using fluorescent mounting medium (Dako). Cells were visualized on a spinning-disc confocal microscope and images were acquired and analysed using Volocity software (PerkinElmer).

2.7 – Co-immunoprecipitation

HEK293 cells were grown in 10 cm dishes to 90% confluency. If transient transfection was required, this was performed 24 hours prior to the co-immunoprecipitation protocol. Cells were washed twice in cold PBS, then scraped into 1 mL cold lysis buffer (50 mM Tris, pH 8.0, 50 mM KCl, 5 mM EDTA, 1% Triton X-100, 1x protease inhibitor cocktail (Roche)), and centrifuged at 16, 100 g for 10 minutes at 4°C. The lysate was then split into two equal aliquots, and primary antibody was added to one sample while control antibody was added to the other. Samples were incubated at 4°C while rotating for one hour. During the incubation, 50 µL of Protein A-sepharose bead slurry (Sigma) was washed three times in lysis buffer containing no detergent. The lysate samples were then added onto the beads and incubated at 4°C while rotating for one hour. The beads were then centrifuged at 13000x g for 2 minutes and washed three times in lysis buffer containing 0.5% Triton X-100. After the final wash, the supernatant was removed and the beads were eluted by adding 40 µL SDS-loading buffer and boiling for 5 minutes. The samples were then subjected to SDS-PAGE and Western blot analysis.

For the co-immunoprecipitation experiments performed in the presence of the chemical cross-linker Dithiobis[succinimidyl propionate] (DSP) (Thermo Scientific), intact cells were first scraped into cross-linking buffer (PBS, pH 8.0, 1 mM DSP, 10 mM iodoacetamide, 0.2 mM phenylmethylsulfonyl fluoride) and incubated on ice for 2 hours. Cross-linking was terminated by adding 10 mM glycine in PBS and incubating on ice for one hour before proceeding with the co-immunoprecipitation protocol. At the conclusion of the protocol, prior to loading the samples onto the acrylamide gel, the cross-link was cleaved by adding 80 mM dithiothreitol (DTT), boiling for 5 minutes, incubating at 45°C for 2 hours, boiling again for 5 minutes, before subjecting the samples to SDS-PAGE and Western blot analysis.
For the co-immunoprecipitation experiments performed in the presence of the NSF inhibitor N-ethylmaleimide (NEM), cells were first incubated in lysis buffer containing 1 mM NEM on ice for 15 minutes. The reaction was stopped by adding 2.5 mM DTT and incubating on ice for 15 minutes before proceeding with the co-immunoprecipitation protocol.

2.8 – Sucrose gradient centrifugation

Sucrose gradients were prepared using a two-chamber gradient pouring apparatus. The inner chamber was filled with 40 mL of 5% (w/v) sucrose in gradient buffer (10 mM HEPES, pH 7.4, 143 mM KCl, 5 mM MgCl₂, 1 mM DTT) containing 0.5% (v/v) Triton X-100 and 0.1% (v/v) protease inhibitor (Sigma). The outer chamber was filled with 40 mL of 20% (w/v) sucrose in gradient buffer containing 0.5% (v/v) Triton X-100 and 0.1% (v/v) protease inhibitor. The two chambers were allowed to combine while being pumped via a motor into 14 mL ultracentrifuge tubes (Beckman), creating a 5-20% sucrose gradient. 40 mL of Dami cells were centrifuged at 1500x g for 5 minutes, the supernatant was removed and cells were lysed in 500 μL gradient buffer containing 0.5% Triton X-100 and 1x protease inhibitor (Roche). Molecular weight standards (4 mg bovine serum albumin, 2 mg catalase, 2 mg apoferritin) were dissolved in 500 μL gradient buffer. The cell lysate and molecular weight standards were each layered on top of one 5-20% sucrose gradient, which were loaded onto an SW-41 swinging bucket ultracentrifuge rotor and centrifuged at 39000 rpm for 16 hours at 4°C (no brake on deceleration). The gradients were then eluted from the top and collected in 600 μL aliquots. These fractions were concentrated down to 300 μL volumes by spinning in a DNA110 DNA Speed Vac® (Savant) for 3 hours, and dialysed (3000 MWCO) overnight against distilled water to remove sucrose from the samples. The samples were then concentrated down in the DNA110 DNA Speed Vac® to 100 μL volumes and 20 μL of each fraction was loaded onto a 10% agarose gel. The presence of the molecular weight standards in each fraction were visualized via Coomassie staining, while the cell lysate fractions were subjected to SDS-PAGE and Western blotting, probing for the presence of STX6, VPS33B, and VPS16B.
2.9 – Mutagenesis

All mutagenesis experiments were performed according to the protocol outlined in the Stratagene QuikChange® Site-Directed Mutagenesis Kit. All primers for engineering point mutations were designed using the mutagenesis primer design tool on the Stratagene website. Primers were designed for identical regions on both the sense and anti-sense DNA strands, and all point mutations listed in this section refer to the DNA or amino acid sequence of VPS33B. For the L30P mutation, a DNA mutation of T89C (codon change from CTG to CCG) was engineered. For the S243F mutation, a DNA mutation of C728T (codon change from TCC to TTC) was engineered. For the G249V mutation, a DNA mutation of G746T (codon change from GGC to GTC) was engineered. For the D252E mutation, a DNA mutation of T756G (codon change from GAT to GAG) was engineered.

PCR reactions were set up using the mutagenic primers and the non-strand displacing PfuTurbo® DNA polymerase (Agilent). pEG202-VPS33B (wildtype) was used as the template DNA for all reactions. At the conclusion of the PCR reaction, samples were digested with the restriction enzyme DpnI (10 units), which recognizes methylated DNA and cuts at the sequence GA(methyl)^TC, allowing for digestion of the methylated, wild-type template DNA and leaving only the non-methylated, mutated DNA. This DNA containing the desired mutation was then transformed into DH5α competent cells and plated. Colonies were picked and the DNA was extracted via a QIAprep Spin Miniprep kit (Qiagen). All mutations were confirmed by sequence analysis.

2.10 – Sequence of mutagenic primers and VPS33B siRNA

The sequence of the primers used to generate point mutations in VPS33B are given below. All nucleotide and amino acid numbering listed refers to the DNA or amino acid sequence of VPS33B. Non-complementary nucleotides included to generate the required point mutations are shown in RED. Though only the sense strand primer is listed for each reaction, complementary primers for the anti-sense strand were included in each case.
L30P: AGCTCATCTATCTGC\textsuperscript{CGGAGCAGCTTCCTGG}\textsuperscript{104}
S243F: CTTTGACAGCACCTTTGCTTCCAAGTGGTTATGAGG\textsuperscript{745}
G249V: CCAAGTGGTTATGAGGTCTAGTAGATGACACCT\textsuperscript{763}
D252E: TGAGGCCTAGTAGAGGACACCTTCCGCA\textsuperscript{T771}

The sequence of siRNA targeted against VPS33B is given below. Nucleotide numbering refers to the DNA sequence of VPS33B.
VPS33B siRNA: CCAAGCAGGATTTCCAGGAGCTAAT\textsuperscript{1091}

2.11 – Phagocytosis/phagosomal maturation assay in RAW cells

Raw cells were grown on coverslips in each well of a 12-well plate. Fluorescein-labelled \textit{E.coli} BioParticles\textsuperscript{®} (Invitrogen) were reconstituted in PBS, according to the manufacturer’s instructions. The BioParticles were sonicated 3x for 20 seconds to remove clumps, and 1 μL of the slurry was added to each well. After a 10 min incubation at 37°C, the medium was removed and replaced with fresh medium. Samples were chased for 15, 30, 45, 60, and 120 min and subsequently fixed, stained, and examined by spinning-disc confocal microscopy.
Chapter 3

Results

3.1 – Yeast two-hybrid screen of VPS33B against all syntaxins

As a member of the SM protein family, it stands to reason that VPS33B interacts with members of the SNARE protein family. Further, any SNAREs identified to interact with VPS33B may also play an important role in α-granule biogenesis. To date, the identity of these SNARE proteins has not been elucidated. However, since other SM proteins have been shown to interact in a one-to-one manner with members of the syntaxin sub-family of SNARE proteins, I decided to focus my attention on the syntaxin sub-family when proceeding with attempts at identifying any VPS33B-SNARE interactions. In order to do this, I decided to perform a yeast two-hybrid screen of VPS33B against all known syntaxins. Munc18-1 was included in the screen as a positive control, as it is known to interact with syntaxins 1, 2, and 3.

3.1.1 – Yeast two-hybrid cloning and syntaxin truncations

The SM proteins, Munc18-1 and VPS33B, were cloned into the vector pEG202, encoding for the DNA-binding domain of LexA. These LexA-SM protein fusions were considered the “bait” proteins for the screen, and these constructs were subsequently transformed into the yeast strain EGY48. For the syntaxins, cDNA encoding for the full-length proteins was first engineered through PCR to create C-terminal truncation mutants such that each syntaxin lacks its transmembrane domain. The computer program TMpred (http://www.ch.embnet.org/software/TMPRED_form.html) was used to predict transmembrane regions in order to determine at which residue each syntaxin should be truncated. The C-terminal truncation for each syntaxin is listed in Table 2. Note that STX11 lacks a transmembrane domain, and thus the full-length protein was cloned. These PCR products were then cloned into the vector pJG4-5, encoding for the acid blob activation domain of B42, also containing an HA epitope tag. These B42-HA-syntaxinΔTM fusion proteins were considered the “prey” proteins for the screen, and these constructs were subsequently transformed into the yeast strain RFY206.
### Table 2. Residue number for the C-terminal truncation of each syntaxin to eliminate the transmembrane domain.

<table>
<thead>
<tr>
<th>Syntaxin</th>
<th>Number of amino-acid residues in full-length protein</th>
<th>Number of amino-acid residues in truncated protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>STX1A</td>
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<td>266</td>
</tr>
<tr>
<td>STX1B</td>
<td>288</td>
<td>265</td>
</tr>
<tr>
<td>STX2</td>
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<td>264</td>
</tr>
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</tr>
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</table>

#### 3.1.2 – Yeast two-hybrid results

Each “bait” yeast strain was mated with each “prey” strain in the presence of galactose and X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside). The expression of the “bait” and “prey” are galactose-inducible, and successful interaction leads to the expression of β-galactosidase, which metabolizes X-gal to produce a blue colouration. The yeast two-hybrid screen is shown in **Figure 13**. Note that due to difficulties in cloning, syntaxins 2, 5, and 13 were not included in the final screen.
Figure 13. Yeast-two hybrid screen of VPS33B against syntaxins. VPS33B displays a robust interaction with STX6 and STX11, and a slight interaction with STX7 and STX10. Included as a control, Munc18-1 interacts with STX1A, STX1B, and STX3, as expected. Note that STX2, STX5, and STX13 were omitted from the screen.

It can be seen from Figure 13 that, as expected, Munc18-1 interacts with STX1A, 1B, and 3, while a robust interaction can be seen between VPS33B and STX6, as well as slight interactions with STX7 and 10. Interestingly, both Munc18-1 and VPS33B display a robust interaction with STX11. Since STX6 displayed a robust and specific interaction with VPS33B, I decided to try to further characterize this interaction, and assess any role it may play in α-granule biogenesis. However, I repeated the yeast two-hybrid screen to include LexA-VPS33A as one of the “bait”
proteins. As mentioned in Section 1.3, VPS33A and VPS33B function in separate and distinct pathways in megakaryocyte granule biogenesis. To be sure that any syntaxins identified to interact with VPS33B may participate in α-granule biogenesis, but do not participate in dense granule biogenesis, it was important to confirm that any syntaxins shown to interact with VPS33B do not also interact with VPS33A. The yeast two-hybrid screen including LexA-VPS33A is shown in Figure 14.

**Figure 14.** Yeast two-hybrid screen repeated to include VPS33A. While some interaction can be seen between STX6 and VPS33A, the interaction is much more robust with VPS33B.

VPS33A may interact with STX1A, 6, 11 and 16. However, the interaction between STX6 and VPS33B is much more robust than that with VPS33A. It should also be noted that in
Drosophila, dVPS33A has been shown to interact with dSTX16 (Akbar et al., 2009). Interestingly, STX11 is shown once again to interact with all three SM proteins, suggesting it may be a universal interactor of SM proteins, or these may all be false-positive interactions.

Based on the evidence provided by these yeast two-hybrid screens, I decided that STX6, displaying a robust and specific affinity for VPS33B, would be the best candidate to pursue in my attempts to identify SNARE proteins important for megakaryocyte α-granule biogenesis, and I proceeded with attempts to further characterize this interaction.

3.2 – VPS33B and STX6 co-immunoprecipitate in a mammalian cell system

In order to further characterize the interaction between VPS33B and STX6, I sought to confirm the yeast two-hybrid results by proving the interaction of these two proteins in a mammalian cell system. To do this, I tested whether overexpressed myc-VPS33B can immunoprecipitate overexpressed STX6 in HEK293 cells. The plan was that once a successful and consistent interaction was proven, I could begin making mutations in VPS33B and then determine, via these co-immunoprecipitation experiments, any important syntaxin-binding region(s) of VPS33B. As mentioned in Section 1.3, putative syntaxin-binding regions have been mapped on VPS33B, but these are based on the Munc18-1/STX1A structure and have not been experimentally examined (Gissen et al., 2005).

3.2.1 – Co-immunoprecipitation experiments

I first attempted these co-immunoprecipitation experiments by over-expressing both STX6 and VPS33B through transient transfection of HEK293 cells for 24h. Experiments were performed using either HA-tagged STX6 lacking its transmembrane domain (HA-STX6ΔTM), or the full-length, untagged protein, together with myc-VPS33B. Complexes were immunoprecipitated using α-myc. Initially, it was shown that VPS33B co-immunoprecipitates with both HA-STX6ΔTM and full-length STX6, as seen in Figure 15A and B. However, these results could not be consistently reproduced, and more often, these same co-immunoprecipitations were unsuccessful, as seen in Figure 15C and D. It is possible that these inconsistencies are the result
of poor immunoprecipitation of myc-VPS33B by the α-myc antibody, but as shown in Figure 15E, myc-VPS33B is efficiently immunoprecipitated by α-myc.

Figure 15. VPS33B co-immunoprecipitates with STX6, but not consistently. HEK293 cells were transfected with myc-VPS33B and either (A, C) HA-STX6ΔTM or (B, D) full-length STX6. Immunoprecipitation was attempted with α-myc and blots probed with α-STX6. In (A) and (B), STX6 was successfully co-immunoprecipitated with myc-VPS33B, however, in (C) and (D) repeat experiments are unsuccessful. In (E), α-myc efficiently immunoprecipitates myc-VPS33B.
Since I had shown these co-immunoprecipitations to be successful, but not consistently, I decided to test different experimental conditions in an attempt to improve the success rate of these experiments. First, I repeated the experiment using a C-terminally tagged STX6 construct lacking the transmembrane domain (STX6ΔTM-GFP). It was thought that including an epitope tag on the N-terminus of STX6 may interfere with binding to VPS33B, and since STX6 normally has a transmembrane domain at its C-terminus, placing a GFP epitope tag here would be unlikely to cause any such interference. However, attempts to co-immunoprecipitate this protein with myc-VPS33B were not successful, even in the presence of the detergent digitonin instead of Triton X-100, which was used in all other co-immunoprecipitation attempts (Figure 16).

![Figure 16](image_url)

**Figure 16.** myc-VPS33B fails to co-immunoprecipitate with STX6ΔTM-GFP. HEK293 cells were transfected with each construct and immunoprecipitation was attempted with α-myc while immunoblotting with α-GFP. This experiment was performed in the presence of 1% digitonin, while all other co-immunoprecipitation experiments were performed in the presence of 1% Triton X-100.

### 3.2.2 – VPS33B and STX6 co-immunoprecipitation after chemical cross-linking

It is evident from the inconsistencies in the co-immunoprecipitation experiments that the interaction between VPS33B and STX6 is likely quite transient in nature. Therefore, I attempted these experiments in the presence of the chemical cross-linker DSP. After treating HEK293 cells with a range of DSP concentrations, I saw the appearance of large protein complexes (>250kDa) when cells were treated with 1 mM DSP for 2h on ice. These complexes did not form if samples were treated with dithiothreitol (DTT), which breaks the cross-links. Nevertheless, attempts to
co-immunoprecipitate over-expressed, full-length STX6 using α-VPS33B under these cross-linking conditions were not successful (Figure 17).

Figure 17. DSP cross-linking yields high molecular weight complexes, but co-immunoprecipitations performed after cross-linking were unsuccessful. (A) Treatment of intact HEK293 cells with 0.1-1.0 mM DSP yields large molecular weight (>250kDa, red asterisk) complexes. These complexes are not seen when the cross-link is broken by treatment with DTT. (B) Immunoprecipitation of VPS33B after cross-linking is unable to pulldown STX6.
3.2.3 – VPS33B and STX6 co-immunoprecipitation after treatment with N-ethylmaleimide

It has been shown that some SM proteins, such as Sec1 in *Saccharomyces cerevisiae*, do not interact in a one-to-one manner with a syntaxin, but rather bind to the four-helix SNARE complex (Carr et al., 1999; Togneri et al., 2006). In addition, as described in Section 1.2.4, most SM proteins contain a large groove capable of binding an N-peptide motif present on their cognate syntaxins. VPS33B does not contain this groove and STX6 does not contain the N-peptide motif. Thus, I thought it may be that VPS33B interacts with STX6 only when it is part of a SNARE complex. In order to enrich the cell with these complexes, I treated cells with N-ethyl maleimide (NEM), which disables NSF, the ATPase responsible for breaking apart SNARE complexes after fusion has occurred (Sollner et al., 1993a). After treatment of cells with 1-5 mM NEM for 15 min on ice, STX6 becomes enriched in higher molecular weight complexes, indicating that I am able to trap STX6 in a SNARE complex. As expected, these complexes are disrupted if the samples are boiled. However, co-immunoprecipitation experiments performed in the presence of 5 mM NEM, while over-expressing myc-VPS33B and immunoprecipitating native STX6, were again unsuccessful, as seen in Figure 18.
Figure 18. STX6 forms high molecular weight complexes when treated with NEM, but co-immunoprecipitations performed under these conditions were unsuccessful. (A) Treatment of intact HEK293 cells with 1-5mM NEM causes STX6 to form large molecular weight SNARE complexes. These complexes are not seen when the complexes are disrupted by boiling the samples. (B) Immunoprecipitation of myc-VPS33B after NEM treatment is unable to pulldown STX6.
3.2.4 – Co-immunoprecipitating with α-STX6

All previous co-immunoprecipitation experiments performed to this point had immunoprecipitated VPS33B, and probed for the presence of STX6 on a Western blot. I decided to try the inverse experiment, immunoprecipitating with α-STX6 and probing for the presence of VPS33B on a Western blot. Specifically, I attempted to pull-down endogenous STX6 and co-immunoprecipitate over-expressed myc-VPS33B. As seen in Figure 19, the co-immunoprecipitation of myc-VPS33B was initially unsuccessful.

Figure 19. Immunoprecipitation of STX6 is initially unsuccessful at pulling down myc-VPS33B. HEK293 cells were transfected with only myc-VPS33B, while endogenous STX6 was immunoprecipitated.

Following these results, I hypothesized that the N-terminal myc tag on VPS33B could abrogate binding to STX6. As stated in Section 1.3, our lab has run cell lysates and non-denaturing Blue Native gels, and it can be seen from these experiments that endogenous VPS33B exists in 480 and 720 kDa complexes. However, when these same experiments are performed with cells stably expressing VPS33B with an N-terminal streptavidin-binding peptide (SBP) epitope, it was found that SBP-VPS33B is part of only a 242 kDa complex, suggesting the N-terminal epitope tag may interfere in the binding of VPS33B to some of its partners (unpublished data). Taking advantage of the fact that we had constructs encoding the SBP tag on either the N-terminus or the C-terminus of VPS33B, I repeated the co-immunoprecipitation experiment using α-STX6 in the presence of either over-expressed SBP-VPS33B or VPS33B-SBP. Under these conditions, I was initially able to co-immunoprecipitate VPS33B-SBP but not SBP-VPS33B. In repeating this experiment, the co-immunoprecipitation failed in both cases, while in a second repetition, the
experiment was successful in both cases. In a final attempt, the co-immunoprecipitation was again successful in the presence of VPS33B-SBP but not SBP-VPS33B, shown in Figure 20. Though these experiments were most consistently successful under this system, I could still not rely co-immunoprecipitate STX6 and VPS33B.

![Figure 20](image)

**Figure 20.** The N-terminal epitope tag on VPS33B may abrogate binding to STX6. The most consistently successful co-immunoprecipitations were acquired when endogenous STX6 was immunoprecipitated in cells transfected with C-terminally tagged VPS33B. HEK293 cells were transfected with SBP-VPS33B (left) or VPS33B-SBP (right), immunoprecipitated with α-STX6, and blots probed with α-SBP.

Since it was my desire to test whether mutations in VPS33B abrogate binding to STX6, it was necessary to be able to consistently co-immunoprecipitate these proteins. As such, I decided to pursue experiments to determine the probability that this was indeed possible.

### 3.3 – Co-fractionation of STX6 with VPS33B on a sucrose gradient

In order to determine if a co-immunoprecipitation of STX6 and VPS33B would be likely, I required a means to determine the relative amount of the total pool of STX6 that exists with VPS33B inside the cell. To do this, I decided to run a cell lysate on a 5-20% sucrose gradient, and determine whether STX6 and VPS33B co-fractionate. This experiment was performed using a lysate prepared from Dami cells, a human pro-megakaryocytic cell line established from the blood of a patient with megaryoblastic leukemia (Greenberg et al., 1988). After running the gradient, fractions of equal volume were collected from the top of the gradient and subjected to SDS-PAGE. Subsequent Western blot analysis was performed to probe for the presence of STX6 and VPS33B in each fraction. As a control, I also probed for VPS16B, the only protein
that has been confirmed to interact with VPS33B. A gradient loaded with proteins of known molecular weight was run in parallel in order to construct the standard curve used to determine approximate molecular weights of the protein complexes present in each fraction. Fractions from this molecular weight-standard gradient were run on SDS-PAGE and subsequently Coomassie-stained. The Dami lysate gradient is shown in Figure 21.

**Figure 21.** The majority of STX6 does not form high molecular weight complexes that co-fractionate with VPS33B in a 5-20% sucrose gradient. The fraction at which peak levels of each of the molecular weight standards elute is indicated.

The majority of STX6 is present in the early-eluting, low-molecular weight fractions. This is presumably the monomeric form of STX6. STX6 levels decrease dramatically in subsequent fractions, followed by a slight rise in STX6 levels peaking around fraction 9. VPS33B is also present in low-molecular weight fractions, likely representing the monomeric pool of VPS33B. However, compared to STX6, a much more substantial fraction of VPS33B is present in higher molecular weight fractions, consistent with the idea that VPS33B exists in high molecular weight complexes. As expected, VPS16B co-fractionates with VPS33B in the higher molecular weight fractions. From this sucrose gradient, it can be seen that while STX6 and VPS33B do co-fractionate somewhat, it seems the majority of STX6 exists in the monomeric form, and very little is present in higher molecular weight fractions. Thus, it is likely that while VPS33B and STX6 do interact with one another, the relative amount of each protein that is involved in this interaction is quite small. The chances of catching this interaction in a co-immunoprecipitation experiment, therefore, will be low.
To further test the possibility of capturing an interaction between VPS33B and STX6 in a mammalian cell system, I attempted colocalization experiments in Dami cells stably-transfected to express GFP-VPS33B cells, and co-stained for GFP-VPS33B and STX6 to examine whether these two proteins localize to similar compartments in mammalian cells. Although the N-terminal GFP tag may disrupt interaction with STX6, this cell line currently provides the only means of examining the staining patterns of VPS33B and STX6, since our lab does not possess an antibody against endogenous VPS33B that is suitable for immunofluorescence experiments. As can be seen in Figure 22, no significant colocalization can be seen between GFP-VPS33B and STX6, suggesting that the majority of the cellular pools of VPS33B and STX6 are present in different cellular compartments, and the relative fraction of each of these proteins that is interacting with the other is small.

![Figure 22](image.png)

Figure 22. STX6 and VPS33B do not co-localize in a mammalian cell system. HEK293 cells were stained for STX6 (green) and VPS33B (red), with the merge at right. Scale bar = 5 μm.

With this result, taken together with the data obtained from the sucrose gradient experiments, I decided that while it is most desirable to characterize the interaction between STX6 and VPS33B in a mammalian cell system, it would be best to perform these experiments by returning to the yeast two-hybrid system, which had delivered consistent and repeatable results.

3.4 – Yeast two-hybrid experiments involving VPS33B mutants

I decided to use the yeast two-hybrid screen system in order to further characterize the interaction between VPS33B and STX6. As noted in Section 1.4, several point mutations and
premature truncations identified in VPS33B are known to cause ARC syndrome. I tested two ARC point mutants (L30P, S243F) and three ARC premature truncations (1-438, 1-502, 1-532) (Gissen et al., 2005; Gissen et al. 2006) for their ability to bind STX6. Also included in the screen was a random mutant (E385G) generated due to DNA polymerase error during cloning. I also included two point mutants known to cause disease phenotypes in *Drosophila* and mouse VPS33A (G249V and D251E respectively) (Sevrioukov et al., 1999; Suzuki et al., 2003), and made the analogous mutations in VPS33B (D251E is D252E in VPS33B). In addition, as three putative syntaxin-binding regions have been identified in VPS33B (Gissen et al., 2005), I decided to test whether truncating VPS33B to lack at least one of these putative binding sites (70-617 and 1-290 mutants) is sufficient to disrupt binding to STX6. Mutants in which either a N- or C-terminal truncation was made but no syntaxin-binding regions were eliminated (17-617 and 1-350 mutants), were also included. A description of each VPS33B mutation tested is detailed in Table 3, and shown schematically in Figure 23.

<table>
<thead>
<tr>
<th>VPS33B point mutation/truncation</th>
<th>Description of truncation</th>
</tr>
</thead>
<tbody>
<tr>
<td>L30P</td>
<td>ARC point mutation</td>
</tr>
<tr>
<td>S243F</td>
<td>ARC point mutation</td>
</tr>
<tr>
<td>G249V</td>
<td><em>Drosophila</em> VPS33A point mutation</td>
</tr>
<tr>
<td>D252E</td>
<td>Mouse VPS33A point mutation (D251E in VPS33A)</td>
</tr>
<tr>
<td>E385G</td>
<td>Random mutation</td>
</tr>
<tr>
<td>17-617</td>
<td>N-terminal truncation (all syntaxin-binding domains intact)</td>
</tr>
<tr>
<td>70-617</td>
<td>Lacks syntaxin binding domain ‘A’</td>
</tr>
<tr>
<td>1-290</td>
<td>Lacks syntaxin binding domain ‘C’</td>
</tr>
<tr>
<td>1-350</td>
<td>C-terminal truncation (all syntaxin-binding domains intact)</td>
</tr>
<tr>
<td>1-438</td>
<td>ARC truncation</td>
</tr>
<tr>
<td>1-507</td>
<td>ARC truncation</td>
</tr>
<tr>
<td>1-532</td>
<td>ARC truncation</td>
</tr>
</tbody>
</table>

Table 3. Description of each VPS33B point mutant or truncation tested for binding to STX6.

Each mutant, along with wildtype VPS33B, was crossed in a yeast two-hybrid screen against STX6. In addition, each mutant was also crossed against VPS16B. Once again, Munc18-1 and
STX1A were included in the screen as a positive control. This screen, along with a Western blot confirming the proper expression of each mutant protein, is shown in Figure 24.
All of the VPS33B point mutants and truncations fail to interact with STX6, save for the randomly-generated E385G mutant. Note that results involving the 17-617, 1-502 and 1-532 mutants are not shown, but these did not bind to either STX6 or VPS16B. Interestingly, only the D252E mutant is still able to bind to VPS16B. All mutants were expressed, and at equivalent levels. Taken together, this suggests that all mutations, save for D252E, may cause a misfolding of VPS33B that abrogates binding to all partners, while the D252E mutation specifically disrupts the VPS33B-STX6 interaction.

To further emphasize the importance of the VPS33B-STX6 interaction, I wanted to pursue knockdown-rescue experiments, wherein some phenotypic defect in cells lacking VPS33B could be scored for, and the ability of either VPS33B(WT) or VPS33B(D252E) to rescue this phenotypic defect would be analyzed. I hypothesized that if the defect could be rescued by expression of VPS33B(WT) but not VPS33B(D252E), this would provide further evidence for the importance of the VPS33B-STX6 interaction.

3.5 – Examination of ARC fibroblasts for phenotypic defects

In order to pursue the knockdown-rescue experiments described above, I first required a suitable assay to score any phenotypic defects. As described in Section 1.4, megakaryocytes of ARC patients, with mutations in VPS33B, completely lack α-granules. However, work in our lab has shown that shRNA-knockdown of proteins in primary megakaryocyte cells is not trivial. Thus, I decided to attempt to find an alternative phenotype which I could use to score my knockdown-rescue experiments. To this end, I cultured control and ARC fibroblast cells possessed by our lab, and stained these cells with different subcellular markers in an attempt to identify some phenotypic defect in the ARC fibroblasts. The different subcellular markers tested included TGN46 (trans-Golgi network), AP1 (trans-Golgi network), lysobisphophatidic acid (LBPA) (late endosomes), Lamp1 (late endosomes and lysosomes), Rab5 (early endosomes), Rab7 (late
endosomes), CD63 (lysosomes), and STX6 (trans-Golgi network and late endosomes). Staining of each of these subcellular markers in control and ARC fibroblasts can be seen in Figure 25.

(A)  

(B)  

(C)
Figure 25. No phenotypic defect could be detected in ARC fibroblasts as compared to control fibroblasts. Subcellular markers tested were: (A) TGN46; (B) AP1; (C) LBPA; (D) Lamp1; (E) Rab5; (F) Rab7; (G) CD63; (H) STX6. Scale bar = 10 μm.

None of the subcellular markers tested exhibit a marked difference in staining in the ARC fibroblasts compared to the control. While there may be some slight variations in the staining patterns, none are such that one could quantify the differences in staining without invoking a high degree of subjectivity. Images of 20-25 cells were examined for each subcellular marker. Based on these results, I decided that I could not use this system to identify phenotypic defects caused by lack of VPS33B.
3.6 – Examination of HeLa cells treated with siRNA against VPS33B for phenotypic defects

Since no phenotypic differences were observed between the control and ARC fibroblasts for any of the subcellular markers tested, as an alternative I continued the search for some phenotypic defect in HeLa cells treated with siRNA against VPS33B. siRNA-mediated knockdown of VPS33B was confirmed by Western blot, shown in Figure 26. Once again, no phenotypic defects were observed in siRNA-treated cells for any of the subcellular markers tested (LBPA, Rab7, STX6) (Figure 27).

![Figure 26. siRNA-mediated knockdown of VPS33B in HeLa cells. Cells were harvested 48h post-transfection.](image)
Figure 27. No phenotypic defect could be detected in HeLa cells treated with siRNA against VPS33B compared to those treated with scrambled siRNA. Sub-cellular markers tested were: (A) LBPA; (B) Rab7; (C) STX6.
3.7 – Investigating the role of VPS33B in phagosome-lysosome fusion

As noted in Section 1.4, *Drosophila* VPS33B has been shown to play a role in phagosomal maturation, specifically the fusion of phagosomes with lysosomes. Wild type hemocytes are able to engulf and fully digest fluorescein-conjugated *E. coli* BioParticles®, while those treated with siRNA against VPS33B can engulf the BioParticles but cannot digest them (Akbar et al., 2011). I first attempted to reproduce these results using HeLa cells transfected with the FcγRII-receptor to allow these cells to become phagocytic. While these transfected cells are able to efficiently phagocytose opsinized latex beads, they did not efficiently phagocytose opsinized *E. coli* BioParticles®, as required in order to enumerate the degradation of the phagocytosed material. I also tried using RAW cells, a mouse macrophage cell line, to assess phagocytosis and phagosome-lysosome fusion. Unfortunately, while wild type RAW cells are able to efficiently phagocytose the BioParticles, degradation does not occur on the timescale reported in the Drosophila hemocytes experiments (45 minutes). As shown in Figure 28, RAW cells are unable to degrade the BioParticles after 120 minutes, and no evidence of degradation was observed at longer timepoints (up to 6 hours). Thus, pursuing the knockdown-rescue experiment in this cell line was not attempted. Attempts to optimize this experiment, or perhaps find a mammalian cell line suitable for proceeding with these experiments, is currently ongoing.

![Figure 28](image.jpg)

**Figure 28.** RAW cells are unable to efficiently degrade fluorescein-conjugated *E. coli* BioParticles®. Cells were treated with BioParticles for 10min, then chased and incubated a further 120min.
3.8 – STX6 co-localizes with markers of the α-granule biogenesis pathway in Dami cells

At the onset of this project, my desire was to better understand the role of VPS33B in megakaryocyte α-granule biogenesis. In an effort to provide some evidence that the interaction of VPS33B with STX6 may be important for this process, I decided to carry out immunofluorescence studies to examine the localization of STX6 in Dami cells, the pro-megakaryocytic cell line. In order to induce Dami cells to become more megakaryocytic in nature, cells were treated with the phorbol ester phorbol-12-myristate-13-acetate (PMA) and the cytokine thrombopoietin (TPO) for three days. Representative images of STX6 stained against various subcellular markers can be seen in Figure 29.

STX6 co-localizes well with both TGN46 and AP-1, both markers of the trans-Golgi network, while little to no co-localization is seen with markers of the early endosome (EEA1), late endosome and lysosome (Lamp-1), or dense granule and lysosome (CD63). Initially, only moderate co-localization is seen against von Willebrand Factor (VWF), an α-granule marker (Figure 29F). Dami cells, which normally exist in suspension, adhere to uncoated glass coverslips when treated with PMA and TPO, and the majority of cells treated for three days retain their round shape and tend to not flatten on the surface. The cells represented in Figures 29A-F display this cellular shape. However, some cells treated with PMA and TPO become flatter, more spread out, and exhibit thin extensions, similar to those that would be produced from a megakaryocyte during pro-platelet formation. In Dami cells observed during this stage, STX6 co-localizes quite well with VWF (Figure 29G).
Figure 29. STX6 co-localizes with markers of the α-granule pathway in Dami cells. Distribution of STX6 was compared to that of (A) TGN46; (B) AP1; (C) EEA1; (D) Lamp1; (E) CD63; (F) VWF in rounded Dami cells; (G) VWF in flat, spread out Dami cells. Scale bar = 10μm.
As described in Section 1.1.3, α-granules are derived from the trans-Golgi network, possibly through the late endosome/multi-vesicular body. Since STX6 co-localizes quite well with trans-Golgi markers, and at later stages of Dami cell development, with the α-granule marker VWF, it is reasonable to suggest that the interaction of STX6 with VPS33B plays an important role in megakaryocyte α-granule biogenesis.
Chapter 4

Discussion

4.1 – Further understanding of VPS33B requires the identification of its interactions with SNAREs

It has been shown that mutations in VPS33B, a member of the SM protein family, causes ARC syndrome, a multisystem disorder characterized by a failure to thrive and death within the first year of life (Gissen et al., 2004). We have shown that platelets of ARC patients completely lack α-granules, due to a lack of α-granule biogenesis in megakaryocytes, the large polyploid cells found primarily in the bone marrow that give rise to platelets (Lo et al., 2005). Over 300 different stored proteins are secreted from α-granules upon platelet activation (Coppingier et al., 2004), and play an important role in platelet function. Studies in our lab (unpublished), as well as others (Pulipparacharuvil et al., 2005; Zhu et al., 2009; Cullinane et al., 2010) have shown that VPS33B interacts with VPS16B. We have shown that VPS33B-VPS16B is part of a high-molecular weight, multi-subunit complex, similar to the HOPS and CORVET complexes in yeast that include Vps33p (unpublished data). Studies in mice have shown that mutations in VPS33A cause a deficiency in dense granules (Suzuki et al., 2003), while in ARC patients this process is unaffected. In addition, studies in Drosophila have shown that dVPS33A interacts specifically with dVPS16A while dVPS33B interacts specifically with dVPS16B, while loss of dVPS33A function is not restored by expression of dVPS33B (Pulipparacharuvil et al., 2005). Based on this evidence, the VPS33B-VPS16B-containing complex is also thought to be separate and distinct from that formed by VPS33A which is required for dense-granule biogenesis in megakaryocytes.

As a member of the SM protein family, it is assumed that VPS33B functions in vesicle trafficking events through its interaction with SNARE proteins. However, no such interactions have been identified to date. Given the role of VPS33B in megakaryocyte α-granule biogenesis, it is reasonable to expect that any SNAREs identified to interact with VPS33B may also play a role in this process. Identification of the SNAREs that make up the SNARE complex required for α-granule biogenesis is key for further understanding the role of VPS33B and its specific
molecular interactions with SNAREs, especially given the wide variance of SNARE-binding modes within the SM family. Does VPS33B function similarly to yeast Sec1, which is thought to only interact with intact SNARE complexes? Or does it interact in a manner more like that of the mammalian homologue of Sec1, Munc18-1, binding in a one-to-one manner to a syntaxin before facilitating SNARE complex formation? To date, all mammalian SM proteins have been identified to interact with at least one syntaxin, with the exception of VPS33A and VPS33B (see Table 4, adapted from Toonen and Verhage, 2003). Thus, it seemed a reasonable starting point to investigate whether any of the syntaxin sub-family of SNAREs are capable of interacting with VPS33B.

Table 4. List of known syntaxin interactions for each SM protein. Adapted from Toonen and Verhage, 2003.

<table>
<thead>
<tr>
<th>SM protein</th>
<th>Syntaxin interactions</th>
</tr>
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<tbody>
<tr>
<td>Munc18-1</td>
<td>STX1, 2, 3</td>
</tr>
<tr>
<td>Munc18-2</td>
<td>STX1, 2, 3</td>
</tr>
<tr>
<td>Munc18-3</td>
<td>STX2, 4</td>
</tr>
<tr>
<td>Sly1</td>
<td>STX5, 18</td>
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<tr>
<td>VPS45</td>
<td>STX16</td>
</tr>
<tr>
<td>VPS33A</td>
<td>Not determined</td>
</tr>
<tr>
<td>VPS33B</td>
<td>Not determined</td>
</tr>
</tbody>
</table>

4.2 – Binding of VPS33B to STX6 is transient in nature

Based on the results of the original yeast two-hybrid screen (Figure 13), it can be seen that both STX6 and STX11 interact with VPS33B. However, only the interaction with STX6 seems to be specific to VPS33B, as STX11 interacts with Munc18-1 and VPS33A, and may be capable of universally binding SM proteins. This screen did not include STX2, STX5, and STX13, leaving the possibility that these syntaxins may also interact with VPS33B. However, this is unlikely the case for each of these proteins. STX2 localizes to the plasma membrane (Hong, 2005), while VPS33B is known to localize to the trans-Golgi network and endosomal structures. Syntaxin 5 localizes to the cis-Golgi network (Hong, 2005). However, our studies in megakaryocytes have shown that VPS33B co-localizes with α-granule markers and late endosomal markers, but not with markers of the cis-Golgi (Lo et al., 2005), making STX5 an unlikely candidate. Since STX13 localizes to early endosomes (Hong, 2005), it is possible that some interaction of STX13 with VPS33B occurs. However, a previous yeast two-hybrid screen of all seven mammalian SM proteins paired against most of the syntaxin sub-family showed no interaction between VPS33B
and STX13 (Yamaguchi et al., 2002). Notably, neither STX6 nor STX11 were tested during this screen.

While a common caveat of yeast two-hybrid screens is the propensity for false-positive interactions, there is additional compelling evidence presented here that VPS33B and STX6 interact, and that this interaction may be important for megakaryocyte α-granule biogenesis. First, the yeast two-hybrid screen which includes VPS33A (Figure 14) shows that while there may be a slight interaction between VPS33A and STX6, this interaction is much less robust than the interaction between STX6 and VPS33B. Indeed, this minor interaction between VPS33A and STX6 may in fact be a false-positive interaction. To this point, it seems VPS33A interacts with STX1A as well, although STX1A localizes to the plasma membrane, while VPS33A is thought to act, like VPS33B, around endosomal structures, as it has been implicated in megakaryocyte dense granule biogenesis (Suzuki et al., 2003). Verification of the VPS33B-STX6 interaction can be seen in the results of the yeast two-hybrid screen of the various VPS33B point mutants and truncations screened for binding to both STX6 and VPS16B. If the VPS33B-STX6 interaction were a false-positive interaction, one would expect that any mutations not affecting the folding of VPS33B would also be observed as positive interactions with STX6. While most mutant proteins tested in this screen do not yield a positive interaction with VPS33B, most also fail to bind to VPS16B, and thus these mutations likely cause misfolding, and do not provide any evidence verifying the existence of a true VPS33B-STX6 interaction. However, the same cannot be said for the VPS33B(D252E) mutation. This mutant protein binds to VPS16B; the interaction between VPS16B and VPS33B(D252E) is equally robust as with VPS33B(WT). This observation suggests that VPS33B(D252E) folds correctly, which is not unexpected given the similar chemical structure of aspartic acid relative to glutamic acid. However, this mutation does abrogate binding to STX6, suggesting that a) the interaction between VPS33B(WT) and STX6 is indeed real and is not likely a false-positive interaction, and b) there are certain amino acid residues within VPS33B which are essential for the binding to STX6, and perhaps for binding to all SNAREs. This mutation, which occurs in a region conserved between VPS33A and VPS33B, has been identified in VPS33A to cause the buff mouse phenotype, characterized by a loss of melanosomes in melanocytes, and dense granules in megakaryocytes and platelets.
(Suzuki et al., 2003), therefore this interaction site must be functionally important in both VPS33A and VPS33B. It has not been shown that this mutation specifically abrogates binding of VPS33A to SNAREs, though one would reason that this is a likely explanation.

While the data obtained from my yeast two-hybrid screens provides ample evidence of an interaction between VPS33B and STX6, co-immunoprecipitation experiments were inconsistent in the verification of this interaction. One could make an argument that, based on the co-immunoprecipitation data presented alone, the VPS33B-STX6 interaction is simply an artefact; the data obtained from the original yeast two-hybrid screen represents a false-positive interaction. However, as explained above, additional evidence has been shown that suggests this interaction is indeed real, and moreover, the lack of a consistent co-immunoprecipitation can be explained. First, it is important to stress that the co-immunoprecipitation of VPS33B with STX6 was not unsuccessful, but rather, inconsistent. Immunoprecipitation of myc-VPS33B is able to successfully bring down either HA-STX6ΔTM or full-length STX6 when overexpressed in HEK293 cells, but this result is not consistently repeatable. I required wildtype VPS33B to be consistently immunoprecipitated with STX6 in order to further pursue one of the aims of my study, namely to examine whether mutants of VPS33B can bind STX6. However, even though these inconsistencies prevented me from pursuing these experiments, the fact remains that these co-immunoprecipitations were, at times, successful (Figure 15A,B and 20), suggesting a specific interaction between VPS33B and STX6 does indeed exist. This binding interaction is likely transient in nature and is therefore difficult to capture in a co-immunoprecipitation system. In addition, successful co-immunoprecipitation became more frequent when endogenous STX6 was immunoprecipitated and probed for the presence of transiently transfected, C-terminally tagged VPS33B-SBP (Figure 20), and indeed, there may be a structural role for the N-terminus of VPS33B in the binding of STX6. As stated in Section 1.3, our lab has run cell lysates on non-denaturing Blue Native gels, and it can be seen from these experiments that endogenous VPS33B exists in 480 and 720 kDa complexes. However, when these same experiments are performed with cells stably expressing VPS33B with an N-terminal SBP epitope, it was found that SBP-VPS33B is part of only a 242 kDa complex, suggesting the N-terminal epitope tag may interfere in the binding of VPS33B to some of its partners (unpublished data). Successful co-
immunoprecipitations were far less frequent when similar experiments were performed with SBP-VPS33B.

The VPS33B-STX6 interaction is most likely very transient in nature, unlike interactions between other SM-STX pairs such as Munc18-1 and STX1A. The interaction between Munc18-1 and STX1A has most often been characterized using brain lysates, as Munc18-1 binding to STX1A is required for synaptic vesicle fusion with the plasma membrane in neurons. This is an extremely regulated system, and specific extracellular signals are required to initiate this process. Munc18-1 is likely required to keep STX1A in its “closed” conformation until such signals are received, at which point Munc18-1 transitions STX1A to an “open” conformation, allowing for synaptic vesicle fusion to occur. In this system, Munc18-1 and STX1A will be bound to each other for relatively long amounts of time, and indeed these two proteins can be readily co-immunoprecipitated from brain lysates (Zhang et al., 2000). VPS33B, on the other hand, is involved in the processes of post-trans-Golgi network trafficking, such as α-granule biogenesis in megakaryocytes. This type of trafficking event is likely a continually occurring process which is not dependant on any specific extracellular signal. As such, VPS33B likely does not remain bound to STX6 for prolonged periods, since these two proteins only interact to facilitate vesicle fusion to a target membrane, with little or no interaction in any pre- or post-fusion state. The transient nature of this type of interaction can explain the inconsistencies seen in the co-immunoprecipitation experiments. In some samples, the conditions are such that enough VPS33B is interacting with STX6, allowing the interaction to be captured and visualized. However, in many instances, the transient nature of the interaction does not allow one to capture the interaction in sufficient quantities to permit visualization.

To try to capture and stabilize the transient interaction between VPS33B and STX6, I attempted to cross-link these two proteins by treating cells with the chemical cross-linker DSP, which crosslinks lysine residues that lie within approximately 15 Å of one another, then performing immunoprecipitations. However, these attempts failed to provide a successful co-immunoprecipitation of these two proteins (Figure 17). One can argue that the lack of successful co-immunoprecipitation of VPS33B and STX6 after treatment with DSP as further
evidence that no interaction exists between these two proteins. After treatment with 0.1-1.0 mM DSP, STX6 can be found in large-molecular weight complexes (>250 kDa). Even if this large complex is the result of some non-specific cross-linking in the cell, one would expect that this also includes all specific binding partners of STX6, and if VPS33B is indeed one of them, it would be expected to co-immunoprecipitate. As no crystal structure of VPS33B bound to STX6 exists, it is not known whether any lysine residues on VPS33B lie within 15 Å of lysine residues on STX6. Assuming that cross-linking with DSP is indeed possible, the lack of detectable crosslinked VPS33B-STX6 can be investigated by analyzing the fractionation of VPS33B and STX6 on a sucrose gradient. In an attempt to determine what percentage of the cellular pools of VPS33B and STX6 are actually present together in the cell, I ran a 5-20% sucrose gradient to examine the degree to which these two proteins co-fractionate. As can be seen in the 5-20% sucrose gradient (Figure 21), under native conditions, very little of the cellular pool of STX6 exists in any high-molecular weight complex; it can be found predominantly in its monomeric form. Thus, the relative fraction of STX6 interacting with, and that therefore would be cross-linked to, VPS33B after DSP treatment is minimal compared to the total cellular pool, and therefore the interaction is undetectable in a co-immunoprecipitation system. The cross-linking of STX6 into >250kDa structures (as seen in Figure 17A) may be the result of STX6 monomers cross-linking to one another. It has been shown for STX1A that syntaxins can exist as self-associated “clusters” on membranes (Sieber et al., 2007).

In order to further determine the relative amount of VPS33B and STX6 that may be interacting with one another, I stained for STX6 in Dami cells stably transfected to express GFP-VPS33B (Figure 22). The results suggest that VPS33B and STX6 exist predominantly in separate cellular compartments. While there appears to be a few puncta of co-localization between STX6 and GFP-VPS33B, the majority of these two proteins do not co-localize. One caveat to this experiment is that, as explained previously, the N-terminal GFP tag may disrupt interactions with STX6. Unfortunately, our lab does not possess high quality antibodies against VPS33B to observe endogenous VPS33B in immunofluorescence experiments, thus I used our Dami cell line stably-transfected with GFP-VPS33B in order to make an attempt at visualizing both VPS33B and STX6 within the cell. The results obtained from both the sucrose gradient and
immunofluorescence experiments points to the notion that, while an interaction between VPS33B and STX6 does exist, this interaction is likely transient in nature. Only a small percentage of the cellular pools of each protein are interacting with one another at a given time. This may explain the inconsistencies observed in the co-immunoprecipitation experiments, and provides a rationale behind my hypothesis that although VPS33B and STX6 do not consistently co-immunoprecipitate, an interaction between these two proteins does indeed exist.

Since some SM proteins, such as yeast Sec1, have been shown to bind to intact SNARE complexes (Carr et al., 1999), I tested whether the interaction between VPS33B and STX6 may be enhanced if STX6 is part of an intact SNARE complex. Treating cells with NEM, which inhibits NSF, the AAA-ATPase responsible for breaking apart intact cis-SNARE complexes after membrane fusion has occurred, stabilizes the SNARE complexes (Sollner et al., 1993a). However, co-immunoprecipitation of VPS33B and STX6 was not observed after treatment with NEM (Figure 18). The failure of NEM-treatment to aid in successful co-immunoprecipitation can be easily explained. It was hoped that, since VPS33B may interact with STX6 only when it is part of a SNARE complex, treatment with NEM would allow these complexes to stay intact, and perhaps increase the amount of VPS33B bound to STX6. However, if VPS33B interacts with STX6 only in a SNARE complex, this interaction very likely occurs when STX6 is part of a trans-SNARE complex, before membrane fusion has occurred. Treatment with NEM only results in an increase of intact cis-SNARE complexes, which have already completed membrane fusion, and thus all the SNAREs are present on the same membrane. At this point in the fusion process, VPS33B has more than likely dissociated from STX6, and thus treatment with NEM will not affect the amount of VPS33B bound to STX6.

4.3 – Binding of VPS33B to STX6 likely represents a novel binding mode for SM-STX pairs

One major question that remains unanswered by this study centres around the exact binding mode of VPS33B to STX6. The most well-characterized SM-STX pair remains Munc18-1-STX1A, with Munc18-1 originally having been shown to bind STX1A in its “closed” conformation (Misura et al., 2000). Other SM proteins, such as SLY1 and VPS45, were shown
to bind their cognate STXs via the STX N-terminus bound to a large groove present on the SM protein (Dulubova et al., 2002; Yamaguchi et al., 2002), and Munc18-1 has also been shown to bind STX1A in this manner (Shen et al., 2007; Dulubova et al., 2007). However, based on the crystal structure of the STX6 N-terminus, it is predicted that STX6 cannot adopt a “closed” conformation (Misura et al., 2002), and thus the original Munc18-1-STX1A binding mode is impossible in the VPS33B-STX6 pair. Likewise, STX6 does not contain an N-terminal peptide motif and VPS33B is predicted to not contain the groove required for binding of SM proteins to their cognate STX’s N-terminus (Hu et al., 2007). Therefore, VPS33B must be binding to STX6 in some novel, previously undescribed manner. This is not entirely surprising, given that STX6 is a Qc-SNARE, and not a “true” Qa-SNARE syntaxin like all the other syntaxins that have previously been identified to bind to SM proteins. Other SM proteins are thought to only interact with STXs as part of a SNARE complex. Yeast HOPS complex containing Vps33p interacts with the syntaxin Vam3p and these proteins can be co-immunoprecipitated in yeast lysates but no direct Vps33p-Vam3p interaction has been shown (Sevrioukov et al., 1999; Price et al., 2000; Dulubova et al., 2001). This mode of binding may not be conserved in VPS33B, however, as the yeast two-hybrid data presented here suggests VPS33B and STX6 interact in a direct manner. In addition, conservation of STX-binding modes among SM homologs may not occur, as indicated by the fact that yeast Sec1 binds its cognate STX only as part of a SNARE complex, while its mammalian homolog Munc18-1 binds directly to STX1A (Carr et al., 1999; Misura et al., 2000). What is likely to be conserved between Vps33p and VPS33B is the requirement for forming a multisubunit complex to facilitate SNARE binding. As previously described, Vps33p interacts with Vps11, 16, and 18 to form the class C-Vps core complex, and both mammalian homologs form similar multisubunit complexes. It is therefore likely that VPS33B is part of a multisubunit complex in order to facilitate binding to STX6. This may also explain why these two proteins do not co-immunoprecipitate consistently when both are overexpressed; in such a system, VPS33B would be present at levels about 1000-fold higher than its partner constituents of the multisubunit complex, and thus would be present in mostly a monomeric form. It is possible that monomeric VPS33B has a weak affinity for STX6 that is strengthened, likely through structural re-arrangements, when VPS33B is part of a larger complex. Thus, in the overexpressed system, the affinity of VPS33B for STX6 would be weak and unlikely to be captured. Interaction between
these proteins in co-immunoprecipitation experiments performed using endogenous protein levels, where most of VPS33B would be present in a complex, may be below the level of detection. In the yeast-two hybrid system, where VPS33B should only be present in its monomeric form, the weak interaction between VPS33B and STX6 may be sufficient to be detected as a positive hit in this system, or perhaps VPS33B may be able to substitute for Vps33p and form a complex with Vps11, 16, and 18 that is capable of facilitating the interaction with STX6.

Additionally, in contrast to evidence supporting the role of Munc18-1 as a molecular chaperone of STX1A, it is unlikely that VPS33B acts as a molecular chaperone of STX6. Munc18-1 knockdown causes mislocalization and decreased expression of STX1A (Verhage et al., 2000; Voets et al., 2001; Arunachalam et al., 2008; Han et al., 2009). As shown in Figure 25H and 27C, STX6 localization and expression levels are not affected in either ARC fibroblasts or in VPS33B knockdown HeLa cells.

It is also interesting to note that all previous SM-STX interactions identified have included STXs that are part of the Qa-SNARE family, while the VPS33B-STX6 interaction represents the first example of a direct interaction between an SM protein and a Qc-SNARE. All STXs other than STX6, 8, and 10 are Qa-SNAREs, and these are also the only known Qa-SNAREs. Since STX6 must form a SNARE complex with a Qa-, Qb-, and R-SNARE, one of the Qa-SNARE STXs must be part of a SNARE complex with STX6. Whether VPS33B interacts with the intact SNARE complex or has indirect interaction with the Qa-SNARE syntaxin remains to be seen.

STX6 has been implicated in playing a role in the formation of specialized granules in other cell types besides megakaryocytes. In endocrine and neuroendocrine cells, STX6 has been shown to be required for homotypic fusion of immature secretory granules (ISGs) to form mature secretory granules, the storage compartment for secretory proteins such as hormones or neuropeptides (Wendler et al., 2001). Like megakaryocyte α-granules, ISGs are formed from the trans-Golgi network, and VPS33B may also interact with STX6 in this cell system to facilitate this homotypic fusion event. Notably, yeast Vps33p, as part of the HOPS complex, is required
for another homotypic fusion event, that of yeast vacuoles, which are also formed from the *trans*-Golgi network (Sato et al., 2000; Seals et al., 2000).

4.4 – Studies of molecular defects that cause ARC syndrome

The yeast two-hybrid screen was utilized to interrogate different VPS33B mutations and the consequence of these mutations to interact with STX6 and VPS16B to possibly explain the defect observed in ARC patients. Our group has shown that ARC patients with the nonsense mutation R438X do not express VPS33B protein in fibroblasts (Lo et al., 2005). However, it is not known if all ARC mutations cause VPS33B protein to be absent, or if the protein exists in the cell in a misfolded, malfunctioning form. Nonsense mutations causing premature stop codons may undergo mRNA decay such that no protein is ever made (Hentze and Kulozik, 1999), while others that cause misfolding may lead to protein degradation. Some missense mutations may also cause misfolding, leading to protein degradation, while others may result in normal expression levels of a non-functioning protein. As demonstrated in my studies, yeast two-hybrid analysis shows that VPS33B(1-438) fails to interact with VPS16B. Unlike in ARC patients, this mutant is properly expressed, indicating that the lack of binding to VPS16B is likely due to the misfolding of the protein. In ARC patients, it may be that this misfolding results in the failure of the protein to be expressed. Similarly, all other ARC mutations tested in this study, including the missense mutants L30P and S243F, as well as the nonsense mutants R507X and R532X, fail to bind VPS16B. Presumably, these mutants are also misfolded in ARC patients. Several other nonsense and frameshift mutations have been identified in ARC patients (Gissen et al., 2006), but these were not tested in this screen. **Table 5** summarizes known ARC mutations, and indicates which of these mutants were tested in these studies.
Table 5. List of VPS33B mutations identified in ARC patients. X = stop codon; fs = frameshift. Adapted from Gissen et al., 2006.

<table>
<thead>
<tr>
<th>VPS33B mutation</th>
<th>Tested in this screen?</th>
</tr>
</thead>
<tbody>
<tr>
<td>L30P</td>
<td>Yes</td>
</tr>
<tr>
<td>R51X</td>
<td>No</td>
</tr>
<tr>
<td>R93X</td>
<td>No</td>
</tr>
<tr>
<td>R97X</td>
<td>No</td>
</tr>
<tr>
<td>S116fsX136</td>
<td>No</td>
</tr>
<tr>
<td>C123X</td>
<td>No</td>
</tr>
<tr>
<td>L145fsX151</td>
<td>No</td>
</tr>
<tr>
<td>L175fsX219</td>
<td>No</td>
</tr>
<tr>
<td>S243F</td>
<td>Yes</td>
</tr>
<tr>
<td>L403fsX414</td>
<td>No</td>
</tr>
<tr>
<td>R438X</td>
<td>Yes</td>
</tr>
<tr>
<td>R507X</td>
<td>Yes</td>
</tr>
<tr>
<td>R532X</td>
<td>Yes</td>
</tr>
</tbody>
</table>

The results of the yeast two-hybrid screen can be examined in more detail by making use of threading programs such as Phyre (http://www.sbg.bio.ic.ac.uk/~phyre) to acquire a hypothetical structure of VPS33B, based on the known structure of Munc18-1. This can be seen in Figure 30, where all point mutants tested in the yeast two-hybrid screen are highlighted in red, the putative syntaxin binding regions in yellow, and the residues truncated from the VPS33B(1-532) ARC mutant in green.
Figure 30. Theoretical structure of VPS33B based on threading of structure of Munc18-1. Point mutants tested in the yeast two-hybrid screens are shown in red, putative STX-binding regions in yellow, and residues truncated from the VPS33B(1-532) ARC mutant in green.

As can be seen from the structural model, all of the point mutants tested, L30P, S243F, G249V, and D252E, are internal residues which are likely to form interactions with neighbouring residues that are important for the overall fold of the protein. In contrast, the randomly-generated E385G mutant represents a residue that extends into the cytoplasm, and thus disruption of this residue is unlikely to cause any defects in folding. This seems to be the case as this mutant is able to bind to both VPS33B and STX6 (Figure 24). In the case of L30P, S243F, and G249V, these mutations likely disrupt inter-residue interactions and lead to misfolding, as suggested by the fact these mutants do not bind to VPS16B or STX6. Since aspartic acid and glutamic acid are similar in chemical structure, the D252E mutation likely does not cause a disruption in these interactions, and indeed, the protein folds properly, as evidenced by the fact
that it is still able to bind VPS16B. Thus, D252 seems to be specifically required for binding to STX6, and though this residue lies outside any of the putative STX-binding regions, based on the structural model, this sidechain extends into the deep cavity in VPS33B that has been shown to be the location of STX-binding on other SM proteins.

None of the N- or C-terminal truncation mutants were able to bind STX6. They were also all unable to bind VPS16B, suggesting they too undergo misfolding. Even the 17-617 mutant, with only a small (16 amino acid) N-terminal truncation and containing all putative syntaxin binding regions, was unable to bind STX6 and VPS16B. This points to an important structural role for the N-terminus of VPS33B. It is in keeping with our studies of complexes (as previously described) on non-denaturing Blue Native gels, where endogenous VPS33B forms 480 and 720 kDa complexes, while the N-terminally-tagged SBP-VPS33B forms only a 242 kDa complex, indicating the tag on the N-terminus may disrupt binding of VPS33B to some of its partners. Given this result, it is not surprising that the more N-terminally truncated 70-617 mutant, which lacks putative STX-binding region ‘A’, is unable to bind either STX6 or VPS16B. The C-terminally truncated 1-290 mutant, which lacks putative STX-binding region ‘C’ and 1-350 mutant, which has all putative STX-binding regions intact, also likely misfold, as they are also unable to bind STX6 and VPS16B. This is not surprising, given the fact that none of the less drastic C-terminal truncations found in ARC patients, 1-438, 1-502, and 1-532, are able to bind STX6 or VPS16B. As can be seen from Figure 30, residues 532-617, shown in green, form a series of small α-helices and a β-sheet that extends into the deep cavity of VPS33B where the interaction with STX6 or other SNAREs likely occurs. Thus elimination of these residues likely disrupts the structural integrity of this cavity, causing the protein to misfold. It would be interesting in future experiments to make point mutations, rather than extensive truncations, at some of these N- or C-terminal residues to gain a further understanding of which of these residues are required for the structurally integrity of VPS33B, and also to identify residues that, in addition to D252, seem to be specific for binding to STX6.

The results obtained in the yeast-two hybrid screen with the VPS33B(D252E) mutant are intriguing. This mutant does not bind STX6, but does bind VPS16B, suggesting that it folds
correctly, and the mutation specifically abrogates VPS33B binding to STX6, and perhaps other SNAREs as well. Indeed, the mutation of aspartic acid to glutamic acid should not cause any significant structural changes to the protein, and folding should remain unaltered. This mutation was included in the screen because the analogous mutation in VPS33A, D251E, comprising a conserved region in both VPS33A and VPS33B, has been shown to cause the *buff* mouse phenotype, characterized by a deficiency of dense granules in platelets and melanosomes in melanocytes, causing coat-color hypopigmentation (Suzuki et al., 2003). This has suggested VPS33A as a candidate gene for some cases of human Hermansky-Pudlak syndrome (HPS), a disorder in organelle biogenesis in which oculocutaneous albinism, bleeding, and occasionally pulmonary fibrosis result from defects of melanosomes, platelet dense granules, and lysosomes. Life expectancy of HPS patients is 40-50 years (Huizing et al., 2008). Since the yeast two-hybrid screens revealed that VPS33B(D252E) still binds to VPS16B and thus no major misfolding is likely to occur, it is presumed that VPS33A(D251E) is expressed in *buff* mice and not simply absent as is likely the case for VPS33B in many ARC patients, although this was never tested.

No such VPS33B(D252E) has been identified in ARC patients. This leads one to wonder if this mutation would even cause ARC syndrome. If VPS33B is present in the cell, even though it is unable to bind STX6 or other SNAREs, would this cause the same phenotype that results from a total lack of VPS33B? Or would some milder but distinct phenotype result from this mutation? Perhaps VPS33B, as part of a large multisubunit complex, plays an important structural role in the integrity of this complex. This complex is quite likely important for other cellular functions apart from SNARE binding. Complete absence of VPS33B might prevent this complex from assembling, while a structurally-sound VPS33B(D252E) mutant would still allow for assembly and proper function, presumably with the exception of SNARE binding, of this complex. This hypothesis is supported by the fact that mutations in VPS16B have also been shown to cause ARC syndrome (Cullinane et al., 2010). Here, absence of VPS16B would also prevent assembly of the multisubunit complex, causing the exact phenotype as that seen when lacking VPS33B. Cells expressing the properly-folding mutant VPS33B(D252E) would likely only be defective in the fusion events mediated by VPS33B, and these likely involve specialized granules and
organelles in specific cell types. Cells lacking VPS33B altogether would not be able to form the multi-subunit complex that includes VPS33B and VPS16B, and this would cause defects related to all cellular functions this complex is involved in, including, but not limited to, VPS33B-mediated fusion events.

One method for testing whether the above-stated hypothesis is correct would be to carry out the knockdown-rescue experiments that were previously described, namely, examining the ability of VPS33B(D252E) to rescue defects in phagosomal maturation caused by knockdown of wildtype VPS33B. If VPS33B(D252E) can in fact rescue these defects, then this would suggest that perhaps this mutant, while it does not bind SNARE proteins, can still facilitate the complex formation that is essential for other cellular functions. This would further implicate the failure of this complex assembly as the cause of ARC syndrome.

4.5 – VPS33B is likely required specifically for fusion events involving the trafficking of specialized organelles

The original goal of this project was centred on identifying SNAREs important for megakaryocyte α-granule biogenesis. One method to accomplish this is to first identify SNAREs that interact with VPS33B, then knockdown these SNAREs in megakaryocytes and examine if this affects the formation of α-granules. To this end, several studies have developed protocols for retroviral-based transduction of CD34+ stem cells (megakaryocyte progenitor cells) to achieve ectopic expression of target proteins (Wilcox et al., 2003; Shi et al., 2004). Moreover, studies in our lab and others (Gilles et al., 2009) have been focused on developing a lentiviral-based transduction of CD34+ cells to achieve shRNA-mediated knockdown of target proteins. However, work in our lab has shown that achieving this knockdown in primary megakaryocytes is not trivial; technical problems such as limited transfection efficiency, the relatively long maturation process of CD34+ stem cells into megakaryocytes (~12 days), and the inability of transduced megakaryocytes to grow in media containing antibiotics for selection such as puromycin, are just a few of the problems faced in this system. Furthermore, I have attempted knockdown of STX6 in HeLa cells using several siRNA sequences, and to date have not found a sequence capable of appreciably decreasing STX6 levels in these cells. Based on these
observations, I decided to alter my strategy somewhat, focusing on the fact that I have identified a mutant of VPS33B, VPS33B(D252E), that is not capable of binding to STX6, but still binds VPS16B, the only other protein that has been identified to bind to VPS33B. The aim was to perform experiments in which I would knockdown endogenous VPS33B in cells and rescue with either VPS33B(WT) or VPS33B(D252E). I hypothesized that the knockdown phenotype would be rescued by VPS33B(WT) but not VPS33B(D252E), thus implicating the binding of VPS33B to STX6 as an important interaction within the cell. As explained in the previous section, if VPS33B(D252E) can restore the wildtype phenotype, this would implicate the failure of the multi-subunit complex that contains VPS33B and VPS16B to assemble, rather than the interaction between VPS33B and STX6, as a main cause of ARC syndrome.

Although numerous tissue abnormalities have been observed in ARC patients, including liver and kidney abnormalities (Gissen et al., 2004), the defects observed in these cells are centred around hepatocyte accumulation of lipofuscin granules, as well as the mislocalization of membrane proteins which are normally trafficked to either the apical/canalicular or basolateral membrane of these polarized cells. Instead they are localized ubiquitously across the plasma membrane, which can be difficult to study in a cell culture system. The advantage of our cell culture system is that lack of VPS33B can be readily assessed by the absence of α-granules in megakaryocytes and platelets; in this system, granules are either present or absent. However, given the technical difficulty of performing knockdown-rescue experiments in primary megakaryocytes, I needed another readout of a readily-observable phenotypic defect in cells where VPS33B function has been altered in order to proceed with these experiments. Fortunately, our lab possesses both control and ARC fibroblast cell lines, allowing me to examine the staining pattern of different sub-cellular markers in a cell line lacking VPS33B. One might expect that ARC fibroblasts may experience defects in trafficking, specifically in post-trans-Golgi trafficking steps, and thus there may be some mislocalization of organelles which participate in these events. However, as seen in Figure 25, all markers of the trans-Golgi network, early endosomes, late endosomes, and lysosomes exhibit no defects in the ARC fibroblasts as compared to the control cells. Similarly, it was thought that HeLa cells treated with siRNA against VPS33B may also experience post-trans-Golgi trafficking defects, but as
seen in Figure 27, these cells showed no defects compared to those treated with scrambled siRNA.

The lack of any phenotypic defects in either ARC fibroblasts or HeLa cells treated with siRNA against VPS33B may be explained by the idea that VPS33B plays a specific role in the fusion events of specialized organelles in different cell systems, such as α-granule biogenesis in megakaryocytes, correct sorting of lipofuscin granules and in liver and kidney cells, or perhaps homotypic fusion of ISGs in endocrine cells (a STX6-mediated fusion event). Trafficking events involving ubiquitous organelles such as lysosomes may be mediated by different SM proteins. To this end, VPS45 is known mediate fusion events involving traffic at the trans-Golgi network, while Munc18-1, Munc18-2, and Munc18-3 all act at the plasma membrane and would likely be involved with the secretion of any organelles or granules with the cell surface (Hong, 2005).

To date, defects identified in ARC patients include: accumulation of lipofuscin granules in liver and kidney hepatocytes, abnormal localization of membrane proteins in liver and kidney polarized epithelial cells which may be due to sorting defects occurring at the late endosomal stage (though this has not been tested), and the lack of α-granule biogenesis in megakaryocytes. In addition, VPS33A mutations lead to defects in megakaryocyte dense granule biogenesis, and biogenesis of melanosomes in melanocytes. Late endosomes, melanosomes, lipofuscin and platelet storage granules all belong to a class of lysosome-related organelles that share their biogenesis pathway (Huizing et al., 2008). However, none of these defects have been shown to specifically be the result of a lack of SNARE-interaction. Given that VPS33B is ubiquitously-expressed, and ARC syndrome is a multi-system disorder, these defects, and defects occurring in cell types that lack these specialized lysosome-related organelles, may not necessarily be due to the failure of some fusion event to occur. VPS33B is part of a multi-subunit complex that presumably has a role in several cellular functions, and mutations in VPS16B, which is not an SM protein and may have no direct role in fusion events, also causes ARC syndrome. This leads one to speculate whether the absence of these proteins disrupts the structural integrity of the complex, and it is this disruption that causes defects to occur in cell types lacking specialized granules, rather than the failure of any specific fusion event to occur.
It has recently been shown that VPS33B plays a role in phagosomal maturation, specifically the fusion of the phagosome and lysosome. This is in keeping with observations that ARC patients suffer from multiple infections (Jang et al., 2009). Upon infection of host cells, \textit{M. tuberculosis} secretes the tyrosine phosphatase PtpA, of which VPS33B is a substrate, which prevents phagosome-lysosome fusion (Bach et al., 2008). Also, when treated with \textit{E. coli} BioParticles, cells lacking VPS33B can phagocytose but not degrade the bacteria, also due to a failure of phagosome-lysosome fusion (Akbar et al., 2011). Thus, it seems I may have a suitable phenotypic assay in which to perform knockdown-rescue experiments. Unfortunately, untreated RAW cells did not efficiently degrade bacteria, and thus these experiments could not be performed in this cell line. Efforts are ongoing to optimize this assay (in either RAW cells or the human monocyte THP-1 cells) including using opsonised bacteria to improve the efficiency of phagocytosis, or using opsonised latex beads in order to study phagosome-lysosome fusion. Although latex beads cannot be degraded and so will not provide a suitable endpoint for my assay, I have been able to observe efficient phagocytosis of these beads when using HeLa cells transfected with the F\(_{\gamma}\)RII-receptor, and thus these beads can be used to get a sense of the time frame required by macrophages to phagocytose and allow for phagosome-lysosome fusion.
Chapter 5

Conclusions and Future Directions

In this study, it was shown through a yeast two-hybrid screen of VPS33B against twelve syntaxins that VPS33B displays a robust interaction with STX6. The interaction with STX6 was specific to VPS33B. However, co-immunoprecipitation experiments were not consistently successful, and sucrose gradient and co-localization experiments revealed that VPS33B and STX6 are rarely part of the same complex. Taken together this suggests that, while an interaction between VPS33B and STX6 does exist, it is likely quite transient in nature. VPS33B mutants, either ARC mutants or other mutants truncated to lack at least one putative syntaxin-binding region, failed to bind STX6, however, they also failed to bind VPS16B, suggesting that these mutants do not fold correctly. One mutant based on a missense mutation identified in VPS33A and affecting biogenesis of melanosomes and platelet dense granules, D252E, did not interact with STX6, but did interact with VPS16B, suggesting this protein does fold correctly, and the mutation specifically abrogates binding to STX6. STX6 co-localizes well with markers of the α-granule biogenesis pathway in Dami cells, providing further evidence for the interaction of VPS33B with STX6. From these experiments, it can be concluded that VPS33B interacts with STX6, but the nature of this interaction is still unclear. The yeast two-hybrid data suggests a robust one-to-one interaction, however data from co-immunoprecipitation experiments suggests that this interaction is highly transient and may require other factors in order to be stabilized.

The mode of binding between VPS33B and STX6 is very likely novel and unique from any other previously-identified SM-STX pair. Based on co-localization studies in Dami cells, STX6 seems to be involved in the α-granule biogenesis pathway. Further studies are required in order to verify the requirement of STX6 for α-granule biogenesis, and to identify other SNAREs required for this process.

In order to allow one to investigate the function of VPS33B further, there are a few areas of research that need to be addressed. Firstly, a clear phenotypic defect in cells lacking VPS33B needs to be identified to allow for studies such as knockdown-rescue experiments to be explored. Although the lack of α-granule biogenesis in megakaryocytes and platelets provides for a good
readout of VPS33B function there are some key experimental difficulties. Primary megakaryocytes are difficult to work with as they can only be obtained in limited cell numbers and do not replicate readily, and because they are difficult to transfect, do not grow in selection media containing antibiotics, and undergo a relatively slow maturation process form CD34+ stem cell to megakaryocyte, shRNA-mediated knockdown of proteins is not trivial. Dami cells, while useful as a pro-megakaryocytic cell line, are not fully megakaryocytic in nature; they do not form typical pro-platelets, and they do not form ultrastructurally recognizable α-granules. Furthermore, we have found that expression of α-granule proteins such as VWF is not ubiquitous amongst a population of cells in culture. Excitingly, the newly discovered role of VPS33B in phagosomal maturation may provide alternative model systems to be utilized. As shown here, mouse macrophage RAW cells are not the most desirable, as they fail to efficiently degrade bacteria. However, other cell lines such as the human monocyte THP-1 cells may prove to be more suitable. Additionally, it would be interesting to investigate whether VPS33B plays a role in the homotypic fusion of immature secretory granules in PC12 cells, as STX6 has been identified as playing a key role in this process (Wendler et al., 2001). This could be tested by treating PC12 cells with siRNA against VPS33B and examining the effect on homotypic fusion of these granules. If siRNA-mediated knockdown of VPS33B in these cells were to disrupt this homotypic fusion event, then this would provide further evidence towards the significance of the VPS33B-STX6 interaction.

Second, it will be important to identify other SNARE proteins that interact with VPS33B, as the identification of these proteins may facilitate the discovery of a complete SNARE complex that interacts with VPS33B and facilitates some trafficking event. Based on the yeast two-hybrid assay of the syntaxin subfamily, VPS33B may only interact directly with STX6, and no other members of the SM protein family have been shown to interact in a one-to-one manner with non-syntaxin SNAREs. Thus, it is likely that any other SNAREs that are part of a VPS33B-interacting SNARE complex do not actually directly interact with VPS33B. However, it is likely that STX6 would be part of such a complex would be high, and therefore, these SNAREs would interact directly with STX6. It would be useful to perform an immunoprecipitation of STX6 and perform a mass spectroscopy analysis of the sample in order to identify these SNAREs. This
procedure would also be aided if one were to first treat cells with NEM before performing the experiment, as this would ensure STX6 is held in intact SNARE complexes, as I have shown with the experiments presented in Figure 18A. Once these other SNAREs that interact with STX6 have been identified, it will be necessary to prove that they participate in fusion events mediated by VPS33B. One method involves making use of SNARE proteins lacking their transmembrane domain as dominant-negative mutants. Here, cytosolic t-SNAREs can be introduced into a cell and will form a SNARE complex with their cognate v-SNAREs, while excluding the membrane-bound t-SNAREs from the complex. This prevents vesicular membranes from coming into close contact with target membranes, and no fusion events occur. In my studies, since STX6 is a Qc-SNARE, it is unlikely that it forms a SNARE complex with one of the non-membrane bound SNAP-23, SNAP-25, or SNAP-29, since these all contain one Qc-SNARE motif. Instead, a SNARE complex containing one Qa-SNARE and one Qb-SNARE present with STX6 on the target membrane is more likely. In this case, dominant-negative versions of each of these three t-SNAREs would likely need to be transfected into cells in order to prevent some VPS33B-mediated fusion event from occurring. For example, mass spectrometry analysis could be used to identify Qa-, Qb-, and R-SNAREs that interact with STX6 in these THP-1 cells or some other macrophage cell line. Transient transfection of these cells with dominant-negative forms of the Qa-SNARE, Qb-SNARE, and STX6 may prevent phagosome-lysosome fusion from occurring. Since this fusion event has been shown to be mediated by VPS33B (Akbar et al., 2011), then these results would a) identify a SNARE complex containing STX6, and b) show that fusion of this STX6-containing SNARE complex is mediated by VPS33B.

As discussed in the previous chapter, it will also be important to further examine the molecular interactions of the VPS33B(D252E) mutant, and its ability to rescue wildtype phenotypes after loss of VPS33B. If this mutant is unable to rescue these phenotypes, it implies that loss of SNARE binding may be an underlying cause of the cellular phenotypes observed in ARC patients. If this mutant can rescue the wildtype phenotypes, it gives strength to the idea that the underlying cause of ARC syndrome lies in the failure of the multisubunit complex containing VPS33B to assemble. It is also conceivable that VPS33B(D252E), while unable to bind
monomeric STX6, may still be able to help facilitate fusion of a trans-SNARE complex containing STX6. Making further mutations in VPS33B, perhaps missense mutations in the putative syntaxin-binding regions, will be important to identify additional residues that are essential for SNARE binding, and examining the effect of these mutations on the ability of VPS33B to bind a protein such as VPS16B will allow us to further study the specific molecular interactions of VPS33B.

As one of the original goals of this project was to further our understanding of megakaryocyte and platelet function, it remains important to pursue future work that is specific to these cells. When it was shown that VPS33B is required for α-granule biogenesis in megakaryocytes (Lo et al., 2005), it was thought that VPS33B was not present in platelets. Work in our lab has since revealed that VPS33B is indeed present in platelets (unpublished data), and I have shown that STX6 is present in both megakaryocytes and platelets (unpublished data). While I have hypothesized that in megakaryocytes, STX6 plays a role in α-granule biogenesis, the VPS33B-STX6 interaction may be important in platelets as well. Some SNAREs that play a role in the secretion of α-granules from platelets have been identified (Flaumenhaft et al., 1999), though this has not been extensively studied. STX6 may play a role in this process, and the VPS33B-STX6 interaction may be important for this fusion event to occur. To examine the role of this interaction in platelets, one can make use of a system that allows for the introduction of exogenous proteins into platelets by tagging a desired protein with the Tat(48-60) peptide sequence. This small peptide facilitates the uptake of the tagged protein across the platelet membrane (Dimitriou et al., 2009). One could introduce the VPS33B(D252E) mutant into platelets, and identify any defects that may occur. For instance, if this would cause a reduction in α-granule secretion, one would expect to observe decreased levels of secreted α-granule proteins in the cell media upon platelet activation. One would expect a similar result if platelets were treated with dominant-negative forms of STX6 and the other STX6-interacting t-SNAREs.

Alternatively, a mouse model has been developed which expresses Cre-recombinase exclusively in the megakaryocyte lineage, allowing for megakaryocyte-specific knockout of a desired gene (Tiedt et al, 2007). This system has been used previously to knockout the MYH9 gene encoding
for nonmuscle myosin heavy chain IIA in megakaryocytes (Leon et al., 2009). This system could be applied to create STX6 megakaryocyte knockouts, and these cells could be harvested and examined for any defects in α-granule biogenesis.

These studies have shown that a VPS33B-STX6 interaction exists, however, the significance of this interaction remains unclear. Determining the significance of this interaction will be a crucial step in furthering our understanding of megakaryocyte platelet function.
References


