The Exocyst Subunit Sec6 Interacts with Assembled Exocytic Snare Complexes: A Dissertation

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THE EXOCYST SUBUNIT SEC6 INTERACTS WITH ASSEMBLED EXOCYTIC SNARE COMPLEXES

A Dissertation Presented

By

MICHELLE LYNNE DUBUKE

Submitted to the Faculty of the University of Massachusetts Graduate School of Biomedical Sciences, Worcester in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

December 18th, 2015

Biochemistry and Molecular Pharmacology
Signature Page

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December 18th, 2015
Dedication

This work is dedicated to my absolutely amazing family and friends without whose constant encouragement I never would have made it this far. Thank you for believing in me, even when I didn’t.
Acknowledgements

First and foremost, I want to thank my advisor Mary Munson. It’s never easy to let a graduate student develop her own ideas, especially when they begin to conflict with your already established hypothesis; thank you for believing in me, and giving me the chance to show you my point of view. This project has been a long time in development, and you’ve been with me and worked with me every step of the way.

Thank you to the members of the Munson Lab (past and present) for all of their help, encouragement, and useful advice over the years. Given all of the stumbling blocks in this project, without those discussions I may have been stuck with no way forward years ago. Especially, thank you Maggie Heider for being my co-student the whole 6 years and putting up with our differences in opinion and methodology, even when it wasn’t easy.

Thank you to Scott Shaffer and all members of the Proteomics and Mass Spectrometry Facility here at UMass, especially Stephanie Maniatis. The early stages of this project depended upon the recent developments in technology and new tools that you brought to the university. Additionally, all of the technical support and useful discussions over the years helped to shape how we think about these two proteins. Thank you to the faculty, staff, students, and post docs of the BMP department. This is an amazing department to work in, and all of the help and advice over the years (from Chalk Talk to TRAC meetings to random discussions in the hallways, both science and otherwise) have been invaluable.
Thank you to the members of my TRAC committee and DEC. Your experimental advice over the years, and constant encouragement, made this project possible and truly shaped me into the critical thinker I’ve become over the years.

Finally, thank you to my family and friends – you’ve always been there, and I can’t thank you enough.
Abstract

In eukaryotic cells, membrane-bound vesicles carry cargo between intracellular compartments, to and from the cell surface, and to the extracellular environment. Many conserved families of proteins are required for properly localized vesicle fusion, including the multi-subunit tethering complexes and the SNARE complexes. These protein complexes work together to promote proper vesicle fusion in other trafficking pathways. Contrary to these other pathways, our lab previously suggested that the exocyst subunit Sec6, a component of the exocytosis-specific tethering complex, inhibited Sec9:Sso1 SNARE complex assembly due to interactions \textit{in vitro} with the SNARE protein Sec9 (Sivaram et al., 2005).

My goal for this project was to test the hypothesis that Sec6 inhibited SNARE complex assembly \textit{in vivo}. I therefore chose to generate Sec6:Sec9 loss-of-binding mutants, and study their effect both \textit{in vitro} and \textit{in vivo}. I identified a patch of residues on Sec9 that, when mutated, are sufficient to disrupt the novel Sec6-SNARE interaction. Additionally, I found that the previous inhibitory role for Sec6 in SNARE assembly was due to a data mis-interpretation; my re-interpretation of the data shows that Sec6 has a mild, if any, inhibitory effect on SNARE assembly. My results suggest a potential positive role for Sec6 in SNARE complex assembly, similar to the role observed for other tether-SNARE interactions.
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Figure 3.5 was produced by me, and published in Molecular Biology of the Cell. No permission needed for authors to use this material.

List of Symbols, Abbreviations, or Nomenclature

MTC, Multisubunit Tethering Complexes; IDP, intrinsically disordered protein; COG, Conserved Oligomeric Golgi; GARP, Golgi-Associated Retrograde Protein; HOPS, HOmotypic fusion and vacuole Protein Sorting; CORVET, Class C CORe Vacuole/Endosome Tethering; TRAPP, TRAnsport Protein Particle; CATCHR, Complexes Associated with Tethering Containing Helical Rods; EDC, 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride; ts, temperature sensitive. GST, Glutathione S-Transferase.
Preface

All of the work presented here was completed in Dr. Mary Munson’s Laboratory at the University of Massachusetts Medical School, with the exception of the cross-linking studies presented in Chapter II. These studies were performed at the Proteomics and Mass Spectrometry Facility at the University of Massachusetts Medical School under the direction of Dr. Scott Shaffer.

A version of Chapter II has been published in the Journal of Biological Chemistry. Dr. Scott Shaffer designed the mass spectrometry experiments, which were performed and optimized by Stephanie Maniatis. The Sec6-ternary SNARE complex interaction was originally identified by Dr. Francesca Morgera, which I subsequently characterized. I performed all other experiments, completed all data analysis, and completed the first stage of manuscript composition. Dr. Mary Munson was the corresponding author on this project, and was involved throughout with concept formation, experimental design, and final manuscript composition.

Sections of Chapter I and Chapter III will be submitted to the Frontiers Journal as a review article; I will be the primary author of that article, with Dr. Mary Munson as corresponding author.
CHAPTER I: INTRODUCTION
Intracellular Trafficking

Intracellular trafficking, the movement of membrane-bound or soluble cargo between distinct cellular compartments, is an essential and highly conserved process in all eukaryotic cells. It requires a vesicle budding off a donor organelle, being transported to its site of fusion, and subsequently fusing with the target membrane (Herrmann and Spang, 2015). These pathways are required for the overall health and function of the cell and the organism; defects in various trafficking pathways can result in kidney disease, defective neuronal outgrowth, and various lysosomal storage disorders (Horton et al., 2005; Platt et al., 2012; Schaeffer et al., 2014).

One of the best studied organisms for all stages of intracellular trafficking is the budding yeast Saccharomyces cerevisiae. The high level of conservation of these pathways, coupled with the genetic tractability of yeast as a model organism, suggests that these processes can be dissected in yeast with the findings being transferred to higher eukaryotes (Feyder et al., 2015). In yeast, intracellular trafficking encompasses several distinct trafficking steps (Figure 1.1). The first, endoplasmic reticulum (ER) to Golgi complex trafficking, is required for the initial movement of secreted or membrane

![Figure 1.1 – A schematic representation of the various intracellular trafficking stages in the budding yeast Saccharomyces cerevisiae. MVB – multi vesicular body, ER – endoplasmic reticulum.](image-url)
proteins from their folding in the ER to their maturation in the Golgi. Once these proteins arrive at the Golgi, they can follow one of two pathways – ER resident proteins are returned via an ER retrograde pathway, and proteins moving on through the secretory pathway are matured in the Golgi via an intra-Golgi pathway. After Golgi maturation is complete, the proteins are sorted; plasma membrane and secreted proteins are brought to the cell surface by the exocytic trafficking pathway, while vacuolar proteins passing through the endosome are trafficked by the Golgi-endosome trafficking pathway. Arrival at the endosome can also occur during endocytic recycling – this is required for retrieval of plasma membrane proteins, which can then be brought back to the Golgi for another round of secretion after ligand removal or degraded through trafficking to the vacuole. The end-stage of trafficking is from the endosome, through the multi vesicular body, and to the vacuole for protein degradation by the homotypic vacuolar fusion pathway (Feyder et al., 2015).

Each of these distinct trafficking pathways are tightly regulated, as mis-sorting of cargo or mis-localization of fusion events can lead to severe cellular defects (Olkkonen and Ikonen, 2006). Additionally, each intracellular route is mediated by members of conserved protein families including (but not limited to) small GTPase proteins on both membranes, specific interactions with lipids, the Soluble N-ethylmaleimide Attachment protein Receptor (SNARE) complexes, the Sec1/Munc18 family of SNARE regulatory proteins, and tethering complexes (discussed in detail below). These protein families work together, by mechanisms not yet fully characterized, to ensure the overall health of the cell/organism.
Once the cargo-containing vesicle arrives at the proper membrane, the process of vesicle fusion occurs in a stepwise manner (Figure 1.2). The fidelity of this process is as important as cargo sorting and vesicle trafficking, and defects in these final stages of trafficking cause additional severe cellular defects (Olkkonen and Ikonen, 2006). The tethering factor localized at the fusion site is thought to form the initial contact between the vesicle and the target membrane through interactions with Rab and Rho/Ral GTPases on one or both membranes. These prenylated small proteins interact with either GTP or GDP; GTP-bound GTPases are associated with the membrane, and therefore function as a “switch” for membrane recruitment of downstream effectors. Activation of the GTPase, and subsequent cleavage of GTP into GDP, results in the removal of the GTPase from the membrane (Segev, 2011). In order to facilitate another round of membrane binding, guanine nucleotide exchange factors (GEFs) exchange the non-functional GDP for a new GTP molecule; this process can be inhibited by GDP dissociation inhibitors (GDIs). This initial interaction bridges the distance between the vesicle and the target membrane and allows the SNARE proteins to engage, which is sufficient to drive membrane fusion and

![Figure 1.2 - Steps of vesicle fusion, depicted for exocytic trafficking. A vesicle arrives at its site of fusion, and first contacts the tethering complexes. The SNARE proteins then engage, which is sufficient to drive membrane fusion. During this process, the SM proteins interact with both the tethering complex and the SNARE proteins/SNARE complex in a variety of ways depending on the trafficking step. The interactions that occur between each of these protein families during this process are specific to each trafficking step, and contribute to the overall regulation of the spatial and temporal delivery of vesicles.](image_url)
release the contents of the vesicle (Jahn and Scheller, 2006). Prior to SNARE assembly, the SM protein family functions to regulate SNARE assembly through a generally uncharacterized mechanism (Südhof and Rothman, 2009). Each trafficking step requires specific members of these conserved protein families, and conserved interactions between these families (described in detail below) contribute to the overall regulation of vesicle fusion (Bonifacino and Glick, 2004).
**SNARE Complexes**

The SNARE complex is a stable four-helix bundle that is the core of the membrane fusion apparatus (Jahn and Scheller, 2006). Formation of this stable complex is sufficient to overcome the energy barrier to membrane fusion, and has been shown to be sufficient for membrane fusion in simplified *in vitro* systems (van den Bogaart et al., 2010; Weber et al., 1998). These proteins are conserved through higher eukaryotes and required for all stages of membrane trafficking through the secretory pathway in all organisms. Mutations in individual SNARE proteins, changes in SNARE protein expression levels, and changes in SNARE complex assembly all result in various cellular and organismal defects from yeast through humans (Garcia-Reitböck et al., 2010; Johnson et al., 2008; Kama et al., 2011).

The various SNARE proteins from each trafficking step are identified by their “SNARE motif”, a characteristic coiled-coil heptad repeat of hydrophobic residues generally followed by a transmembrane domain (Rossetto et al., 1994). Many SNARE proteins also contain N-terminal domains that provide additional functions; these domains can slow or inhibit formation of the SNARE complex, bind critical interaction partners, or perform other not-yet-characterized actions (Jahn and Scheller, 2006).

For the majority of trafficking steps, SNARE assembly proceeds in two stages as shown in Figure 1.2. First, the SNAREs localized on the target membrane (t-SNAREs) assemble into an “acceptor” complex. Then, the vesicle-bound SNARE proteins (v-SNAREs) “zip” into the acceptor complex from N-terminus to C-terminus (Fasshauer, 2003). Each of the helices provide one ionic residue at the center of the assembled four-
helix bundle: three glutamine residues from the t-SNAREs, and one arginine residue from the v-SNARE (Sutton et al., 1998). These charged residues at the center of an otherwise hydrophobic complex core are required for complex stability; loss of the glutamine residues, or rotation of the charges, results in destabilized or non-fusogenic complexes (Katz and Brennwald, 2000; Ossig et al., 2000; Scales et al., 2000). The assembled SNARE complex requires the ATPase activity of the Sec17 and Sec18 complex (α-SNAP and NSF in mammalian systems, respectively) for disassembly (Hayashi et al., 1994; Söllner et al., 1993a). Due to the energetic favorability of assembling the SNARE complex and the stability of the assembled complex, regulation of vesicle fusion necessitates the regulation of SNARE complex assembly.

In exocytosis, the yeast t-SNAREs Sso1/2 (syntaxin family in mammals) and Sec9 (SNAP-25 family in mammals) form a binary acceptor complex that binds the v-SNAREs Snc1/2 (VAMP/synaptobrevin family in mammals) to drive membrane fusion (Aalto et al., 1993; Brennwald et al., 1994; Gerst et al., 1992; Söllner et al., 1993b; Weber et al., 1998) (Figure 1.3). Prior to
complex formation, both Sec9 and Snc2
are intrinsically disordered proteins (IDPs)
and fold into their helical SNARE
conformations during assembly of the
SNARE complex (Fasshauer et al., 1997;
Nicholson et al., 1998; Rice et al., 1997).

When the SNARE proteins were
first identified, it was hypothesized that
SNARE pairing was sufficient to encode
the specificity required for membrane
fusion (Söllner et al., 1993b). However,
several lines of evidence indicate SNAREs are not the sole specificity determinants and
that specific control of SNARE complex assembly is required to prevent the formation of premature, mislocalized, or non-specific SNARE complexes that may result in the
correct delivery of important cargo. First, yeast SNAREs can form fusion-competent
complexes promiscuously in vitro (McNew et al., 2000). Vesicles containing the exocytic
Sso1:Sec9 t-SNARE complex can fuse with vesicles containing v-SNAREs other than
Snc1/2, and vesicles loaded with the vacuolar t-SNARE complex (containing Vam3,
Vti1, and Vam7) can fuse with vesicles containing non-vacuolar v-SNAREs including the
exocytic/endocytic Snc2 (Izawa et al., 2012). This promiscuity extends to mammalian
cells – SNAREs from various intracellular compartments can form stable non-cognate
SNARE complexes in vitro (Fasshauer et al., 1999; Hohenstein and Roche, 2001; Yang et
al., 1999). Secondly, at least in exocytosis, protein localization is not sufficient to promote specific SNARE pairing. The exocytic t-SNAREs Sec9 and Sso1 are not restricted to the yeast bud tips and mother-daughter necks, where secretion is occurring, but rather are distributed along the plasma membrane (Brennwald et al., 1994) (Figure 1.4). One example of regulation comes from Sso1; like many syntaxins, Sso1 contains an autoinhibitory domain that can prevent premature binary Sso1:Sec9 complex assembly (Dietrich et al., 2003; Weimbs et al., 1997). However, this autoinhibition is not absolute because SNARE complex assembly can proceed in vitro, without the addition of putative “opening” factors, albeit at non-physiological rates (Munson et al., 2000; Nicholson et al., 1998). Similarly, the mammalian exocytic SNAREs can form non-fusogenic SNARE complexes at the Golgi if not inhibited prior to trafficking to the cell surface (Medine et al., 2007). Therefore, other levels of activation and/or inhibition are necessary to prevent inappropriate complex formation and subsequent vesicle fusion. These are likely provided by the protein families that are properly localized at sites of secretion, such as the SM proteins and the tethering factors.
SNARE Regulatory Proteins

Sec1/Munc18 (SM) Proteins

The Sec1/Munc18 (SM) protein family members function as SNARE regulators. These proteins interact with SNARE proteins or SNARE complexes in 4 distinct modes – through interactions with the autoinhibited conformation of syntaxin, an N-terminal peptide found on the N-terminal regulatory domain of syntaxin, the fully assembled SNARE complex, and the SNARE domain of the v-SNARE (Baker et al., 2015; Carr and Rizo, 2010; Furgason et al., 2009; Toonen and Verhage, 2007) and references therein. However, despite years of careful study, the exact mechanism of action for the SM proteins remains elusive; it is not clear whether they primarily function to stabilize individual SNARE proteins, promote the formation of cognate SNARE complexes, stabilize properly assembled complexes, help stimulate membrane fusion after SNARE assembly, prevent formation of non-cognate SNARE complexes, or some combination of all of these roles.

The SM proteins were identified in both the worm Caenorhabditis elegans and in the earliest secretory screen in yeast; however, it wasn’t until later that these proteins were found to be part of a related family required for synaptic transmission and various intracellular trafficking pathways (Aalto et al., 1992; Hosono et al., 1992; Novick et al., 1980). Once the yeast exocytic homologue Sec1 was cloned and studied in vitro, it was found to bind fully assembled exocytic SNARE complexes and increase the rate of fusion of liposomes reconstituted with exocytic SNARE proteins (Carr et al., 1999; Scott et al., 2004). However, mechanistic insight into how Sec1 promotes this increased fusion rate
has been difficult to obtain. Therefore, genetic and biochemical binding information has provided the bulk of the mechanistic analysis to date.

There are lines of evidence of specific protein-protein and genetic interactions for each SM family member; however, they have not yet revealed a common mechanism of action, or a thorough understanding of how the family promotes SNARE assembly. Mutational analysis of the exocytic SM protein Sec1 suggests that it functions both before and after SNARE complex assembly. One class of mutations affects the ability of SNARE complexes to assemble prior to vesicle fusion, while a second class affects binding to the assembled SNARE complex. Both classes cause growth and secretion defects (Hashizume et al., 2009). The neuronal Sec1 homologue, Munc18, binds the autoinhibited conformation of its syntaxin and stabilizes that conformation; it then binds to partially assembled SNARE complexes to mediate vesicle priming prior to fusion, a neuronal-specific adaptation for fast vesicle release (Deák et al., 2009). Vps45, the SM protein involved in pre-vacuolar/endosomal trafficking, directly regulates the protein levels of its cognate syntaxin Tlg2. Deletion of Vps45 results in reduced protein levels of Tlg2, which in turn results in decreased levels of assembled SNARE complexes (Bryant and James, 2001). Additionally, it promotes formation of SNARE complexes by relieving the inhibition of the N-terminal autoinhibitory domain of the mammalian Tlg2 homologue (Struthers et al., 2009). Sly1 and Vps33 (the yeast SM proteins required for Golgi and endo-vacuolar fusion, respectively) work together with the Sec17/Sec18 disassembly machinery to protect cognate SNARE complexes from disassembly prior to fusion, resulting in an increased level of assembled SNAREs (Lobingier et al., 2014).
Vps33 has also been shown to interact with both the t-SNARE Vam3 and the v-SNARE Nyv1 (Lobingier and Merz, 2012; Sato et al., 2000). However, the most compelling mechanistic evidence of SM protein function was published earlier this year, based on a pair of crystal structures – if Nyv1 and Vam3 interact with Vps33 simultaneously, the SNARE proteins would be correctly aligned for initial zippering of the SNARE complex up to the ionic layer (Baker et al., 2015). Most of the SM proteins also interact with their cognate tethering complexes, either as a transient interaction or as a stoichiometric component of the intact complex (discussed more below). Therefore, the SM proteins are crucial positive regulators of SNARE assembly, although it remains to be determined whether they function by the same mechanism.

**Lethal giant larvae (Lgl) proteins Sro7 and Sro77**

The lethal giant larvae (Lgl) family are another class of SNARE regulatory proteins that were originally identified as tumor suppressors in Drosophila melanogaster, and later in mammalian cells where they contributed to establishing cell polarity (Gateff, 1978; Manfruelli et al., 1996). Members of this family have been shown to interact with both the assembled SNARE complex and the t-SNARE Sec9 in yeast (Lehmberg et al., 1999). In mammalian cells, they interact with the t-SNARE syntaxin and the partially assembled “acceptor” SNARE complex (Fujita et al., 1998). While the overall mode of these interactions are different, they serve a similar function – slow down or prevent SNARE complex assembly, either by sequestering one of the t-SNARE components or competing with the v-SNARE for binding (Ashery et al., 2009; Hattendorf et al., 2007).
However, as the deletion of these proteins in yeast is not lethal, it is unclear if this is an essential function in polarized exocytosis (Lehmberg et al., 1999).

The Sro7/77 proteins in yeast physically interact with other members of the late secretory pathway including the vesicle-associated GTPase Sec4, the class V myosin motor Myo2, and the exocyst tethering complex component Exo84 (Gangar et al., 2005; Grosshans et al., 2006; Rossi and Brennwald, 2011; Watson et al., 2015; Zhang et al., 2005). The interaction with Sec4 has been implicated in vesicle clustering prior to vesicle associate with the plasma membrane, suggesting a role in tethering, while the interaction with Myo2 has been implicated in recruiting Sro7 to vesicles; however these roles are in contrast to the localization of the protein, which is primarily membrane peripheral similar to Sso1 (Lehmberg et al., 1999). Additionally, deletion of Sro7/77 results in a change in vesicle cargo, suggesting a role in cargo sorting at the Golgi, which also does not agree with the primary protein localization (Forsmark et al., 2011; Wadskog et al., 2006). Therefore, while the role of Sro7/77 as a SNARE regulator seems relatively established in vitro, it is unclear what the primary function of these proteins are in vivo.
Tethering Complexes

The Multisubunit Tethering Complexes (MTCs) are proposed to promote the initial interaction between a vesicle and its target membrane via interactions with lipids and small GTPases on both membranes. Most stages of trafficking are associated with a tethering complex (Figure 1.5), and while these complexes differ in overall subunit composition and number, they each interact with the same families of proteins – small GTPases, SM proteins, and either SNARE proteins or SNARE complexes. Many of these tethering complex also interact with the specific GEF for their GTPases. Additionally, these complexes have been shown to generally function upstream of SNARE assembly, and therefore upstream of vesicle fusion (Bröcker et al., 2010).

Despite the differences in subunit number and general complex architecture, a subset of tethering complexes has been defined based on the secondary structure of the individual subunits - the Complexes Associated with Tethering Containing Helical Rods (CATCHR) family (Yu and Hughson, 2010). As the name suggests, these complexes contain proteins that are either shown or predicted to be composed of helical bundles, suggesting divergent evolution. The remaining complexes have a more diverse subunit

Figure 1.5 - Overview of each tethering complex in budding yeast, and where each complex acts in the trafficking pathways
composition, and are therefore classified as “non-CATCHR” complexes; however, they still share many of the same protein family interactions, suggesting commonalities across all of the complexes.

**Transport Protein Particle (TRAPP) Complexes**

The TRAPP complexes, of which there are three identified in yeast, are putative tethering factors that function in ER to Golgi transport (TRAPPI), intra-Golgi trafficking (TRAPPII), and autophagosome formation (TRAPPIII) (Barrowman et al., 2010), and references therein). The best evidence of tethering for this complex is that ER-derived vesicles do not associate or fuse with Golgi membranes *in vitro* in the absence of whole cell lysate containing the TRAPPI subunit Bet3 (Barrowman et al., 2000). However, as this whole cell lysate may have provided additional required factors, this is not conclusive evidence of tethering by the complex.

The TRAPP complexes share the least similarity with the other tethering complexes based on their interacting partners. They have been shown to interact with coat proteins and have specific subunits that differentiate ER-derived COPII coated vesicles from the Golgi-derived COPI coated vesicles (Sacher et al., 2001). Additionally, TRAPPI and TRAPPII can function as a GEF for Ypt1, a small Rab GTPase found on ER-derived vesicles and required for fusion with the Golgi (Wang et al., 2000). However, no interaction has been demonstrated between TRAPP and either an SM protein or SNARE proteins/complexes. Therefore, the role of TRAPP complexes as an MTC is the least evident based on its interacting partners.
**Homotypic Fusion and Vacuolar Protein Sorting (HOPS) Complex and Class C Core Vacuolar/Endosomal Tethering (CORVET) Complex**

The HOPS and CORVET complexes are required for early endosomal homotypic fusion, early to late endosomal fusion, and vacuolar/lysosomal fusion. As these subcellular compartments can be purified, as well as the tethering complexes associated with them, these are some of the few trafficking steps that have been fully re-constituted *in vitro* and are therefore well-characterized (Balderhaar et al., 2013; Conradt et al., 1992; Haas, 1995; Stroupe et al., 2006). They are also the only tethering factors that have been shown to be *bona fide* tethers.

HOPS and CORVET are unique amongst MTCs because they are composed of a shared core of four subunits; each has two additional unique subunits that promote binding to specific Rab family GTPases. HOPS interacts with the Rab7 homologue Ypt7, and primarily promotes fusion with the Ypt7-positive lysosomal/vacuolar compartment (Price et al., 2000; Seals et al., 2000). CORVET interacts with the Rab5 homolog Vps21, and primarily promotes early endosomal fusion and fusion between the early and late endosomes, both of which are Vps21/Rab5 positive (Peplowska et al., 2007). In addition, unlike other tethering factors, HOPS/CORVET incorporate an SM protein (Vps33) into the complex rather than recruiting it when needed (Seals et al., 2000). It is through this subunit that many of the SNARE interactions occur (discussed more below). Another common interaction that HOPS shares with other tethering factors is between the tether and the GEF for its Rab GTPase. To this end, one of the HOPS-specific subunits has been suggested to function as a GEF for Ypt7 in yeast, and the HOPS complex has been shown
to interact with the Ccz1/Mon1 GEF dimer (Nordmann et al., 2010; Wurmser et al., 2000); however, in mammalian systems no evidence of GEF activity has been shown for the HOPS complex (Peralta et al., 2010). These interaction similarities suggest that HOPS shares conserved functions with the other tethering complexes.

**Dependence on Sly1 (Dsl1) Complex**

The CATCHR family Dsl1 complex is the smallest of all known tethering complexes with only three core subunits – Dsl1, Tip20, and Dsl3/Sec39 (Spang, 2012). It is localized at the ER and is required for specific recognition of COPI coated Golgi-derived vesicles prior to fusion with the ER (Andag et al., 2001; Reilly et al., 2001); mutations in the Tip20 subunit lead to ER-derived COPII vesicles re-fusing with the ER, suggesting that the complex is no longer able to differentiate between COPI and COPII coats (Kamena, 2004).

The Dsl1 complex shares many of the same interactions with other tethering factors, but is lacking one notable interaction - interactions with GTPases on either membrane have not been shown. However, the Dsl1 subunit interacts with the COPI coat of the incoming vesicle, while the Dsl3 and Tip20 subunits interact with SNARE proteins on the target membrane (Zink et al., 2009). Therefore, while the switching action of a GTPase is likely not used to modulate the interaction with the vesicle, the un-coating of the vesicle may provide a similar mechanism for proceeding to SNARE complex assembly (Zink et al., 2009). Additionally, it has been postulated that this un-coating is required to access the v-SNARE, which would help to prevent SNARE complex
assembly prior to specific un-coating of the vesicle (Zink et al., 2009). The complex also interacts with the SM protein Sly1 – the same SM protein that functions in retrograde Golgi trafficking through interactions with the COG complex (Kraynack et al., 2005). Therefore, it participates in a portion of the same interactions that tie together the functionality of the various tethering complexes.

**Golgi Associated Retrograde Protein (GARP) Complex**

GARP is a late-Golgi-localized MTC with subunits that are primarily helical bundles, placing it into the CATCHR family of MTCs (Bonifacino and Hierro, 2011). It was first identified as the three subunit Vps52/53/54 complex in yeast, and is required for protein sorting at the late Golgi (Conibear and Stevens, 2000); a fourth subunit, Vps51, was later identified (Conibear et al., 2003). This complex has also been identified in mammalian cells in two forms – GARP containing the Vps54 subunit, and EARP containing a novel protein syndetin (Schindler et al., 2015). The EARP complex localizes to recycling endosomes rather than the late Golgi, and depletion of the syndetin subunit results in delayed recycling back to the cell surface. Therefore, GARP and EARP are required for various stages of post-endosomal trafficking based on their localization patterns.

As observed for most MTCs, GARP interacts with the Golgi-localized Ypt6 GTPase, and several domains of GARP subunits have been identified as important for interactions with the incoming vesicle; however, the interacting partner(s) on the incoming vesicle have yet to be identified (Siniossoglou, 2005; Siniossoglou and Pelham,
2001). Similarly, no physical interaction has yet been identified between any of the GARP subunits and an SM protein. However, one group identified a synthetic genetic interaction between the Vps53 subunit and Sly1, the SM protein that functions at the Golgi, suggesting that Sly1 may also be involved in Golgi retrograde transport (VanRheenen et al., 2001). GARP also has not been shown to physically interact with any GEFs, but several different studies have identified synthetic genetic interactions in high throughput screens between components of GARP and the Ric1/Rgp1 dimeric GEF for Ypt6 (Costanzo et al., 2010; Hoppins et al., 2011; Tong, 2004).

**Conserved Oligomeric Golgi (COG) Complex**

The COG complex is the MTC necessary for retrograde transport between Golgi compartments, and is one of the best studied CATCHR complexes. It is composed of 8 subunits that form two separate “lobes” connected through the COG1 and COG8 subunits (Willett et al., 2013b, and references therein). Unlike other members of the CATCHR family, work in cell-free systems suggested that COG can tether vesicles prior to fusion (Cottam et al., 2013). Additionally, COG has been shown to be required upstream of vesicle fusion: depletion of various COG subunits results in a build-up of vesicles at the Golgi (Wuestehube et al., 1996; Zolov and Lupashin, 2005).

To provide its tethering function, COG has been shown to interact with small GTPases on both the vesicle membrane and target membrane. In yeast, these interactions are limited to two Golgi-associated Rab-family GTPases (Suvorova et al., 2002; Yu et al., 2008). Mammalian COG interacts with a more extensive suite of Rab family GTPases,
including the Rab family homologues from yeast (Fukuda et al., 2008; Miller et al., 2013). The COG4 subunit has also been shown to physically interact with the SM protein Sly1 (Laufman et al., 2009), and several COG subunits genetically interact with the GEF for Ypt6, Ric1 (Costanzo et al., 2011; Hoppins et al., 2011; Schuldiner et al., 2005; Tong, 2004). Finally, COG has several conserved interactions with its cognate SNARE proteins and assembled SNARE complexes (discussed more below).

The Exocyst Complex

The exocyst complex is the MTC proposed to recognize and tether secretory vesicles to the plasma membrane (Heider and Munson, 2012) and references therein. The exocyst appears to function prior to SNARE assembly and vesicle fusion, as temperature-sensitive (ts) mutants of individual exocyst subunits result in vesicle accumulation in the bud in yeast (Grote et al., 2000; Novick et al., 1980). In addition, the exocyst has been implicated in other essential membrane trafficking processes such as autophagy, ciliogenesis, and pathogen invasion, likely due to either a “hijacking” or relocalization of the exocyst’s putative tethering function (Bodemann et al., 2011; Farré and Subramani, 2011; Nichols and Casanova, 2010). Although there is no high resolution structure of the entire complex, structures of domains of the individual exocyst subunits Sec6 (Sivaram et al., 2006), Sec15 (Wu et al., 2005), Exo70, and Exo84 (Dong et al., 2005; Hamburger et al., 2006; Moore et al., 2007) reveal that they are composed of evolutionarily conserved helical bundles, placing it into the CATCHR family. The remaining subunits are predicted to have similar structures (Croteau et al., 2009).
Consistent with a putative upstream tethering role, three of the eight exocyst subunits interact with lipids and small Rab and Rho family GTPases on the opposing membranes, although tethering has not yet been directly demonstrated (Adamo et al., 1999; Baek et al., 2010; Brunet and Sacher, 2014; Guo et al., 1999; He et al., 2007; Wu et al., 2010; Yamashita et al., 2010; Zhang et al., 2001; 2008). The exocyst also interacts with the SM protein Sec1 through its Sec6 subunit (Morgera et al., 2012; Wiederkehr et al., 2004). Currently, no loss-of-binding mutants exist to test the function of the Sec1:Sec6 interaction in vivo, but work in our lab and others suggests that this interaction takes place in the context of the whole exocyst complex (Morgera et al., 2012; Wiederkehr et al., 2004). The exocyst also interacts with Sec2, the GEF for the vesicle-specific GTPase Sec4, through its Sec15 subunit (Medkova et al., 2006). Finally, Sec6 has been shown to interact with both the t-SNARE Sec9 and the v-SNARE Snc2 (Shen et al., 2013; Sivaram et al., 2005). Together, these indicate that the exocyst participates in the same set of interactions common to almost all tethering complexes, as detailed above.
Tethering Complex-SNARE Complex Interactions

Interactions between tethering factors and SNARE complexes have been observed for almost all trafficking steps, and generally serve to promote formation of proper and stable SNARE complexes (Hong and Lev, 2014). However, only a few of these steps have been studied in enough detail to begin to understand the mechanism by which these interactions regulate SNARE complex assembly (summarized in Table 1.1). Work on well-established systems such as HOPS and COG suggests a common mechanism of action for the tether-SNARE interactions, despite the low structural similarities between CATCHR and non-CATCHR tethering factors.

The HOPS complex interacts with many of the individual SNARE proteins, and the assembled SNARE complex, required for endosomal/lysosomal fusion (Krämer and Ungermann, 2011; Lobingier and Merz, 2012; Lürick et al., 2015; Stroupe et al., 2006). These interactions are important for several levels of regulation. First, HOPS recruitment by the t-SNARE Vam7 maintains proper HOPS localization at sites of vacuole fusion, and loss of Vam7 membrane binding results in reduced HOPS enrichment; this suggests that SNARE proteins enrich HOPS at sites of vacuole fusion (Wang et al., 2003).

Secondly, HOPS is required to recruit the t-SNARE Vam7 to sites of fusion after disassembly of post-fusion SNARE complexes; these two interactions together result in a positive feedback enrichment of the various fusion machinery (Zick and Wickner, 2013). Thirdly, HOPS competes with the disassembly machinery (Sec17/Sec18) for binding to the assembled SNARE complex and preferentially binds to trans-SNARE complexes; this protects the pre-fusion trans-SNARE complex from disassembly (Collins and Wickner,
2007; Collins et al., 2005; Xu et al., 2010). Finally, HOPS has a higher affinity for properly-formed SNARE complexes than for non-cognate SNARE complexes, suggesting that it may "proofread" the SNARE complex prior to fusion (Starai et al., 2008). This suggests that HOPS is important in all stages of SNARE assembly.

Although less well characterized, COG:SNARE interactions operate using similar mechanisms of SNARE regulation as the HOPS:SNARE interactions (Kudlyk et al., 2012; Laufman et al., 2009; 2011; 2013; Shestakova et al., 2007; Suvorova et al., 2002; Willett et al., 2013a). Knockdown of individual COG subunits in mammalian cells leads to an increase in uncomplexed SNAREs, changes in SNARE localization, and a decrease in overall SNARE stability (Fotso et al., 2005; Oka et al., 2004; Shestakova et al., 2007). Re-localization of the COG complex results in a relocalization of Golgi-destined vesicles, suggesting that a properly localized COG complex is required to recruit the proper vesicles to their sites of fusion (Willett et al., 2013a). Interestingly, COG has a higher affinity for the assembled SNARE complex than the individual SNAREs, suggesting a mechanism of protection similar to that of HOPS (Shestakova et al., 2007). Also similar to HOPS, regulation of SNARE pairing relies on interactions with the SM protein Sly1 (Laufman et al., 2009; Willett et al., 2013a); the Vps33 SM subunit of HOPS mediates many of the HOPS:SNARE interactions (Baker et al., 2015; Lobingier and Merz, 2012; Lürick et al., 2015).

While the mechanisms have not been as thoroughly tested for the remaining tethering complexes, they all have been shown to interact with their cognate SNAREs with the exception of the TRAPP complexes. CORVET interacts with
endosomal/vacuolar SNARE proteins and SNARE complexes through the Vps33 subunit it shares with HOPS (Balderhaar et al., 2013; Subramanian et al., 2004). The Dsl1 complex interacts with t-SNARE proteins on the ER, potentially in lieu of interactions with small GTPases, and functional Dsl1 subunits are required for formation of the assembled t-SNARE complex (Kraynack et al., 2005; Meiringer et al., 2011). Finally, the GARP complex interacts with the N-terminal regulatory domain of the syntaxin homolog Tlg1, as well as the SNARE domains of several mammalian Golgi SNAREs. The mammalian GARP homologue also interacts with the assembled SNARE complex, and SNARE assembly is reduced when GARP is depleted (Conibear et al., 2003; Pérez-Victoria and Bonifacino, 2009; Siniosoglou and Pelham, 2001; 2002). Together, these interactions suggest an overall positive role for the tether-SNARE interactions in promoting proper SNARE assembly prior to vesicle fusion.

The outlier to this model for tether-SNARE interactions was the exocyst complex. The exocyst subunit Sec6 interacts with two SNARE proteins – the t-SNARE Sec9, and the v-SNARE Snc2. The Snc2 interaction may function in recruitment of the exocyst to sites of secretion, as mutants in Snc2 which disrupt the interaction with Sec6 result in a mild depolarization of the exocyst complex, and a general growth defect (Shen et al., 2013). The interaction with Sec9, however, was suggested to be a negative regulator of SNARE assembly in vitro (Sivaram et al., 2005); this was in direct contrast to all other known tether:SNARE interactions, and meant the exocyst did not fit into the emerging model of tether:SNARE interactions. I therefore sought to dissect the seemingly
inhibitory Sec6:Sec9 interaction, and determine if reducing the rate of SNARE complex assembly is the function of this interaction in vivo.

<table>
<thead>
<tr>
<th>Tethering Complex</th>
<th>SNARE Interaction</th>
<th>Function</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td>HOPS</td>
<td>Vam7 (t-SNARE)</td>
<td>Promotes HOPS/SNARE localization</td>
<td>Wang et al., 2003</td>
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<tr>
<td></td>
<td>Vam3 (t-SNARE)</td>
<td>Promotes vacuolar fusion</td>
<td>Zick and Wickner, 2013</td>
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<td></td>
<td>Properly assembled SNARE complexes</td>
<td>Protection from disassembly</td>
<td>Saita et al., 2008</td>
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<td></td>
<td>Pre-fusion SNARE complexes</td>
<td>Protection from disassembly</td>
<td>Collins et al., 2005</td>
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<td></td>
<td></td>
<td></td>
<td>Xue et al., 2010</td>
</tr>
<tr>
<td>CORVET</td>
<td>Pep12 (t-SNARE)</td>
<td>Unknown function</td>
<td>Subramanian et al., 2004</td>
</tr>
<tr>
<td></td>
<td>Assembled SNARE complexes</td>
<td>Unknown function</td>
<td>Balderhaar et al., 2013</td>
</tr>
<tr>
<td>Dsl1</td>
<td>Unknown partner</td>
<td>Promotes SNARE assembly</td>
<td>Meiring et al., 2011</td>
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<tr>
<td></td>
<td>Sec22 (v-SNARE)</td>
<td>ER Localization?</td>
<td>Kräusnack et al., 2005</td>
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<td></td>
<td>Sec20 (t-SNARE)</td>
<td>ER Localization?</td>
<td>Kräusnack et al., 2005</td>
</tr>
<tr>
<td>GARP</td>
<td>Unknown partner</td>
<td>Promotes SNARE assembly</td>
<td>Pérez-Victoria and Bonifacino, 2009</td>
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<tr>
<td></td>
<td>Tlg1 (t-SNARE)</td>
<td>Unknown function</td>
<td>Conibear et al., 2003</td>
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<td></td>
<td>SNARE domains of various SNARE proteins</td>
<td>Unknown function</td>
<td>Simossiouglou and Pelham, 2001</td>
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<td></td>
<td>Assembled SNARE complexes</td>
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<td>Pérez-Victoria and Bonifacino, 2009</td>
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<td></td>
<td>Simossiouglou and Pelham, 2001</td>
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<tr>
<td>COG</td>
<td>Unknown partner</td>
<td>Stabilizes SNARE proteins/protein levels</td>
<td>Futos et al., 2005</td>
</tr>
<tr>
<td></td>
<td>Sed5 (t-SNARE)</td>
<td>Stabilizes assembled SNARE complexes</td>
<td>Shestakova et al., 2007</td>
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<tr>
<td></td>
<td>Unknown partner</td>
<td>Promotes proper SNARE localization</td>
<td>Oka et al., 2004</td>
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<td></td>
<td>Assembled SNARE complexes</td>
<td>Protection from disassembly?</td>
<td>Willett et al., 2013a</td>
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<td>Shestakova et al., 2007</td>
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<tr>
<td>Exocyst</td>
<td>Snc2</td>
<td>Promotes exocyst polarization</td>
<td>Shen et al., 2015</td>
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<td></td>
<td>Sec9</td>
<td>Reduces rate of SNARE assembly</td>
<td>Sivanam et al., 2005</td>
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Table 1.1 – Summary of tether/SNARE interactions. The functional implications of the various tether-SNARE interactions in yeast and mammalian cells are summarized above. Anywhere “unknown” is present in the SNARE interaction field, it represents a functional consequence on the SNAREs without a known binding partner. Where the function is “unknown,” it means a binding interaction has been identified but not characterized.
CHAPTER II: THE EXOCYST SUBUNIT SEC6

INTERACTS WITH ASSEMBLED EXOCYTIC SNARE COMPLEXES
Significant Background and Experimental Rationale

Our lab previously showed that the yeast exocyst subunit Sec6 interacts with the C-terminal SNAP-25 domain of the plasma membrane t-SNARE Sec9 (Sec9CT; residues 414-651). This interaction was identified by an in vitro pull-down using GST-Sec9 and purified full-length Sec6 (Sivaram et al., 2005). Subsequently, we showed that the interaction also occurs in whole cell lysate by a co-precipitation assay using an HA-tagged Sec6 construct and both endogenous and over-expressed levels of Sec9. Additional lysate gel filtration experiments suggested that the Sec6:Sec9 interaction takes place outside the assembled exocyst complex, as the majority of the Sec9 identified was found at a lower molecular weight than the exocyst (Morgera et al., 2012).

The identification of this binary interaction led to the hypothesis that the exocyst, like other MTCs, participates in assembly of the exocytic SNARE complex. As discussed in Chapter I, the assembly of the SNARE complex proceeds in two stages – the t-SNAREs assemble into a binary “acceptor” complex, and the v-SNARE is then incorporated upon vesicle arrival to provide the energy for membrane fusion. In budding yeast exocytic trafficking, the t-SNARE Sso1 adopts an auto-inhibited conformation prior to binary complex assembly; this results in in vitro assays reporting a non-physiological rate of assembly, with complete complex assembly taking 72+ hours (Munson and Hughson, 2002; Munson et al., 2000; Nicholson et al., 1998). Our lab and others have proposed the presence of an opening factor that would allow assembly to proceed at a more physiologically relevant rate, and upon discovery of the Sec6:Sec9 interaction it was proposed that Sec6 may provide that function. However, when the effect of Sec6 was
tested on the rate of SNARE assembly Sec6 appeared to inhibit, rather than promote or stabilize, in vitro formation of the Sso1:Sec9 binary SNARE complex - the rate of assembly, as measured by an in vitro gel filtration assembly assay, was four-fold slower in the presence of Sec6 (Sivaram et al., 2005). Therefore, our lab proposed that the Sec6:Sec9 interaction prevented premature assembly of the Sso1:Sec9 SNARE complex, and that assembly of the exocyst complex would recruit Sec6 and release Sec9 to form fusogenic SNARE complexes. In order to test these hypotheses, I sought to disrupt the Sec6:Sec9 interaction through mutagenesis of specific binding site residues. These residues were selected to avoid disrupting other critical protein-protein interactions that Sec6 or Sec9 participate in, and the mutant proteins were then used to test the importance of this interaction in vivo.

Prior to formation of the assembled SNARE complex both Sec9 and Snc2 (the v-SNARE for exocytosis) are intrinsically disordered proteins (IDPs), and adopt their helical conformations upon complex formation (Fasshauer et al., 1997; Nicholson et al., 1998; Rice et al., 1997). IDPs are an intriguing challenge for biology – it is becoming increasingly apparent that many important regulatory proteins contain some degree of disordered sequence, yet current structural biology methods that rely on detection of static structures are often unable to capture the binding state of these proteins (Jurneczko et al., 2012; Tompa and Fuxreiter, 2008; Wright and Dyson, 2015). Additionally, due their inherent binding “fuzziness,” individual mutations are often unable to disrupt the native binding state of an ordered-disordered protein interaction as the disordered protein may use multiple low-affinity binding interactions in lieu of one tight binding site. This
makes it difficult to use systematic mutagenesis approaches to identify the critical binding residues (Uversky, 2002; 2013b; 2013a; Wright and Dyson, 2015).

Therefore, because Sec9 is an IDP, the process of identifying the critical residues for the Sec6:Sec9 interaction was not straightforward. Additionally, previous studies to identify a minimal region of Sec9 necessary for the interaction with Sec6 found that the linker region between the two SNARE domains was required; further characterization, including limited proteolysis and truncation experiments, was hindered by the protease sensitivity of Sec9 (Sivaram et al., 2005). Identification of the minimal binding region of Sec6 was similarly hindered, this time by the insolubility of the N-terminal domain of Sec6. While the C-terminal domain is fully soluble, and has a solved crystal structure (Sivaram et al., 2006), it was shown to be insufficient for binding; the insolubility of the N-terminal domain meant it was not possible to determine if that region was sufficient for binding (Sivaram et al., 2005).

I therefore chose to use a chemical cross-linking and tandem mass spectrometry approach to define residues necessary for the Sec6:Sec9 interaction. This would allow for identification of specific binding site residues, and also provide information about how an ordered partner interacts with an IDP in the presence of a crosslinker. These experiments highlighted a patch of residues in the linker region of Sec9 that, when mutated, caused a mild exocytic trafficking defect. These residues are not necessary for the Sec6:Sec9 direct interaction, but are required for a novel Sec6:SNARE complex interaction. The identification of this interaction required re-evaluation of the existing inhibition of SNARE assembly data, and I found that the previous data were misinterpreted - Sec6
does not reduce the rate of SNARE assembly to the extent previously observed. This novel interaction, together with the re-evaluation of the SNARE complex assembly data, suggest that the Sec6:Sec9 interaction may serve to positively regulate SNARE complex assembly similar to other MTC:SNARE interactions.
Results

The Sec6:Sec9 interaction has low micromolar affinity

The first step in identifying loss-of-binding mutants for the Sec6:Sec9 interaction was to characterize the wild-type interaction, specifically the dissociation binding constant \(K_d\). Having a precise value would allow us to more precisely design experiments where we needed to use concentrations well above or well below the \(K_d\). Previous attempts to determine this constant relied on gel filtration experiments, and were not able to narrow it down further than the 0.5 – 1.0 µM range (Sivaram et al., 2005). We therefore designed a surface plasmon resonance (SPR) experiment to determine the precise \(K_d\) for the interaction.

The C-terminal SNAP-25 domain of Sec9 was immobilized on a CM5 biacore sensor chip using the cross-linker EDC and stabilizing agent NHS to a final level of 500

Figure 2.1 – Determination of the Sec6:Sec9 \(K_d\) by SPR. Sec9 was immobilized on a CM5 carboxydextran chip, and Sec6 was flowed over at varying concentrations. The RU value just prior to the buffer flow was then fit with a total binding curve containing a variable for linear non-specific binding, and the \(K_d\) was determined from the curve fit. This is the data from three separate flows over the same immobilized Sec9 followed by NaOH regeneration between experiments.
Response Units (RUs). This level of immobilized protein has been previously shown to be sufficient to detect binding (C. Painter, personal communications). Sec6 was then flowed over immobilized Sec9 in three separate experiments, and the saturation level of protein was detected at eight different protein concentrations from 0.1-13 µM (Figure 2.1A). The resulting saturation point for each concentration was fit to a total binding curve with a constant for non-specific binding, and we determined the $K_d$ to be $1.2 \pm 0.2$ µM with a non-specific contribution of 3.6 RUs per µM of Sec6 (Figure 2.1B). These numbers are on the upper edge of the range determined by gel filtration (0.5 – 1.0 µM). There were, however, technical difficulties with the instrument and therefore the experiment could not be repeated with a different chip for confirmation of the calculated values.

**Sec6 specifically cross-links to the IDP Sec9, which identifies potential binding residues**

Once we identified the precise $K_d$ of the interaction, we sought to examine the function of the Sec6:Sec9 interaction *in vivo* by generating specific loss-of-binding mutants in Sec9. Therefore, we needed to identify the residues necessary for the binding interaction. We chose to use the chemical cross-linker EDC followed by mass spectrometry analyses to map this interaction; this would allow us to circumvent difficulties caused by the flexible nature of the IDP Sec9. Chemical cross-linkers create covalent bonds between side chains (usually Lys-Lys or Asp/Glu-Lys) separated by specific distances; EDC reacts with Asp/Glu and Lys residues that are in close proximity.
and creates a peptide bond between the free carboxyl and free amine groups. Purified Sec6 and Sec9 were mixed together, cross-linked by EDC (+/- NHS or Sulfo NHS to stabilize the cross-linker), and a portion of the total reaction was quenched at various time points (1-120 min, depending on the experiment). The reaction from each time point was run on SDS-PAGE and the band corresponding to the 1:1 Sec6:Sec9 complex was excised, trypsinized, and subjected to LC/MS mass spectrometry by varying collision methods. A list of all crosslinks identified is presented in Table 4.1 (Appendix A), along with the experiment in which they were identified and the crosslinking class they were assigned to. The identified cross-linked peptides mapped across the entire length of Sec9 (36 residues out of 63 total Glu/Lys/Asp residues) and Sec6 (68 residues out of 200 total residues).

Figure 2.2 - EDC cross-linking results in a large dataset. All of the cross-links identified in each of the experiments described in the Experimental Procedures were mapped onto the C-terminal structure of Sec6 (aa 411-805) and linear segments representing the N-terminal domain of Sec6 and the SNARE domains of Sec9. Grey boxes indicate helical secondary structure – the known SNARE helices on Sec9, and the predicted helical regions of the Sec6 (aa 1-410) (SOPM algorithm, (Rost et al., 2004)).
Glu/Lys/Asp residues; all Glu/Lys/Asp residues in the Sec6 C-terminal domain are located on the surface (Sivaram et al., 2006), with 152 unique cross-links between them. This large number of cross-links was expected due to the flexible nature of the IDP Sec9. However, only ~50% of the available Asp/Glu/Lys residues on Sec9 and only ~35% of the Asp/Glu/Lys residues on Sec6 were identified as participating in a cross-link (Figure 2.3), indicating that the cross-linking reaction was not random.

Due to the large number of cross-links identified, we sought to limit our analysis to only those likely involved in the binding interaction. Because this experiment was carried out as a time course, we categorized each cross-link based on presence/absence and intensity at different time points (Figure 2.4A). These analyses identified two classes of cross-links: those that had constant intensities over time, and those whose intensities changed over time. The constant intensity cross-links are ones that form early in the
reaction, and those that are more variable form later as cross-links that are secondary to the “early-forming” cross-links (Figure 2.4B). We hypothesized that residues participating in salt bridges in the binding interface are initially protected from EDC; thus, the early-forming cross-links lie outside the core of the protected binding site. After formation of the early cross-links, the now-limited flexibility of the IDP Sec9 and low micromolar affinity of the Sec6:Sec9 interaction will result in disruption of some of the salt bridges, allowing those residues to participate in “late-forming” cross-links. Some residues are capable of participating in both early and late-forming cross-links, likely due to the bulk nature of this assay and the variable accessibility of those residues due to the flexibility of Sec9.

Figure 2.4 - Cross-linking over a time-course results in two classes of cross-linked peptides. A) At each time point in the cross-linking reaction (0-120min, depending on experiment), a portion of the reaction was quenched and the cross-linked peptides identified/quantified by mass spectrometry. The resulting cross-links were grouped into two classes – “early-forming” cross-links (top panel) that form early in the reaction and do not change intensity over time, and “late-forming” cross-links (bottom panel) that increase in intensity over time. B) A model for how an intrinsically disordered protein may interact with a well-folded binding partner over time in the presence of EDC. (1) Sec9 binds to Sec6 and this interaction (red circle) protects binding site residues from EDC. (2) Early-forming cross-links (yellow) form as chemically capable side chains come into contact. (3) Equilibrium dissociation of the local interaction occurs; early forming cross-links (yellow) force the proteins to remain in close proximity despite loss of the original interaction (red). (4) Late forming cross-links (blue) form as chemically capable side chains come into contact through random motion of Sec9– these new cross-links can occur in the binding site (bottom), or outside the binding site (top).
Based on these hypotheses, we focused on residues that participate in late-forming cross-links, specifically those that are located nearby early-forming cross-links (Figure 2.5, red circle). After filtering the data set accordingly, we identified a five amino acid stretch on Sec9 that satisfied the above conditions – the first (Lys-533) and last (Glu-537) residues cross-link to adjacent regions on the Sec6 C-terminal domain (Sec9 Lys-533 to Sec6 Glu-447, Sec9 Glu-537 to Sec6 Lys-516), and these cross-links are nearby early-forming cross-links (Lys-531 and Lys-532 on Sec9). The intervening residues contain a mixture of hydrophobic and electrostatic side chains that are incapable of forming cross-links (Leu-534, Met-535, Arg-536). These residues lie in the middle of the 80-90 residue linker region of Sec9, between the two regions that will fold into the SNARE motif helices upon SNARE assembly; therefore, mutations in these residues will not disrupt the

![Figure 2.5 – Cross-linking time course highlights residues likely necessary for the interaction. Cross-linked residues are colored based on the classes of cross-links (“early-forming”, yellow; “late-forming”, blue; combination residues that participate in both classes, green). Only cross-links between “late-forming” residues are shown. Based on the model in Figure 2., residues likely involved in the binding interaction are circled in red. The sec9-142 mutation is located between two blue residues (Lys-533 and Glu-537) in the circle.](image-url)
stability of the assembled SNARE complex. Additionally, previous studies suggested that this region may be important for the Sec6:Sec9 interaction (Sivaram et al., 2005). To generate a mutant sec9 allele for studying the importance of these residues in the Sec6:Sec9 interaction, the entire stretch was mutated to oppositely charged residues generating the sec9-142 allele (Lys-533 to Glu, Leu-534 to Glu, Met-535 to Glu, Arg-536 to Glu, and Glu-537 to Arg).

**The sec9-142 allele causes growth defects in sensitized backgrounds, but does not disrupt Sec6:Sec9 direct binding**

To test the effect of the sec9-142 allele, we integrated the mutant into the endogenous locus in otherwise wild-type yeast and tested if it could function as the sole copy of SEC9. We predicted that the interaction between Sec6 and Sec9 is necessary, and that disruption of the interaction would result in a growth defect due to disruption of the secretory pathway. After a serial dilution assay to compare the growth of wild-type and sec9-142 yeast at various temperatures (23-37 °C) (WT vs sec9-142; Figure 2.6), we found no observable differences between the two strains. This suggests that the mutant protein is sufficient for growth under otherwise wild-type conditions.

Despite not having a growth defect, the sec9-142 strain may still have a mild disruption in the exocytic pathway that will be exacerbated when combined with other mutations in the secretory pathway. To test for such synthetic effects, we replaced the wild-type SEC9 gene with the sec9-142 allele in temperature sensitive (ts) strains that
Figure 2.6 - The sec9-142 mutant causes synthetic growth defects in combination with late secretory mutants. The sec9-142 mutation was integrated into the genomic SEC9 locus, and tested either alone or in combination with other secretory pathway ts mutants at various temperatures. The sec1-1/sec9-142, sec3-2/sec9-142, sec8-6/sec9-142, and sec15-2/sec9-142 combinations showed synthetic growth defects.

have exocytic defects at non-permissive temperatures: a mutation in an exocytic SNARE regulator (sec1-1), and mutations in individual subunits of the exocyst complex (sec3-2, sec5-24, sec6-4, sec8-6, sec10-2 and sec15-2) (Novick et al., 1980). Each double mutant strain was then tested for growth at various temperatures (23-37 °C). In these sensitized backgrounds, sec9-142 showed synthetic growth defects in combination with sec1-1, sec3-2, sec8-6, and sec15-2 (Figure 2.6). These results revealed that the sec9-142 allele causes a modest exocytosis and cell growth defect.

We then hypothesized that the synthetic effect we observed was due to a disruption of the Sec6:Sec9 interaction, and therefore tested whether these mutations were sufficient to disrupt Sec6:Sec9 direct binding. A low concentration of GST-Sec9 or GST-Sec9-142 (25 nM) was incubated with three different concentrations of Sec6 (1 μM,
0.1 µM, and 0.01 µM), and the extent of binding by a GST pull-down assay was quantified as a fold change over binding to GST alone. We found that Sec6 binds to both GST-Sec9 and GST-Sec9-142 significantly over background at all concentrations tested except 5.00 µM (Figure 2.7), but detected no significant differences in binding between the two Sec9 constructs. This is likely due to the capability of an IDP to bind to an ordered partner with multiple low-affinity binding sites, and our disruption of only one of those sites (Uversky, 2002; 2013b; Wright and Dyson, 2015). Therefore, further tests of this interaction necessitate either generation of more mutants, or testing the Sec9-142 protein in a functional test that requires the Sec6:Sec9 interaction.

**Sec6 does not affect the rate of SNARE assembly in vitro**

While we could detect no significant loss of the Sec6:Sec9 interaction, the function of Sec6 as a SNARE assembly inhibitor may be impacted by the Sec9-142 protein, resulting in the observed synthetic growth defect. To further investigate this, we...
tested the effect of Sec9-142 on SNARE complex assembly in vitro. Our earlier studies had shown a decreased rate of SNARE assembly (~4-fold) as monitored by gel filtration chromatography in the presence of Sec6, as well as reduced levels of assembled SNARE complexes using a native gel mobility shift assay; therefore, if this mutant disrupted the inhibitory function of Sec6, there should be no difference between the conditions with or without Sec6 when combined with the Sec9-142 mutant (Fasshauer et al., 1997; Nicholson et al., 1998; Rice et al., 1997).

To test if the Sec9-142 protein is sufficient to remove the inhibition of Sec6 on SNARE assembly, we adapted the previous native gel mobility shift assay to measure the t-SNARE assembly rate. Previously, these experiments were run on thin, upright, native gels (pH 7.4); due to the lack of a stacking layer, both the SNARE complex band and the uncomplexed Sso1 band were inconsistently resolved. We modified the assay to use a “slab” gel (a thicker, 0.5-1.0 cm polyacrylamide gel polymerized in a horizontal gel box) and lowered the pH of the gel (pH 6.6). These modifications resulted in sharper resolution of the free Sso1 band. The experiment was run with seven time points incubated for up to 72 h, as required by the slow nature of in vitro SNARE assembly with the autoinhibited form of Sso1, and the resulting decreased intensity of the uncomplexed Sso1 bands over time was fit to an integrated second order rate equation (Hayashi et al., 1994; Söllner et al., 1993).

This modified assay was used with various combinations of proteins (Sec9+Sso1, Sec9-142+Sso1, Sec9+Sso1+Sec6, and Sec9-142+Sso1+Sec6) to determine if Sec9-142 affected the rate of SNARE complex assembly. First, we tested if the Sec9-142 mutant
protein has a defect in SNARE assembly by comparing the rates of assembly of the mutant and wild-type Sec9 in the absence of Sec6. After quantification of the free Sso1 band (Figure 2.8A, top left vs. top right panel, and Figure 2.8B, light blue vs. yellow curves), the rate ($k$) of assembly of SNAREs containing Sec9-142 was within two-fold of those containing wild-type Sec9 ($1.7 \pm 0.4 \text{ M}^{-1}\text{s}^{-1}$ for Sec9-142 vs. $0.88 \pm 0.3 \text{ M}^{-1}\text{s}^{-1}$ for Sec9). This difference in rate can be attributed to potential differences in concentration of the two Sec9 constructs. We therefore concluded that Sec9-142 does not have an appreciable effect on the rate of binary SNARE complex assembly. We next tested whether Sec6 was able to inhibit formation of Sec9-142 containing SNARE complexes. Unexpectedly, we did not observe an inhibition of SNARE complex assembly by Sec6 with either Sec9 or Sec9-142, despite a potential decrease in the intensity of the assembled SNARE complex band (Figure 2.8A, bottom left vs. top left panel). When the
loss of uncomplexed Sso1 over time was quantified (Figure 2.8B), there was no appreciable difference between any of the conditions tested (Sec9, \( k = 0.88 \pm 0.1 \, \text{M}^{-1}\text{s}^{-1} \); Sec9-142, \( k = 1.7 \pm 0.4 \, \text{M}^{-1}\text{s}^{-1} \); Sec9+Sec6, \( k = 0.91 \pm 0.3 \, \text{M}^{-1}\text{s}^{-1} \); Sec9-142+Sec6, \( k = 1.4 \pm 0.4 \, \text{M}^{-1}\text{s}^{-1} \)). Curiously, the extent of the reaction appears different in the Sec9+Sec6 condition: at \( t = \infty \), \( \sim30\% \) of Sso1 is calculated to remain unbound when Sec6 is present, while \(<5\% \) of Sso1 is calculated to remain unbound in the other conditions. Therefore, Sec6 does not appear to affect the rate of SNARE assembly, but may be affecting the final equilibrium between the proteins; e.g., SNARE complex assembly may not be able to proceed to completion due to Sec6 sequestering a fraction of Sec9, but not Sec9-142. Several explanations could be responsible for the differences between these results and the previous ones, including the fact that the earlier quantifications were performed on samples analyzed by gel filtration chromatography rather than native gels. To understand these conflicting results, we sought to determine if the differences in experimental design were responsible for the discrepancies, and if one or both experiments were misinterpreted.

**Gel filtration chromatography cannot fully resolve uncomplexed Sec6 and Sso1 peaks**

To examine differences between the gel filtration and native gel SNARE assembly assays, we repeated the gel filtration assay as previously described (McNew et al., 2000). In this assay, we monitored SNARE complex assembly by a loss of the uncomplexed Sso1 A_{280} peak. The free Sso1 peak heights in this experiment appeared
similar to our previous findings ([Figure 2.9A](#), [Izawa et al., 2012](#)). However, when we examined the entirety of the gel filtration profiles, we found that the previous analyses did not account for the contribution of uncomplexed Sec6. Sec6 elutes as a non-symmetrical peak on a gel filtration column, with a right-hand tail, and overlaps with the free Sso1 peak. This causes an increase in the Sso1 peak height when Sec6 and Sso1 are run together compared to Sso1 alone ([Figure 2.9B](#), blue trace vs red trace). When Sec9 is incubated with Sec6 and Sso1, Sec9 binds Sec6 and the Sec6:Sec9 complex migrates as a larger complex that has little to no overlap with the free Sso1 peak ([Figure 2.9B](#), red trace). As SNARE complex assembly proceeds, Sec6 is released and its peak shifts back to the uncomplexed position; this results in increased overlap with the free Sso1 peak ([Figure 2.9B](#), red/orange/yellow traces) and an increase in the apparent Sso1 peak height at later time points. Additionally, due to the propensity of Sec6 to aggregate over time, a fraction of it can be found in a peak that exits near the void volume of the column; Sso1 and Sec9 are also found in this aggregate peak, which affects our ability to quantify the amount of free Sso1. Thus, quantification of Sso1 peak heights in the presence of Sec6 may have previously led to an erroneous interpretation of the rate of binary SNARE assembly.

To circumvent the overlapping peaks problem in the gel filtration experiments, we quantified changes in the amount of free Sso1 in the eluted free peak using protein gels. The three fractions that correspond to the top of the free Sso1 peak from each time point were run on SDS-PAGE gels. All proteins were then detected with high-sensitivity Krypton fluorescent protein stain ([Figure 2.9C](#)) and the free Sso1 band was analyzed to
determine the \( k_{obs} \) for the loss of free Sso1 (Figure 2.9D). This rate is faster than that measured by native gel in Figure 2.8 (0.49 ± 0.4 M\(^{-1}\)s\(^{-1}\) for Sec9, 0.58 ± 0.3 M\(^{-1}\)s\(^{-1}\) for
Sec9+Sec6), but with larger errors. Despite the differences, these data agree that the presence of Sec6 does not appreciably decrease the rate of SNARE complex assembly.

**Sec6 binds both the binary and ternary SNARE complexes, and this interaction is significantly reduced by Sec9-142**

Our results above resolved the difference between our current data and those previously published (Sivaram et al., 2005). However, they did not provide insights into the nature of the Sec6:Sec9 interaction *in vivo*. We therefore tested whether, as observed for other MTC-SNARE complex pairs (Brennwald et al., 1994), Sec6 could bind to binary Sec9:Sso1 or ternary Sec9:Sso1:Snc2 SNARE complexes, and if Sec9-142 was sufficient to disrupt that interaction. As before with the Sec6:Sec9 interaction, we used multiple concentrations of Sec6 (0.05, 0.5, and 5.0 µM) and a low fixed concentration of SNAREs (25 nM), to maximize the signal from the bound complex. At concentrations much higher or much lower than the equilibrium dissociation concentration ($K_d$), the signals will not be significantly different between various conditions due to the absence of binding at low concentrations and increased background binding at high concentrations. At concentrations of Sec6 closer to the $K_d$, differences in signal will be more readily observable. For each binding reaction, the molar ratio of Sec6:GST-protein was normalized to the GST background signal, and all related conditions were tested for significant differences in binding (one-sided ANOVA with multiple comparisons on non-matched data sets).
We chose to test the following constructs for Sec6 binding: GST and GST-Sso1 as negative controls, GST-Sso1:Sec9 and GST-Sso1:Sec9-142 to test binding to the binary SNARE complex, and GST-Sso1:Sec9:Snc2 and GST-Sso1:Sec9-142:Snc2 to test for binding to the ternary SNARE complexes. All Sso1-containing constructs were formed using just the SNARE domain (Sso1-Ctb) to maximize purification of assembled SNARE complexes. As predicted, the binding of Sec6 to SNARE complexes at concentrations ~5-10 fold above (Figure 2.10, 5 µM, left panel) or below (Figure 2.10, 0.05 µM, right panel) the approximate $K_d$ of the Sec6:Sec9 interaction showed no significant difference over background in any of the conditions tested.

When the GST-SNAREs were incubated at concentrations near the $K_d$ (Figure 2.10, 0.5 µM, center panel), the amount of Sec6 bound to SNARE complexes was significantly higher than to GST alone. Sec6 bound both binary GST-Sso1:Sec9 (light green bar) and

![Figure 2.10 - Sec6 binds both the binary and ternary SNARE complex.](image)

Sec6, at the listed concentrations, was incubated with 25 nM GST and GST-SNAREs (GST-Sso1CTb, GST-Sso1CTb:Sec9 or binary SNAREs, GST-Sso1CTb:Sec9:Snc2 or ternary SNAREs). The Sec6 protein that bound to the beads was quantified by densitometry of SDS-PAGE stained with krypton high-sensitivity protein stain. Each experiment is represented as the fold change over the GST-only background binding, and the differences between the pairwise interactions were determined by a one-way ANOVA statistical test on non-matched parametric data with Holm-Sidak correction for multiple comparisons in GraphPad Prism. n=3, graphed as mean plus S.E. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.005, ****p ≤ 0.001.
ternary GST-Sso1:Sec9:Snc2 (light pink bar) SNARE complexes over background (p \leq 0.005). Additionally, there was a significant increase in Sec6 binding to ternary complexes compared to binary complexes, indicating that Sec6 has a greater affinity for the ternary than the binary complex (p \leq 0.005, light green vs. light pink bars). Finally, the binding to binary and ternary SNARE complexes containing Sec9-142 was significantly decreased (p \leq 0.05 for binary SNARE complexes, light green vs. dark green bars, and p \leq 0.001 for ternary SNARE complexes, light pink vs. dark pink bars). Neither binary nor ternary SNARE complexes containing Sec9-142 bound Sec6 significantly over the GST background. These data demonstrate that Sec6 binds both the binary and ternary SNARE complexes. Furthermore, binding of Sec6 to SNARE complexes requires residues in the linker region of Sec9, as the interaction is abrogated in the presence of Sec9-142. Additionally, they demonstrate how the folded and disordered forms of Sec9 vary in their ability to bind Sec6 – while the disordered protein was able to compensate for the loss of the Sec9-142 binding site, the ordered protein in the assembled complex is either no longer able to compensate for those mutated residues, or other potential binding sites are occluded by the assembled complex.
Experimental Procedures

Protein expression and purification

Mutations in recombinant proteins were generated by overlap extension PCR, cloned into the T7-expression vectors pET15b (Sec6 constructs) or pETDuet-1 (Sec9 and Sso1 constructs), and confirmed by sequencing. Full-length yeast Sec6(1-805), Sec9(414-651), and the cytosolic domain of Sso1(1-265) proteins were overexpressed in BL21(DE3) or BL21(DE3) RIL E. coli cells and purified as described (Nicholson et al., 1998; Sivaram et al., 2005). Protein concentrations were determined by a quantitative ninhydrin assay (Rosen, 1957). The C-terminal domain of Sec9 is homologous to the mammalian homolog SNAP-25 and was previously shown to be functional in yeast (Brennwald et al., 1994).

Cross-linking and Protein Digestion

Sec6 and Sec9 were combined 1:1 (7 µM each) in a solution of 10 mM potassium phosphate buffer (pH 7.4), 140 mM KCl, and 4% glycerol and incubated for two hours at room temperature. EDC (1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride; Thermo Scientific) was then added at 1000x molar excess in 5 µL of 10 mM potassium phosphate buffer (pH 7.4). Variations on this reaction were also performed, to improve detection sensitivity: with the addition of either 500x molar excess NHS (N-hydroxysuccinimide; Thermo), or 500x molar excess Sulfo-NHS (N-hydroxysulfosuccinimide; Thermo), to increase the stability of the partially cross-linked complex; and at low concentrations of reactants (1 µM) to detect only early-forming
crosslinks. The reaction proceeded for 15, 30, 60, and 120 min before quenching with Laemmli sample loading buffer (Bio-Rad) and boiling for 10 min. One additional experiment was run at 30 s, 1 min, 5 min, and 10 min to again detect only early-forming crosslinks. Approximately 15 µg of protein from each reaction was loaded and run on a 4-20% Mini-PROTEAN TGX precast SDS gel (Bio-Rad). Bands corresponding to the monomeric Sec6:Sec9 cross-linked complex were excised and destained twice with 200 µL 25 mM ammonium bicarbonate in 50% acetonitrile, reduced in 10 µL of 50 mM DTT for 10 min at 60 °C, then alkylated in 10 µL of 100 mM iodoacetamide for one h in the dark at room temperature. Proteins were digested overnight at 30 °C with 100 ng trypsin (Promega). Following digestion, peptides were transferred into a clean tube, and then further extracted twice with 50% acetonitrile containing 5% (v/v) formic acid. The combined extracts were dried in a SpeedVac and brought to 20 µL with 0.1% (v/v) formic acid for LC-MS/MS.

**LC-MS/MS**

Peptide digests were injected (2 µL) and loaded at 4 µL/min (5% acetonitrile containing 0.1% formic acid) onto a custom packed trap column (100 µm I.D. fused silica with Kasil frit) consisting of 2.0 cm of 200 Å, 5 µm Magic C18AQ particles (Bruker-Michrom) configured to a custom packed analytical column (75 µm I.D. fused silica) packed with 25 cm 100 Å, 5 µm Magic C18AQ particles (Bruker-Michrom) to a gravity-pulled tip. Peptides were separated at 300 nL/min using a Proxeon Easy-nLC (Thermo Scientific) system using a linear gradient from 100% A (5% acetonitrile with 0.1% (v/v)
formic acid) to 35% B (acetonitrile with 0.1% formic acid) in 90 min and eluted directly into an LTQ Orbitrap Velos hybrid mass spectrometer (Thermo Scientific) (Olsen et al., 2009). Data were acquired using a data-dependent acquisition routine of acquiring one mass spectrum from m/z 350-2000 in the Orbitrap (resolution 60,000) followed by tandem mass spectrometry scans of the 10 most abundant precursor ions found in the mass spectrum. Alternate runs collected either collision-induced dissociation (CID) or higher-energy collisional dissociation (HCD) spectra acquired in the Orbitrap mass analyzer. Data-acquisition utilized charge state rejection of singly, doubly, and triply charged ions, and dynamic exclusion was utilized to minimize data redundancy and maximize peptide identification (Olsen et al., 2009).

**Data Analysis**

A concatenated peptide database was generated by xComb (University of Washington) software (Panchaud et al., 2010). The database considered all inter- and intramolecular Sec6 to Sec9 EDC-linked tryptic peptides up to 2 missed cleavages. The raw data was converted to peak lists and searched using the Sequest search engine contained in Proteome Discoverer (Version 1.3; Thermo Scientific). Briefly, no enzyme specificity was considered; parent ion tolerances were set to 15 ppm, fragment ion tolerances were set to 0.05 Da, methionine oxidation was considered as a variable modification, and carbamidomethylation of cysteine was considered as a fixed modification. The peptide results were then filtered by removing hits with an XCorr x Sp product less than 146. Label-free quantitation using peptide extracted ion chromatograms
(XIC) was done using ProteoIQ (Version 2.3.08; NuSep) quantitative analysis software using the replicate method (Radulovic et al., 2004). Cross-linked peptides with a precursor ion intensity of less than $1.0 \times 10^5$ from the mass spectrum in the 120-minute reaction time samples were filtered and removed from the dataset. For the generation of the crosslinking Figures 2.2, 2.3, and 2.5, all experimental designs were combined and examined together to determine the effect of the crosslinker on the Sec6:Sec9 complex.

**Effect of Sec9 mutations in vivo**

The full-length sec9-142 (1-651) mutant gene was cloned into the yeast integration vector pRS306, linearized with EcoRI, and transformed into both BY4741 and BY4742 ((Brachmann et al., 1998); Open Biosystems) using a yeast high efficiency transformation protocol (Gietz and Woods, 2002). The wild-type SEC9 and residuals from the pRS306 plasmid were selected against using 5-fluoroorotic acid (Sikorski and Hieter, 1989), and the mutations were confirmed by sequencing. Double mutant strains containing sec9-142 and exocytic temperature sensitive alleles were generated by mating the above sec9-142 strains to the exocytic temperature sensitive strains stated (Novick et al., 1980), sporulation, and confirmation by temperature sensitivity and sequencing of the sec9 locus. Growth defects were detected by a serial dilution assay, where log phase cultures were diluted to $1.0 \text{ OD}_{600}$ units/mL. This culture was diluted 10-fold over 6 samples, and dilutions were spotted onto plates containing YPD media and incubated for 72 h at the indicated temperature.
SNARE complex assembly monitored by native gel mobility shift assay

Purified recombinant Sso1 (a.a. 1-265) was mixed with the indicated proteins at 10 µM final concentration of each protein in a 15 µL final reaction volume of 10 mM sodium phosphate (NaPhos) pH 7.4, 200 mM sodium chloride (NaCl), 1 mM DTT and incubated at 18 °C for various times (0-72 h). 3 µL of 6x native gel loading dye (0.6% w/v bromocresol green, 30% v/v glycerol, 25 mM histidine, 30 mM MOPS) was added to each reaction in the cold room, and 5 µL was loaded on a 200 mL slab native PAGE gel at pH 6.6 (6.0% acrylamide, 25 mM histidine, 30 mM MOPS, 2.5% w/w glycerol, polymerized with 2.0 mL 10% w/v APS and 200 µL TEMED) that was pre-equilibrated for 1h at 4 °C. The gel was run for 4 h at 100 V at 4 °C in pH 6.6 native gel buffer (25 mM histidine, 30 mM MOPS) (McLellan, 1982), and protein bands visualized by Coomassie blue staining (Wong et al., 2000). The percent free Sso1 was quantified by densitometry (Photoshop, CS5): the density of each band was divided by the Sso1 band from the zero time point. The resulting curve was fit using the derived second-order rate equation (Nicholson et al., 1998) using GraphPad Prism, with Y at t = 0 constrained to 1.0 and Y at t = ∞ constrained to > 0. All four replicates were fit to a global rate constant, k, and presented as +/- the standard error of the fit as reported by GraphPad Prism.

SNARE complex assembly monitored by gel filtration

All proteins were incubated together in 200 µL final volume at 10 µM final concentration in 30 mM NaPhos pH 7.4, 200 mM NaCl, 1 mM DTT for the indicated time (0-72 h), and injected on a Superdex 200 10/30 column (GE LifeSciences) pre-
equilibrated in the same buffer. 200 µL fractions were collected from 0.41-0.71 column volumes (CV) and run on a 10% SDS-PAGE gel. Proteins were visualized by fluorescent Krypton staining (Pierce) and imaged on a Typhoon fluorescence stage with a 532nm laser (GE LifeSciences). Sso1 band intensities of the three fractions corresponding to the top of the A$_{280}$ free Sso1 peak (0.61-0.63 CV) were quantified by densitometry (Photoshop CS5), and each fraction was plotted as a curve across the time course. Since the free Sso1 peak is approximately Gaussian, the resulting curves were fit using the derived second-order rate equation (Nicholson et al., 1998) as described above in GraphPad Prism with each curve fit to a global rate constant with the surrounding fractions (three in total) to control for loading error. A representative fit of two replicate experiments is shown, and the $k$ value is presented as +/- the standard error of the fit as presented by GraphPad Prism.

**Sec6:Sec9 and Sec6:SNARE binding**

GST-Sec9 and GST-Sec9-142 were purified by expression of each construct as previously described (Sivaram et al., 2005). GST-tagged binary and ternary SNARE complexes were purified by mixing *E. coli* lysates containing each of the individual components (Sec9CT, GST-Sso1CTb (residues 179-265), Snc2 (residues 1-92)), expressed as previously described (Nicholson et al., 1998), with a limiting concentration of GST-Sso1CTb. All GST-protein containing lysates were incubated for 1 h at 4 °C with glutathione agarose resin, washed in wash buffer without glutathione (50 mM Tris pH 8.0, 150 mM NaCl, 10% glycerol, 0.5% IPEGAL, 3 mM DTT), and eluted in wash buffer
+ 40mM reduced glutathione. All constructs except GST-Sec9-142 were diluted 2-fold in 10 mM HEPES pH 7.4, loaded onto a MonoQ 10/10 column (GE LifeSciences) pre-equilibrated in 10 mM HEPES pH 7.4 and 100 mM NaCl, and eluted with a gradient from 100 mM – 1 M NaCl. Fractions containing the SNAREs were concentrated and frozen in 10 mM NaPhos pH 7.4, 140 mM NaCl. GST-Sec9-142 was diluted 30x in 10 mM Hepes pH 7.0, 150 mM NaCl, concentrated to 1 mL in an Amicon 10 KDa spin concentrator (Millipore), and frozen in 10 mM NaPhos pH 7.4. The final concentration of the protein was determined by a quantitative ninhydrin assay (Rosen, 1957).

To test for binding to Sec6, 25 nM GST-protein (or GST alone) were incubated with the indicated concentration of Sec6 in binding buffer (30 mM NaPhos pH 7.4, 200 mM NaCl, 5% glycerol, 0.5% IPEGAL, 1 mM DTT) in a final volume of 100 µL for 1 h at 4 °C. 90 µL of the incubated proteins were added to 2 µL of magnetic glutathione resin slurry (Thermo Fischer, 25 % slurry) and incubated for an additional 1 h at 4 °C. The supernatant was removed, and the resin was re-suspended in 1x Laemmli loading buffer before being heated at 95 °C for 5 min. The beads were not washed prior to boiling to maximize the bound protein signal. 10 µL of each sample was loaded on a 12% SDS-PAGE gel, and proteins were visualized by Krypton staining (Pierce) and imaging on a Typhoon fluorescence stage (GE LifeSciences). The extent of binding was measured by calculating the molar Sec6:GST-protein ratio (densitometry of Sec6 divided by the densitometry of the GST-protein, normalized for protein size), and dividing by the Sec6:GST molar ratio to normalize by background binding. Each bar represents three replicates ± standard error, and statistical significance was calculated using an ordinary
one-way ANOVA test with multiple comparisons corrected for by Holm-Sidak’s multiple comparisons test (GraphPad Prism).
CHAPTER III: DISCUSSION
Scientific Questions

My studies sought to understand how the exocyst and its interaction with the SNARE protein Sec9 contribute to the regulation of SNARE complex assembly and vesicle fusion. Additionally, in a broader context we sought to understand how the exocyst fits into the emerging picture of other tethering factors; specifically, if the exocyst shares a common set of interactions with the other tethering complexes involved in protein trafficking.

In terms of the various protein-protein interactions, the exocyst is a typical example of a tethering complex. It interacts with the Rab GTPase Sec4 on the vesicle membrane and the Rho GTPase Cdc42 on the target membrane (although evidence of a direct interaction with Sec4 has yet to be conclusively demonstrated), the SM protein Sec1, and the GEF Sec2. Like other tethering complexes, disruption of the exocyst results in an accumulation of vesicles prior to fusion. However, a major difference between the exocyst and other MTCs is in the exocyst’s interactions with SNARE proteins. Sec6, a component of the exocyst complex, interacts with both the v-SNARE Snc2 and the t-SNARE Sec9. Although the interaction with Snc2 may be required for proper polarization of the complex, the interaction with Sec9 appeared to be inhibitory and reduced the rate of binary t-SNARE complex formation. This was in stark contrast to other tethering-SNARE interactions, which are primarily involved in stabilizing the SNARE proteins and promoting formation of/protecting the assembled SNARE complexes.
The lab’s previous conclusion that Sec6 decreased the rate of binary SNARE assembly was based on two independent lines of evidence – a quantitative decrease in the rate of depletion of one of the reactants, Sso1, and a qualitative observation of less SNARE complex formation on a native gel over time. These two lines of evidence supported each other; however, it was difficult to incorporate exocytic trafficking into the overall world-view of intracellular trafficking in general, and the field was forced to conclude that exocytic trafficking was an outlier. Therefore, I sought to determine if inhibition of SNARE assembly was truly the function of the Sec6:Sec9 interaction.
Major Conclusions

Our biochemical studies identified, for the first time, an interaction between a subunit of the yeast exocyst tethering complex and assembled exocytic SNARE complexes (Figure 2.10). This Sec6:SNARE complex interaction is important for function in vivo, as mutations in Sec9 that disrupt the Sec6:SNARE interaction lead to synthetic growth defects in combination with mutations in exocyst subunits and in the SM protein Sec1 (Figure 2.6).

Due to the disordered and flexible nature of Sec9, identification of residues central to the Sec6:Sec9 interaction required use of the zero-length cross-linker EDC and tandem mass spectrometry analyses. We expanded existing protocols for these experiments to include a time course (Chang et al., 2004); this allowed us to visualize which cross-links formed and how the amount of each cross-link changed over time. These experiments also allowed us to better understand how an IDP interacts with a well-folded partner in the presence of a cross-linker, and highlight the “fuzziness” that is inherent to ordered-disordered protein interactions (Tompa and Fuxreiter, 2008). Our cross-linking analyses identified a stretch of residues in the inter-helical region of Sec9 that are likely involved in the interaction with Sec6 (Figure 2.5), and we mutated this five amino acid stretch to a charged patch to generate the sec9-142 allele (Lys-533 to Glu, Leu-534 to Glu, Met-535 to Glu, Arg-536 to Glu, and Glu-537 to Arg). Our phenotypic analyses suggested that exocytic trafficking is impaired in the sec9-142 yeast strain (Figure 2.6); however, these mutations were not sufficient to disrupt the Sec6:Sec9
direct interaction at our level of detection (Figure 2.7). We decided that a functional test 
in vitro would be a better way to determine the basis of the in vivo growth defect.

Our in vitro quantitative biochemical analyses of the sec9-142 protein and its ability to form SNARE complexes in the presence of the exocyst subunit Sec6 revealed unexpected results that contradicted published findings (Sivaram et al., 2005). We tested the purified wild-type and mutant Sec9 proteins in an optimized SNARE complex assembly assay (Nicholson et al., 1998; Sivaram et al., 2005) and found that Sec6 has no appreciable effect on the rate of SNARE complex assembly regardless of the Sec9 protein variant used (Figure 2.8). One possibility was that exocyst plays a very different role in exocytosis than other MTCs perform at other stages of membrane trafficking; however, subsequent analyses instead suggest that the previous data were misinterpreted (Figure 2.9). We show here that Sec6 can bind both the binary Sec9:Sso1 and ternary Sec9:Sso1:Snc2 SNARE complexes and that these interactions are disrupted by the Sec9-142 mutations (Figure 2.10). We therefore conclude that the synthetic growth defect observed in sec9-142 cells is due to a disruption of the Sec6:SNARE complex interaction rather than a loss of Sec6-mediated SNARE assembly inhibition.
Implications

Tethers and SNAREs in trafficking

Most MTCs, including exocyst, interact with their cognate SNARE complexes or with individual SNARE proteins (Arasaki et al., 2013; Balderhaar et al., 2013; Collins et al., 2005; Conibear et al., 2003; Diefenbacher et al., 2011; Krämer and Ungermann, 2011; Lobingier and Merz, 2012; Lürick et al., 2015; Pérez-Victoria and Bonifacino, 2009; Shestakova et al., 2007; Sivaram et al., 2005). However, the function of these interactions is not well understood. The best studied of the MTC-SNARE complex interactions is between HOPS and its cognate SNARE proteins/SNARE complex, which appears to serve several functions. First, HOPS recruitment to the assembled SNARE complex is important for maintaining its localization at sites of vacuole fusion (Wang et al., 2003). Secondly, HOPS competes with the disassembly machinery (Sec17/Sec18) for binding to the assembled SNARE complex and preferentially binds to trans-SNARE complexes; this likely protects the pre-fusion trans-SNARE complex from disassembly (Collins and Wickner, 2007; Collins et al., 2005). One of the HOPS subunits can bind both the v-SNARE and t-SNARE and these interactions may align the proteins for proper complex formation (Baker et al., 2015). Finally, HOPS has a higher affinity for properly-formed SNARE complexes than for non-cognate SNARE complexes, suggesting that it may “proofread” the SNARE complex prior to fusion (Starai et al., 2008). Other MTC:SNARE interactions appear to have similar functions, but have not yet been studied in as much mechanistic detail.
It is interesting to speculate that exocyst-SNARE complex interactions may have similar roles to the HOPS-SNARE interactions. In support of this hypothesis, a cluster of point mutations in Snc2 (Snc2-M2) that face outward from the assembled SNARE complex, and are therefore exposed when the complex is fully assembled, disrupt a direct interaction between Sec6 and Snc2 resulting in a mild exocyst polarization defect (Shen et al., 2013). Our studies show that Sec6 has a higher affinity for the Sec9:Sso1:Snc2 ternary complex than the Sec9:Sso1 binary complex (Figure 2.10), suggesting that Snc2 may be providing additional binding residues on the surface of the assembled SNARE complex for Sec6 binding. The Snc2-M2 mutant protein may also disrupt Sec6:SNARE complex binding, similar to the disruption seen with the Sec9-142 mutant, and thus the Sec6:SNARE complex interaction may help maintain proper polarization of the exocyst complex.

The fact that Sec6 appears to have a higher affinity for the fully assembled SNARE complex also suggests that Sec6 may serve a protective role, similar to HOPS, and compete with the Sec17/18 disassembly machinery for binding to the complex. This hypothesis would predict that Sec6 may have an even higher affinity for the complex when it is in the trans conformation, rather than the cis-like conformation lacking transmembrane domains tested here. Additionally, Sec6 and the exocyst may cooperate with Sec1 for this function, as the SM proteins have been shown to compete with the Sec17 for binding to assembled SNARE complexes in other stages of trafficking (Lobingier et al., 2014).
The studies presented here provide a crucial step towards deciphering the role of exocyst-SNARE interactions in exocytic growth in yeast and suggest future experiments to test the conservation of this interaction in other model systems. Additionally, as I have only identified a novel interaction here, many critical mechanistic questions remain unanswered. Many other MTC:SNARE interactions take place in the context of the assembled MTC; is the entire exocyst complex involved in the SNARE interaction or just the Sec6 subunit? Mutations in Sec6 cause mislocalization of the entire exocyst complex (Songer and Munson, 2009); is the assembled SNARE complex, through interactions with Sec6, required to polarize the exocyst at sites of fusion? HOPS can also act with its SM protein Vps33 to protect properly formed complexes from the disassembly machinery prior to vesicle fusion, and other SM proteins have been shown to serve a similar role (Lobinger et al., 2014); are Sec6 or the exocyst complex protecting the assembled SNARE complex prior to vesicle fusion, possibly through the SM protein Sec1? These questions will be discussed in more detail in the Future Directions section, below.

**IDP:ordered protein interactions**

During the cross-linking experiments used to identify the critical Sec6:Sec9 binding site residues, we were presented with a larger dataset than expected due to the presence of the IDP Sec9. This required developing hypotheses and methodologies to understand how a disordered protein would interact with an ordered protein during a cross-linking reaction. Unlike other studies using EDC to map binding sites (Chang et al.,
2004), the presence of an IDP in our reaction necessitated the use of a cross-linking time course. If we had examined just the cross-links identified at the end of the reaction, we would have been left with an overwhelming dataset that was impossible to interpret (Figure 2.2). The large number of cross-links identified was likely due to the flexible nature of Sec9; the protein can sample more conformations and activated cross-linkable residues have a higher probability of being proximal. However, by studying the behavior of the cross-linked peptides over time we were able to separate different classes of cross-links and filter the dataset.

The behavior of the secondary crosslinks is also unique to the ordered:disordered protein interaction. In an ordered:ordered system, the cross-links that form later in the reaction are more likely to cause structural changes to one or both binding partners; this may cause them to be less reliable indicators of the binding site. However, because Sec9 remained flexible, a higher fraction of the secondary crosslinks can capture the native state of Sec6. This resulted in more cross-links to examine, but also allowed us to identify individual binding residues rather than binding domain regions. There is, however, the possibility that some of the cross-links destabilized Sec6, but if so, we have no way of identifying which ones in order to exclude them from our analysis.

Finally, the patch we identified and mutated on Sec9 was not sufficient to disrupt Sec6:Sec9 direct binding. This is a hallmark of ordered:disordered interactions, as many predict that IDPs utilize multiple low affinity binding sites to bind their ordered partner (Uversky, 2002; 2013; Wright and Dyson, 2015). This is evolutionarily advantageous – IDPs are able to withstand random mutations without losing their necessary functions or
binding partners (Gitlin et al., 2014), but makes generating specific loss-of-binding mutants challenging. The C-terminal domain of Sec6 alone was not sufficient to bind to Sec9 despite Sec9 cross-linking to this domain; this suggests that the binding sites are scattered across both domains of Sec6 (Sivaram et al., 2005). However, the Sec9 mutations were sufficient to disrupt Sec6 binding once Sec9 was folded into the SNARE complex. This makes sense, as the folded Sec9 will likely have lost the rest of its contacts on Sec6 with only the inter-helical residues remaining exposed.

The loss of binding once the mutant protein is incorporated into the SNARE complex raises an interesting question – is the Sec6:Sec9 direct interaction truly necessary or functional in vivo? Similarly, is the Sec6:binary SNARE interaction necessary or functional in vivo? Or are these interactions a product of studying proteins in isolation, and not physiologically relevant? The flexible nature of Sec9 could result in non-physiological contacts with the free Sec6, and interaction with the binary SNARE complex could be a weaker version of the fully assembled SNARE interaction lacking the additional binding sites provided by Snc2. Similarly, is the Snc2:Sec6 direct binary interaction physiologically relevant in vivo, or a lower affinity version of the Sec6:SNARE interaction occurring due to the disordered nature of Snc2 outside the SNARE complex? Testing this will require additional mutations, likely scattered across the length of the proteins, that maintain the Sec6:SNARE complex binding but disrupt Sec6:Sec9 direct binding.
Interpreting the SNARE complex assembly assay

One of the major conclusions of this study was that the presence of Sec6 does not change the rate of SNARE complex assembly, in contrast to previously published data (Sivaram et al., 2005). This conclusion was based on the foundation of two lines of evidence: quantitative curve fitting from a gel filtration mobility shift experiment, and a qualitative observation of less SNARE complexes forming by a native gel mobility shift. However, by looking at the whole picture of the gel filtration profiles, it became clear that a Superdex 200 column was unable to resolve the free Sec6 and free Sso1 peaks. To confound matters, the percent of overlap between the free Sec6 and free Sso1 peaks changed over time due to the changing binding interactions in solution. The gel filtration assay was sufficient when only two components were present (and one of the components has essentially no extinction coefficient at the concentrations used), but once more components were added the data became difficult to interpret.

However, was the gel filtration assay sufficient even in its original incarnation? Re-working the SNARE complex assembly assay, and re-analyzing the previous gel filtration data, illuminated many unknown factors about the interplay between the various proteins. The equation derived and used in the original study was meant for a simple second order reaction – the SNARE domains of Sec9 and Sso1 react to form the assembled binary complex with rate $k_I$ (Figure 3.1). However, when the full-length Sso1 is used, the equation is

![Figure 3.1 - Simple second order reaction of SNARE assembly.](image-url)
not longer sufficient – it does not take into account that Sso1 is autoinhibited and must interconvert between its closed, non-reactive form and its open, reactive form (Figure 3.2). This means that at any given time, the concentration of reactive Sso1 is much lower than the concentration of reactive Sec9.

This changes the way we mathematically fit the results of the experiment, because we can no longer assume an equal concentration of reactants. However, this is not quite a pseudo first order reaction because while the total concentration of Sso1 is decreasing at a predictable rate ($k_1$) we do not know how the concentration of Sso1$^0$ is changing over time (controlled by $k_2/k_2'$). Therefore, we do not understand how the two components contribute to the rate of the reaction, and are likely measuring $k_2$ rather than $k_1$.

Addition of Sec6 into this reaction changes several additional factors. Because Sec6 can interact with Sec9, it reduces the concentration of available Sec9 at any given time. If the reaction is depended on the concentration of Sec9, instead of the concentration of open Sso1, this may alter the rate of the reaction. If Sso1 is unable to fully compete with Sec6 for Sec9 binding, this may alter the extent of the reaction (what percentage of Sso1 will be in

Figure 3.2 - Sso1 interconverts between an open and closed conformation, with the closed conformation being more prevalent; this changes the amount of open Sso1 available to react with Sec9 at any given time.

Figure 3.3 – Addition of Sec6 changes the amount of available Sec9. Addition of Sec6 to the reaction changes the amount of available Sec9 at any given time. This can change the rate of the reaction (if the rate is dependent on the concentration of Sec9, and if the change in concentration is enough to affect the rate), the extent of the reaction (how much of each reactant is in the SNARE complex at $t=\infty$), or both.
SNARE complexes at \( t = \infty \) (Figure 3.3). As observed in Figure 2.8, the addition of Sec6 results in \( \sim 30\% \) of Sso1 remaining un-complexed at \( t = \infty \), suggesting that the final equilibrium state of all proteins in the reaction involves a portion of Sec9 sequestered by Sec6. Additionally, because Sec6 can interact with the Sec9:Sso1 SNARE complex (as shown here), this may change the amount of free Sec6 over time that can interact with Sec9 (Figure 3.4).

The changes in SNARE complex assembly with the Sec9-142 protein provide some insight into the interplay between the various proteins. In our analysis, the addition of Sec6 causes a reduced extent of SNARE assembly with Sec9, and it appears to have no effect in combination with Sec9-142. This suggests that something about Sec9-142 changes the final equilibrium between all components of the reaction. Because we know that the Sec6:Sec9-142 interaction still occurs, there are two potential explanations for this phenomenon. One possibility is that the direct binding reaction is altered, without substantially changing \( k_3 \) or \( k_3', \) resulting in Sso1 being able to compete Sec9-142 away from Sec6. Another is that the Sec6:Sec9-142 interaction does not interfere with SNARE complex formation, due to changes in which residues of Sec9-142 make contact on Sec6, and that SNARE complex assembly releases Sec6. Therefore, the changes observed when using the Sec9-142 protein suggest that Sso1 cannot compete with Sec6 for Sec9 while the binding site residues in the inter-helical region are present.

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Figure 3.4 – Sec6 can interact with the binary SNARE complex. Sec6 can also interact with the binary SNARE complex, changing the amount of Sec6 available at any given time.
In order to fully understand this assay, several pieces of additional information are needed. First, what is the concentration of active Sso1 at the beginning of the reaction? Dropping the concentration of Sec9, and observing any changes in $k_1$, will begin to answer this question as $k_1$ will depend more on the Sec9 concentration once Sec9 is the limiting reagent. However, because the active Sso1 can be replenished by the autoinhibited Sso1, this will require re-working the equations. Secondly, using an assay that allows direct measurement of the formed complex will be a better way to determine $k_1$ – this has previously been done using circular dichroism, as formation of the SNARE complex results in a large increase in helicity, but the high helical content of Sec6 may result in too much background signal that will swamp the helical change by SNARE complex formation. Despite these problems, this assay is still useful, as any reagent that increases the amount of open Sso1, and therefore increases $k_2$, will result in an increase in $k_1$. Therefore, this assay can be used to screen for proteins that promote “opening” of Sso1 once candidates have been identified.
Short-Term Additional Studies

One of the large remaining questions from these studies is whether the Sec6:SNARE interaction is stand-alone or if it occurs in the context of the entire exocyst complex. Other MTC:SNARE interactions involve either the intact complex or multiple subunits interacting with the SNARE proteins. Until recently, this was a difficult question to solve. Previous attempts showed that a subset of exocyst subunits were unable to interact with Sec9, and whole cell lysate fractionation showed Sec9 outside the pool of assembled exocyst (Figure 3.5, (Morgera et al., 2012)).

However, this experiment was detecting interactions with Sec9 without incubating the yeast with sodium azide/sodium fluoride to prevent disassembly of the SNARE complexes during cell lysis (Grote and Novick, 1999). Now that we know to look for an

![Fig 3.5](image-url)
interaction with the SNARE complex, these experiments can be repeated. Additionally, recent successes at purifying the intact exocyst complex (Heider et al., 2016) provide a tool to test for SNARE binding to the entire complex. These experiment are limited for now, however, by the fact that neither the exocyst nor the SNARE complex can be concentrated high enough for quantitative binding studies.

Another unknown in these experiments is the role of Sec1. The SM protein also binds assembled SNARE complexes, and the linker region has been shown to be required for this interaction (Togneri et al., 2006). Therefore, the Sec9-142 mutation in the linker region may also be disrupting the Sec1:SNARE complex interaction. Similar to the exocyst, it is difficult to concentrate Sec1 high enough for these quantitative binding studies; however this barrier may be overcome easier than concentrating the exocyst, and therefore present an ideal first step in further characterization of the Sec9-142 mutation. A large panel of Sec1 mutations that disrupt SNARE binding are also available and would be ideal for testing for synthetic interactions with the sec9-142 strain in vivo (Hashizume et al., 2009).

As previously mentioned, the Snc2-M2 mutant that disrupts Sec6 binding may also disrupt Sec6:SNARE binding (Shen et al., 2013). This idea can be tested by purification of SNARE complexes containing either the Snc2-M2 mutation alone or both the Sec9-142 and Snc2-M2 mutant proteins in the same complex. Similarly, generation of a sec9-142/snc2-M2 strain will allow testing of synthetic effects of the double mutation. Initial attempts at generating this strain have already begun, but the snc2-M2 strain has defects in sporulation that complicate strain generation. Therefore, genomic replacement
of *SEC9* with the *sec9-142* allele in the *snc2-M2* strain is likely the best method for generating this double mutant moving forward.

Finally, the *snc2-M2* allele results in a mild mislocalization defect of the exocyst complex. If this defect is due to a loss of Sec6:SNARE binding, then the *sec9-142* strain may show a similar mislocalization defect. Therefore, localization studies of the exocyst, Sec1, and Sso1 in the context of the *sec9-142* allele may provide more clues as to the function of the Sec6:SNARE interaction *in vivo*. 
Long-Term Future Directions

Are SNARE complexes the exocyst anchor?

One of the functions of the HOPS:SNARE interaction is to maintain localization of the HOPS complex at sites of vacuole/lysosomal fusion (Wang et al., 2003). HOPS and the SNAREs then recruit each other to achieve the concentrations necessary to mediate fusion. Previous work in the lab identified two highly conserved patches on the surface of Sec6 and mutation of these patches resulted in mis-localization of the intact exocyst complex (Songer and Munson, 2009). These alleles resulted in severe growth and secretion defects \textit{in vivo} that can be rescued by overexpression of individual SNARE proteins. It was hypothesized in that study that the defect was due to loss of binding to a putative “anchor” protein, and that the SNARE overexpression allowed bypass of the tethering function of the exocyst. However, were those mutations in Sec6 disrupting SNARE binding? And was a loss of SNARE binding the cause of the exocyst mislocalization? These mutations were not sufficient to disrupt Sec6:Sec9 direct binding, similar to the work presented here; however, in support of this...
hypothesis, the Sec9-142 residues cross-link to regions adjacent to the these mislocalization patch mutants on Sec6, suggesting that they may be adjacent to potential SNARE binding sites (Figure 3.6).

Some work has already begun to resolve this – specifically, the Sec6 mutants were tested for Snc2 binding defects, and were not sufficient to disrupt binding (Margaret Heider, unpublished data). However, as seen with the Sec9 mutant, it may be that these mutations are not sufficient to disrupt binding to the disordered protein while sufficient to disrupt binding to the assembled SNAREs. And, while the Snc2-M2 mutation was sufficient to disrupt binding to Sec6, this mutation consisted of multiple residues across the N-terminal half of the SNARE motif. This may have provided enough mutations to disrupt several low-affinity sites the IDP Snc2 uses to bind Sec6.

There are several additional studies that are necessary to resolve this question. Firstly, does SNARE overexpression rescue the mislocalization defect of these Sec6 mutants? If overexpression of the SNAREs is truly allowing for bypass of the exocyst’s tethering function, then strains overexpressing SNARE proteins should still have an exocyst mislocalization defect. Similarly, are the Sec6 mutant proteins sufficient to disrupt binding of Sec6 to the assembled SNARE complex? These proteins are well folded in vitro, and therefore would be excellent candidates for future SNARE complex binding studies. Finally, studying the localization of the exocyst in the sec9-142 strain will begin to resolve if the exocyst:SNARE interaction is required for proper exocyst localization.
Do Sec6 or the exocyst protect pre-fusion SNARE complexes from disassembly?

The idea of SNARE binding being a determinant for exocyst polarization is intriguing, but may only be one function of the interaction. While bound, the exocyst may also serve a stabilizing/protective function, similar to HOPS, and compete with the Sec17/Sec18 disassembly machinery for SNARE binding to prevent disassembly of properly formed SNARE complexes prior to vesicle fusion.

Studies have already shown this role for a subset of the the SM proteins. Sly1, the SM protein involved in ER and Golgi fusion events, and the HOPS SM subunit Vps33 can both protect assembled SNARE complexes from disassembly by Sec18 (Lobingier et al., 2014). Does the exocytic Sec1 have a similar function? And does it cooperate with Sec6 or the exocyst to accomplish that role? It is possible to test this using a SNARE disassembly assay previously described, modified to include the exocytic SNARE complex and the SM protein Sec1, Sec6, or both (Lobingier et al., 2014). Additionally, synthetic tests with overexpression of Sec17 and/or Sec18 in combination with either Sec1 mutants defective for SNARE binding (Hashizume et al., 2009), or the Sec9-142 SNARE complexes described here defective for Sec6 binding, will begin to unravel any requirement for SNARE binding when cells more promiscuously disassemble SNARE complexes.
**Liposome fusion assays**

Many studies used to understand SNARE mediated vesicle fusion, at least after binary SNARE complex assembly, have relied upon liposome fusion assays (McNew et al., 2000; Nickel et al., 1999; van den Bogaart et al., 2010). In these assays, liposomes are reconstituted with the SNARE proteins required for the specific fusion event, and the rate of fusion is measured by lipid mixing, content mixing, or a combination of both (Diao et al., 2012; 2013; Kyoung et al., 2013; Xu et al., 2011). While many of these assays have shown a dependence on lipid compositions, and the findings are therefore not necessarily physiologically relevant if using non-native lipid compositions (Tong et al., 2009; Vicogne et al., 2006; Zick et al., 2014), they are an excellent way to determine how addition or loss of various factors can change the rate of fusion. By using whole cell lysate depleted of components or by adding in vitro purified regulatory proteins, it is possible to generate a picture of how each component contributes to the overall regulatory process. Since these assays require low concentrations of reactants, it is also possible to test the contribution of components that are difficult to concentrate – specifically the exocyst and Sec1. Therefore, these assays may be an ideal next step in de-convoluting the contribution of each protein family in overall regulation of SNARE assembly and vesicle fusion.
Final Thoughts

The studies presented here are a first step in understanding whether the exocyst, a multi subunit tethering complex, behaves similarly to the tethering complexes in other trafficking steps. While the common set of interactions has been known for some time, the idea that an exocyst subunit reduced the rate of SNARE complex assembly set the exocyst apart. Now, having identified that the SNARE interaction is not inhibitory to SNARE complex formation, it is possible to design experiments that will begin to dissect the specific functions of this interaction.
Appendix A: Table of all identified Sec6-Sec9 cross-links

The cross-linking experiments (Figures 2.2-2.5) included eight different experimental setups, and one re-analysis of a previous dataset. Each of these experiments produced an overlapping list of cross-linked peptides, which are detailed in Table 3.1. The peptides identified are listed for each protein, and the residue number of the cross-linked amino acid is denoted (where identifiable) in red. In cases where two adjacent residues are chemically capable of cross-linking, both are colored red; the peptide was then duplicated in the sheet with each possible cross-linked residue represented.

The experiments are as follows:

Experiment 1: 7.5 µM Sec6/Sec9, cross-linked with EDC and Sulfo NHS for 0-120 min
Experiment 2: A refinement of the data set from experiment 1.
Experiment 3: 7.5 µM Sec6/Sec9, cross-linked with EDC alone for 0-120 min
Experiment 4: 7.5 µM Sec6/Sec9, cross-linked with EDC and NHS for 0-120 min
Experiment 5: 7.5 µM Sec6/Sec9, cross-linked with EDC and Sulfo NHS for 0-120 min
Experiment 6: 1 µM Sec6/Sec9, cross-linked with EDC alone for 0-120 min
Experiment 7: 7.5 µM Sec6/Sec9, cross-linked with EDC alone for 0-5 min
Experiment 8: 1 µM Sec6/Sec9, cross-linked with EDC alone for 60 min
Experiment 9: 7.5 µM Sec6/Sec9, cross-linked with EDC alone for 0-5 min

The table is sorted according to the reproducibility of each cross-link in varying experiments. Each identified cross-link in each experiment is color coded to represent the class it belongs to, and each residue number is color coded according to which class(es) of cross-links it participates in (yellow, early-forming; blue, late-forming; green, both early-forming and late-forming) (Figure 2.4 and Figure 2.5).
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Table 4.1 – All cross-links identified during cross-linking and mass spectrometry analyses. The peptides identified are listed, with the cross-linked amino acid in red; the corresponding residue number is in the next column. Each cross-link identified in each experiment is color-coded to represent the class it belongs to, and each residue is color coded to represent the classes of cross-links it participates in. The data is sorted so the most reproducible cross-links are listed first.
Appendix B: Sec6-Sec6 cross-links identified in Sec6-Sec9 cross-linking dataset

The data from cross-linking experiment 1 (see Appendix A and Table 4.1) were analyzed for any Sec6-Sec6 cross-links that formed during the Sec6-Sec9 cross-linking reaction. Previous work showed that Sec6 dimerizes in vitro, and that the Sec6:Sec9 interaction is between two copies of each protein (Sivaram et al., 2005). The cross-links identified are shown in Table 4.2, and summarized in Figure 4.1. However, it is not possible to distinguish between intra- and inter-molecular cross-links without labeling one of the two protein copies.

Figure 4.1A shows all cross-linked residues labeled according to the class of cross-link as defined in Appendix A and Figure 2.4. Unlike the Sec6:Sec9 interaction, there is no IDP present in these cross-links; accordingly, each cross-link identified in the C-terminal domain generally contains at least one residue in a flexible loop region of the crystal structure, likely because the flexibility allows the cross-linker activated residues to come into contact. Many of the cross-links in the N-terminal domain also occur in or near regions of predicted flexibility (Figure 4.1B, with the residues removed for better visualization of the predicted helical secondary structure). There are also residues that cross-link to both Sec6 and Sec9, denoted with asterisks above the N-terminal domain structure, suggesting that these residues are exposed and easily cross-linked during the cross-linking reaction.
Surprisingly, only one cross-link occurred between the C-terminal and N-terminal domains. Similarly, there are fewer cross-links in the C-terminal domain than in the N-terminal domain in general. However, because this cross-linking was run in the presence of Sec9, it is not possible to determine if these cross-links are capturing the native state of the Sec6:Sec6 interaction, or even the native conformation of the protein itself.
Table 4.2 - All Sec6-Sec6 cross-links identified during cross-linking and mass spectrometry analyses. The peptides identified are listed, with the cross-linked amino acid in red; the corresponding residue number is in the next column. Each cross-link identified is color-coded to represent the class it belongs to, and each residue is color coded to represent the classes of cross-links it participates in. The data is sorted in residue number order, with the most N-terminal residue in the cross-link in the left-hand column.
Figure 4.1 - Sec6-Sec6 cross-links identified in the Sec6-Sec9 cross-linking reaction. The cross-links are mapped onto the C-terminal domain structure (Sivaram et al., 2006) and a linear segment representing the N-terminal domain. The boxes on the N-terminal domain represent putative helices by secondary structure prediction (SOPM algorithm), and the asterisks denote residues that cross-link to both Sec6 and Sec9. A) Each residue is colored, as in Figure 2.4, according to the class of cross-link it participates in. Each cross-link contains at least one residue in/near a flexible loop region of the protein, suggesting that the protein flexibility greatly contributes to its availability to crosslink. B) The residues are removed from the N-terminal region to allow better visualization of the secondary structure prediction.
Appendix C: Plasmid-based analysis of Sec6/Sec9 point mutations in vivo

The first attempt to identify the residues necessary for the Sec6:Sec9 interaction was via in vivo mutagenesis. CEN LEU2 plasmids bearing a single point mutation in either SEC6 or SEC9, both as an alanine substitution and a charge reversal, were introduced into yeast where the genomic copy was deleted; the deletion was covered by a wild-type plasmid with the URA3 auxotrophic gene. The mutant-containing plasmids were transformed into the yeast, and the wild-type gene was selected against using 5-fluoroorotic acid (5-FOA). Two distinct colony sizes were identified for all mutations, and one colony of each size was selected; each of these individual mutant strains were then assayed for growth defects at varying temperatures on both rich YPD media and minimal synthetic media (Figure 4.2).

While the strain containing the E537R mutation had a growth defect at high temperatures, there was concern about the colony size as small colonies can be indicative of other defects, and may confound the analysis (Dr. Reid Gilmore, personal communications). Therefore, the strain was re-constructed by integrating the mutation into the genome. Upon analysis of the newly generated strain, a growth defect was no longer evident (Figure 4.3). This led to questions regarding the copy number of the yeast CEN plasmids, and if that would compensate for any mild binding defects. Moving forward, all mutations were integrated into the genome (Figure 2.6). However, the Sec9 Glu-537 to Arg mutation was carried forward for in vitro studies (Appendix D), and eventually became the final mutated residue in the sec9-142 allele.
Figure 4.2 – Serial dilutions of Sec6 and Sec9 mutants made based on the early cross-linking experiments. Each mutation position is color-coded based on the class of residue it was later sorted into. Those mutations that showed a growth defect in at least one of the two colonies tested is denoted by a black dot. A) Mutations in Sec6 tested. B) Mutations in Sec9 tested. Sec9 K437 is an N-terminal internal residue in the assembled SNARE complex, and the growth defect observed is likely due to disruption of SNARE complex assembly/reduced SNARE complex stability.

Figure 4.3 – Growth curve of the integrated Sec9 E537R construct. This integrated construct shows no growth defects at any of the temperatures tested, suggesting that the growth defect previously observed was due the plasmid-based strain construction strategy rather than an allele-specific effect.
Appendix D: Alternative methods of quantifying Sec6:Sec9 direct binding

Using gel filtration to detect \textit{in vitro} binding defects

The Sec6:Sec9 interaction was originally studied by size exclusion chromatography; equimolar Sec6 and Sec9 were incubated together for 1 hr at 4 °C, and then run over a Superdex 200 gel filtration column. The purified Sec6 runs as a dimer, eluting with a retention volume of approximately 13.5 mL; when it binds Sec9, it elutes with a retention volume of approximately 12 mL, indicating that the complex now has a larger hydrodynamic radius. Therefore, we hypothesized that changes in the binding affinity would result in a return of Sec6 to its uncomplexed position.

Multiple protein constructs, with mutations in both Sec6 and Sec9, were tested by this assay. Initial experiments showed a mild binding defect with Sec9 (Glu-537 to Arg), Sec9 (Leu-534 to Glu), Sec9-142, and Sec6 (Lys-515 to Asp) (Figure 4.4). However, upon examination of the concentrations of the various constructs, it became clear that the concentration of Sec9 was over-estimated (due to contaminants in the prep) while the concentration of Sec6 was under-estimated (likely due to incomplete hydrolysis during the ninhydrin assay) (Figure 4.5B). When the concentration of Sec9-142 (the most severe disruption of binding mutant by gel filtration) was re-calculated and the assay re-run, the binding defect was lost (Figure 4.5A). Therefore, we concluded that gel filtration was likely not sensitive enough to detect changes in binding affinity.

However, the fact that a binding defect was detected when the proteins were not incubated at equimolar concentrations, despite the wild type proteins showing complete
binding even with the concentration differences, suggests that the mutants that showed weakened binding may still have a mild binding defect. Sec9-142, though, did not show a binding defect by direct binding assays; a full binding curve, and calculation of the $K_d$ for each interaction, may show very mild differences that the six concentration experiment could not detect (Figure 2.7).

**Using Fluorescence Polarization to determine the $K_d$ of the Sec6:Sec9 interaction**

Another potential way of measuring the Kd of the Sec6:Sec9 interaction was using fluorescence polarization. Sec6 is a large protein (~90 kDa) that dimerizes in solution; Sec9 is a much smaller protein (~25 kDa) that is a disordered monomer in
solution. Therefore, the Sec6:Sec9 interaction (which involves two copies of each protein) will be significantly larger and affect the protein’s ability to tumble in solution.

I designed and produced a Sec9 construct with a C-terminal cysteine. Early attempts at purification showed little to no Sec9 after induction; this was fixed by altering the induction method (see modified Sec9 purification protocol in Chapter V). Once the purification was optimized, this construct was soluble, purified similarly to the wild-type Sec9, and was able to be concentrated. I used TCEP instead of DTT as a reducing agent to keep the free cysteine from forming disulfide bonds, as DTT needs to be removed prior to conjugation with a maleimide dye; however, upon addition of the TCEP, a large

Figure 4.5 – The loss of binding observed in previous gel filtration experiments was due to a mis-calculation of the protein concentrations in the assay. A) A gel filtration profile showing Sec9-142 at 10 µM using the concentration measured by ninhydrin (bottom, grey), and a re-measurement of the same proteins when the concentrations were re-done using Krypton staining and a BSA standard. B) Gels showing 0.5 and 0.25 µg of protein according to the ninhydrin calculations with BSA as a loading control.
portion of the protein precipitated and was unable to be re-solubilized. Concentration testing suggested very little protein remained in solution.

Despite the low concentration, I attempted to conjugate an Alexa488 Maleimide dye as described previously (Furgason et al., 2009). After running on a Superdex 200 column, the eluted peak contained a very small amount of labeled Sec9 (Figure 4.6). Upon concentration, the Sec9 remaining precipitated. This avenue was abandoned due to the instability of the C-terminally tagged construct.

While other attempts may have improved the solubility of the construct (adjusting the buffer conditions, different fluorescent dyes, using DTT instead of TCEP followed by buffer exchange), the propensity of the construct to aggregate was concerning as any aggregation might confound the anisotropy results.
Appendix E: Sso1-Cysteine Constructs for Maleimide Labeling in SNARE Complex Assembly Native Gels

The contaminant band from the Sec9 prep confounded the quantification of SNARE assembly by native gel because its migration resulted in partial overlap with the free Sso1 band. Therefore, I attempted to conjugate an Alexa488-maleimide to Sso1, and monitor the fluorescent Sso1 signal in an electrophoretic mobility shift assay (EMSA) as previously described (Furgason et al., 2009). This would allow me to measure the fraction of each time point bound, rather than the fraction of input lost.

I therefore designed constructs with a cysteine at either the N-terminal or C-terminal end of Sso1; the construct lacking the transmembrane domain does not contain any other cysteines. I then purified each construct as previously described (Munson et al., 2000). The constructs purified and concentrated with no issues. They were then labeled and concentrated after labeling with still no complications.

Each of the Sso1 constructs was incubated with Sec6, Sec9, or both, for 0-96 hours. The resulting mixtures were then run on a pH 7.4 slab native gel as previously described (Furgason et al., 2009), and the fluorescently labeled protein was detected by a Typhoon fluorescence stage with the appropriate Alexa-488 filter set (Figure 4.7).

Upon visualization of the native gel’s fluorescence, it became clear that there were problems with the fluorescent Sso1 constructs. The N-terminal Alexa construct showed decent resolution of assembled SNARE complexes; however, two bands are present for uncomplexed Sso1, and while the lower band is at least generally decreasing
over time in the conditions containing Sec9, we are unable to account for the upper band in the free Sso1 region. Finally, as the upper band appears to be increasing in intensity over time, we cannot quantify the total fluorescence in each lane. We therefore concluded that the N-terminal labeled construct is not feasible for these studies.

Different and far more difficult problems accompanied use of the C-terminal fluorescent construct. In these experiments, a partially unresolved band likely due to cleaved or free dye is present at the bottom of each lane. Those lanes that do not contain the free dye did not behave in any predictable manner. We therefore also abandoned use
of the C-terminal labeled construct for these experiments, and turned instead to coomassie labeling of the native gels (Figure 2.8).
Appendix F: Modeling of the *P. patens* Type-V Myosin Cargo Binding Domain

Over the last few years, we have developed a collaboration with Dr. Luis Vidali’s lab at WPI. The Vidali lab studies exocytic trafficking in the model system *Physcomitrella patens*, a moss species that has genetic tractability similar to budding yeast; these similarities allow both homologous recombination and siRNA silencing in the same organism (Cove et al., 2009).

The growth of root tip cells in moss is a form of polarized growth that utilizes the same machinery as polarized growth and secretion in budding yeast: specifically Rab GTPase families, type V myosin motors, and the exocyst complex. Previous work in the lab identified that the type V myosin motor MyoXI is responsible for transport of vesicles to sites of active growth; however, identification of the vesicle-associated Rab GTPase has been elusive (Vidali et al., 2010). Sequence homology studies have narrowed it down to the RabA family, but are unable to further resolve the specific family member.

Using structural homology modeling against the yeast type V myosin Myo2, the Vidali lab generated a set of mutations designed to disrupt binding with the Rab (Pashkova et al., 2006). These constructs were then tested for their ability to complement loss of the wild-type MyoXI *in vivo*, and three mutations were identified. These mutations were then selected for *in vitro* binding studies with candidate RabA proteins, which I worked to develop with two undergraduate students (J. Garbarino and S. Kaptur et al., unpublished data).
Figure 4.8 – Structural homology modeling of MyoXI based on the structure of the human MyoVb cargo binding domain. The mutations previously identified as failing to compliment loss of the wild-type MyoXI in P. patens are highlighted in yellow; two of these mutations appear to be to surface residues, but the third (Val-1418) is likely an internal residue. This agrees with preliminary in vitro data; the Val-1418 to Arg mutation is not soluble.

However, during the early phases of the *in vitro* studies, a new structure of the human MyoVb was released; this protein has higher sequence homology to the MyoXI from *P. patens*, and the paper more thoroughly maps the Rab-interacting surface from several systems. I therefore utilized the Modelr software suite to generate a homology model of the *P. patens* MyoXI cargo binding domain based on the human MyoVb cargo binding domain structure (Figure 4.8).

Based on the new model, it appears that only two of the mutations that were carried into *in vitro* studies are surface exposed; the third is likely a buried hydrophobic. This was confirmed by attempts to purify a Val-1418 to Arg His-tagged protein construct – the protein had low expression levels, and was insoluble after the initial nickel purification (J. Garbarino and S. Kaptur, unpublished data).
REFERENCES


