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Elucidation of the Role of the Exocyst Subunit Sec6p in Exocytosis: A Dissertation

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ELUCIDATION OF THE ROLE OF THE EXOCYST SUBUNIT SEC6p

IN EXOCYTOSIS

A Dissertation Presented

By

Daniel Niron Brewer

Submitted to the Faculty of the University of Massachusetts Medical School of

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November 23rd, 2009

Biochemistry and Molecular Pharmacology

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ABSTRACT

Trafficking of protein and lipid cargo through the secretory pathway in eukaryotic cells is mediated by membrane-bound vesicles. Secretory vesicles are targeted to sites of exocytosis on the plasma membrane in part by a conserved multi-subunit protein complex termed the exocyst. In addition to tethering vesicles to the plasma membrane, the exocyst complex and components therein may also add a layer of regulation by directly controlling assembly of the SNARE complex, which is required for membrane fusion, as well as other regulatory factors such as Sec1p. In the past, we have shown that Sec6p interacts with Sec9p *in vivo* and that that interaction retards binary SNARE complex formation in a SNARE assembly assay. Though many interactions have been mapped using *in vitro* methods, confirming them *in vivo* and placing them into the context of a complete model that accounts for all observed interactions (and lack of interactions) has proven difficult.

In order to address these problems, I have studied the interactions between Sec6p and other factors involved in exocytosis at the plasma membrane via *in vivo* methods. My hypothesis was that Sec6p interaction with Sec9p and subsequent inhibition of SNARE complex assembly *in vitro* was an intermediate state and Sec6p was part of a set of cofactors that accelerated SNARE complex assembly *in vivo*. To test this hypothesis I showed that the interaction between the plasma membrane t-SNARE Sec9p and the yeast exocyst subunit Sec6p can be observed *in vivo* and designed point mutations to disrupt

that interaction. Interestingly, I also showed that Sec6p:Sec9p interaction involves the free pool of Sec6p rather than the exocyst bound fraction of Sec6p.

Point mutations in the N-terminal domain of Sec6p result in temperature sensitive growth and secretion defects, without loss of Sec6p-Sec9p interaction. However, at the non-permissive temperature, the exocyst subunits Sec5p, Sec10p and Sec15p are mislocalized and are absent from the exocyst complex. The resulting subcomplex, containing Sec3p, Sec8p, Exo70p and Exo84p, remains stably assembled and localized at sites of polarized secretion. This subcomplex is likely due to disruption of interaction between Sec6p and Sec5p, and may be similar to that observed at restrictive temperatures in the *sec6-54* temperature sensitive mutant.

Additionally, one of the *sec6* temperature sensitive mutants displays a loss of binding to the yeast regulatory protein Sec1p. *In vitro* binding studies indicate a direct interaction between Sec1p and the free pool of the wild-type Sec6p protein, suggesting close interplay between Sec6p and Sec1p in the regulation of SNARE complexes. A coherent model which incorporates all these interactions has continued to be elusive. However, the results I have found do suggest several hypotheses which should prove testable in the future.

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ABREVIATIONS

SNARE – Soluble NSF Attachment Protein Receptor

COPI – Coat Protein Complex I

COPII – Coat Protein Complex II

COG – Conserved Oligomeric Golgi Complex

HOPS – Homotypic Fusion and Vacuole Protein Sorting Complex

GARP – Golgi Associated Retrograde Protein Complex

ER – Endoplasmic Reticulum

GTP – Guanosine Triphosphate

GDP – Guanosine Diphosphate

GDI – Guanine Nucleotide Dissociation Inhibitor

GEF – Guanine Nucleotide Exchange Factor

TRAPP – Transport Protein Particle Complex

CORVET – Class C Core Vacuole/Endosome Tethering Complex

EM – Electron Microscopy

PREFACE

- Chapter II of this thesis has been resubmitted as part of the following manuscripts in October 2009.

Brewer DN, Carr CM, Munson M: "Disruption of N-terminal exocyst binding residues in Sec6p results in a stable subcomplex localized to sites of exocytosis"

Chapter I:

Introduction

<u>Trafficking and the eukaryotic cell</u>

A defining feature of the eukaryotic cell, from single-celled yeast to complex metazoans like humans, is the organelle. Organelles are compartments that delineate spatially and chemically distinct features and allow both spatial and temporal separation of eukaryotic processes. For example, protein synthesis of proteins destined for secretion or membrane insertion occurs within the endoplasmic reticulum (ER), where mRNA is translated and proteins are folded for secretion or insertion into membrane. Proteins that require post-translational modification may be processed in the Golgi apparatus. Some carbohydrate synthesis also occurs here. Proteins on their way to degradation may then pause in the early and late endosomes, organelles that can mature into lysosomes and also process endocytic trafficking from the plasma membrane to the Golgi. The lysosome, or vacuole in yeast, sequesters and degrades proteins, membranes, and other molecular cargo via resident enzymes, thus providing a "garbage disposal" for the eukaryotic cell. Finally, proteins, membranes, and other cargo destined for secretion find their way to the plasma membrane, the surface of the cell where exocytosis is completed.

Each of these organelles, or compartments, is surrounded by a membrane that sequesters the contents from other organelles and from the cytosol that fills the space between organelles. This compartmentalization allows a lysosome to have a low pH and proteases that would be harmful, if not lethal, to the rest of the cell. However, the

compartmentalization that allows for complex eukaryotic life, like most solutions, creates another problem that must be solved.

The problem created by the strict delineation of membrane-bound organelles is one of transport. How does a cell that is divided into distinct organelles move proteins, macromolecules, and lipids between those compartments to ensure the continuation of essential processes such as growth and secretion? Vesicular trafficking, the movement and delivery of cargo and membrane between spatially and chemically distinct organelles, is an essential and highly conserved process in all eukaryotes [1]. There are several requirements that trafficking must meet if it is to be effective. Trafficking must be fast. It must occur within the timescale of living processes. Trafficking must also be targeted and have high fidelity. Cargoes must reach their destinations and only their destinations. Otherwise, for example, membrane needed for plasma membrane growth risks being degraded in the vacuole. To ensure that these trafficking processes occur efficiently and effectively requires a host of proteins. Vesicular trafficking is highly conserved among eukaryotes. Because of this evolutionary conservation, we can use the budding yeast *Saccharomyces cerevisiae*, a genetically tractable, easily grown eukaryote, as a model organism for research that will apply across the eukaryotic spectrum, including humans. This thesis focuses on vesicular trafficking in *S. cerevisiae*.

Trafficking is a conserved process in eukaryotes

Trafficking can be divided into five conserved steps (Figure 1.1) including (1) budding, (2) transport, (3) tethering, (4) docking, and (5) fusion. I will discuss all of these

Figure 1.1 The five conserved steps of trafficking. The steps of trafficking and classes of proteins are represented schematically. 1) Vesicle buds from the donor organelle membrane. 2) Vesicle is trafficked through the cytosol along the cytoskeleton. 3) Vesicle is loosely associated with target membrane via tethering factors interacting with small GTPases. 4) SNARE complexes are formed and the vesicle docks with the target membrane. 5) SNARE complexes complete formation and membranes fuse, releasing vesicle contents into the target organelle and merging the membranes.

Figure 1.1

(1) Budding

(2) Transport

(3) Tethering

(4) Docking

(5) Fusion

Coat Protein

Small GTPases

v-SNARE   t-SNARE   Syntaxin t-SNARE

Tethering Factor   SM Protein
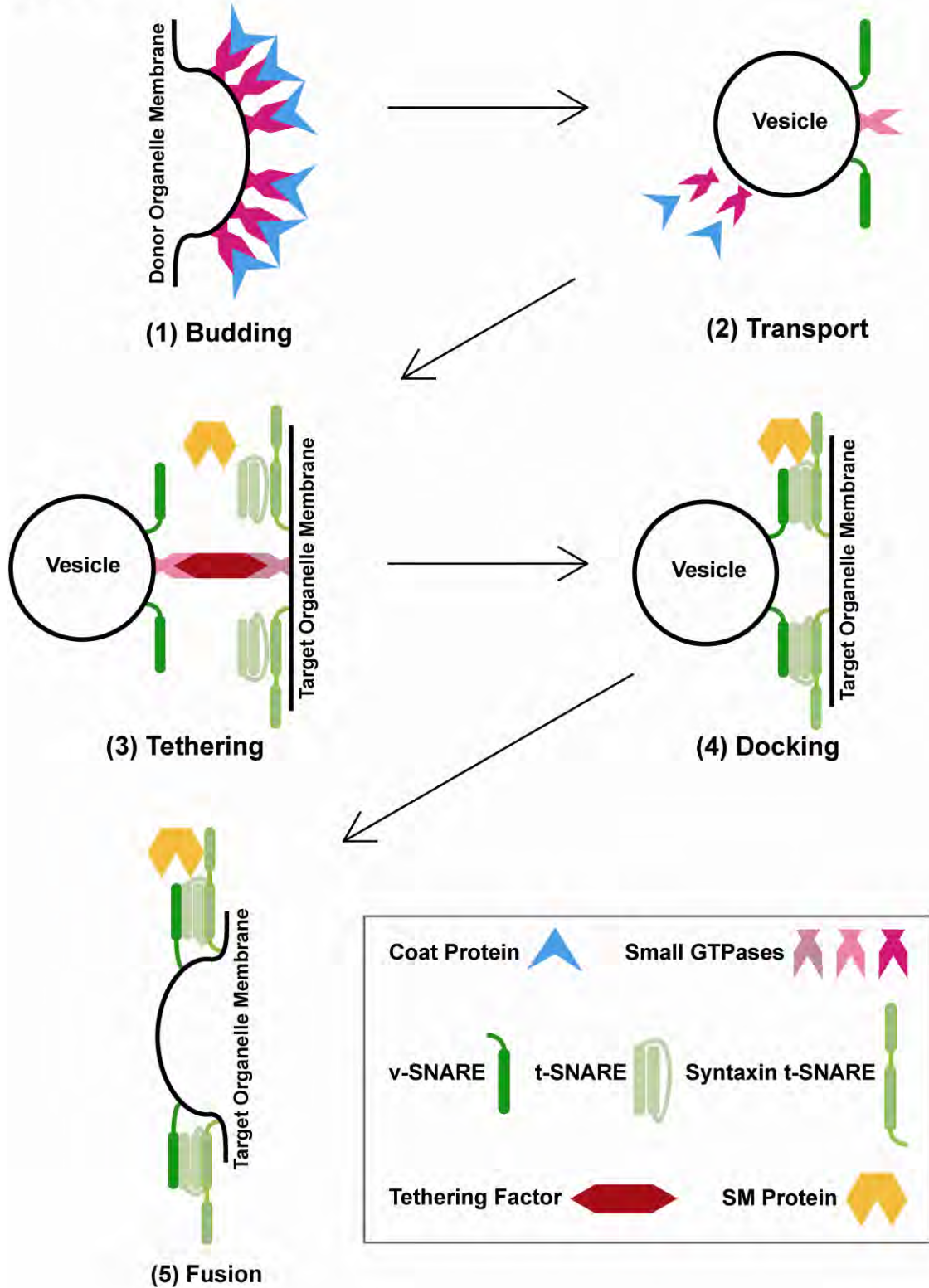
in detail later. Briefly, budding (1) is the formation of a cargo-carrying vesicle from the membrane of a donor organelle. Budding occurs when coat proteins, such as clathrin, COPI and COPII complexes, associate with the donor membrane and oligomerize to form large complexes, shaping a vesicle [1-3]. Transport (2) of the budded vesicles from the donor membrane to the target membrane occurs along either microtubules or actin filaments via interactions with the myosin and dynein classes of molecular motors [4]. Tethering (3) occurs when the vesicle arrives at the target membrane, and is defined as the formation of a reversible, often long distance, interaction between the target and vesicle membrane. This interaction occurs before docking and involves "tethering factors" [1,5,6]. Docking (4) is the formation of an irreversible link between the vesicle and target membrane via the formation of a fusion-competent SNARE (soluble NSF attachment receptor) protein complex [1,7]. Fusion (5) with the target membrane is the final step of trafficking. Cargo is released into the target organelle or extracellular space when the vesicle membrane merges with the membrane of the target organelle [1,7,8].

These basic steps are conserved both between organisms (*S. cerevisiae* trafficking is similar to *H. sapiens* trafficking) and between organelles (Golgi-plasma membrane trafficking is similar to ER-Golgi trafficking). Additionally, anterograde trafficking towards the plasma membrane, exocytosis, is similar to retrograde trafficking towards the nucleus, endocytosis. This poses another challenge for the eukaryotic cell. Not only is trafficking between these sealed organelle compartments essential, but trafficking between different organelles is a structurally very similar process. So how does a cell maintain the fidelity of vesicular trafficking? How does it ensure that cargo destined for

the Golgi apparatus is trafficked only to the Golgi apparatus, and, once the cargo has

arrived, that it fuses only with the Golgi apparatus?

The solution is multiple layers of protein families that are conserved in the same

manner as the trafficking steps themselves. These proteins ensure that cargo is trafficked

to the proper organelle, at the proper time, and fuses in the proper place [1,8-10]. The

majority of these proteins are essential; without them the cell cannot survive even under

ideal conditions. Mutations in most trafficking proteins cause disease or, often, cell death

[11-15]. Due to the fundamental and ubiquitous nature of the trafficking process, it is

unknown how many diseases and malfunctions of the biological system result from

defects in endo/exocytosis. The proteins involved in trafficking are numerous. In this

thesis I will deal largely with the families of proteins involved in the Tethering, Docking,

and Fusion (Figure 1.1 steps 3-5), and more specifically with those proteins involved in

exocytic trafficking from the Golgi apparatus to the plasma membrane: coat proteins,

small GTPases, tethering factors, SNARE proteins, and Sec1/Munc18 (SM) proteins. The

trafficking process begins with budding by coat proteins.

Coat Proteins

The major coat proteins in *S. cerevisiae* are clathrin, COPI, and COPII [1,2]. Coat

proteins provide the primary force for vesicle formation (Figure 1.1 light blue proteins).

Additionally these proteins provide regulation of cargo specificity and targeting through

several mechanisms. First, they aid in the selection of membrane cargo, ensuring that

cargo is loaded into the forming vesicle and non-cargo is excluded. Second, coat proteins

interact with Arf/Sar GTPases, which control both construction and deconstruction of the coat. If the GTPases dissociate from coat proteins before membrane fission, the forming vesicle will not bud and will instead be resorbed by the donor organelle [1,3,2]. Additionally, the coat confers specificity of targeting via direct interactions with motor and tethering proteins [3,9,14]. In addition to being controlled by coat proteins, budding is also regulated by small GTPases.

Small GTPases

Small GTPases play a role in many, if not most, processes in the cell, including all steps of trafficking from coat formation and budding to fusion at the target organelle (Figure 1.1 pink proteins). The superfamily of small GTPases is subdivided into five families, the Ras, Rho/Rac/Cdc42, Rab, Sar1/Arf and Ran families. The small GTPases of the Ras superfamily are involved in processes that range from acting as signaling hubs, to directing coat protein interaction, to regulating all steps of trafficking [16-19].

Each group of small GTPases regulates a specific subset of processes. The founding group of the Ras superfamily, the Ras GTPases are required for signal transduction to the nucleus for cell growth, survival, and differentiation [19,20]. As mentioned above, the Sar1/Arf family of small GTPases regulates vesicle budding via regulation of coat formation [20]. Ran GTPases are essential for trafficking in and out of the nucleus and for mitosis [19,20]. The Rho GTPases are involved in cytoskeletal regulation, gene expression, cell polarity, and motility [19,20]. Finally, the Rab GTPases regulate vesicle trafficking with individual Rabs localized to particular compartments, identifying

particular vesicles for specific target organelles [19]. Recent data has demonstrated they also play crucial regulatory roles as late as the final fusion step of exocytosis. In this paradigm small GTPases function both as spatial regulators and regulators of the target membrane, possibly directly controlling deformation of the membrane prior to fusion [21,22]. Most relevant to this thesis are the Rab and Rho/Rac/Cdc42 families.

Small GTPases cycle between two states: an active GTP-bound (guanosine triphosphate) state and an inactive GDP-bound (guanosine diphosphate) state. While active (GTP-bound), they bind to highly specific protein partners, defined as "effectors" to carry out diverse functions. In exocytosis, these functions include interaction with the forming and collapsing vesicle coat [1], spatial regulation of fusion [22], and interaction with tethering factors [23].

When the GTP associated with the GTPase is hydrolyzed, losing a phosphate group to become GDP (the GTPase then entering the so called "GDP-bound" state), the GTPase dissociates from the effector, effectively "switching off" the process mediated by the effector[19]. Small GTPases are further regulated by GDI (guanine nucleotide dissociation inhibitor) and GEF (guanine nucleotide exchange factor) proteins that regulate the binding and exchange of GTP and GDP [20]. These features make GTPases ideal regulators, allowing both spatial and temporal control of various processes. For example, when the small GTPase Sar1p interacts with its GEF protein and becomes GTP bound, a conformational change occurs. This reveals a small hydrophobic tail, anchoring Sar1p to the membrane. Once bound to the membrane, Sar1p recruits components of the

COPII vesicle coat, initiating budding. The coat can then be disassembled when Sar1p

hydrolyzes bound GTP to GDP and dissociates from the membrane as the hydrophobic

tail retracts [24]. Small GTPases form a first line of regulation. They assure that vesicles

bud when appropriate and bind to the correct motors, via direct and indirect interactions

[17,19,20,25]. This ensures trafficking to the correct organelles, where small GTPases then

interact specifically with appropriate tethering factors so fusion occurs only with the

target organelle.

SNARE Proteins

SNARE proteins form the core of the fusion machinery (Figure 1.1 green

proteins) [26]. The SNAREs are a group of highly conserved proteins that interact via ~60

residue "SNARE motifs" to form a four-helix coiled-coil complex that bridges the vesicle

and target membrane (Figure 1.2) [27-31]. The formation of this complex occurs in a parallel

manner, like a zipper, from the N-termini to the C-termini of the SNAREs and is

proposed to carry energy sufficient to initiate fusion of the vesicle and target membranes

[7,26,32-36]. SNARE complexes always contain four SNARE motifs, but there may be three or

four proteins utilized [26,37,38]. For example in exocytosis between the Golgi apparatus and

the plasma membrane, three SNARE proteins are utilized, one of which, Sec9p, provides

two SNARE motifs to the complex [37]. All complexes, however, are formed from one

SNARE motif associated with the vesicle and three associated with the target membrane

[31]. Most, though not all, SNARE proteins are anchored in the relevant membrane via

Figure 1.2 The yeast exocytic four-helix SNARE complex represents a conserved architecture. The crystal structure of the fusion-competent yeast exocytic SNARE complex, (pdb 3B51) includes the t-SNARE Sec9p, v-SNARE Snc2p, and Syntaxin t-SNARE Sso1p. Sso1p and Snc2p are anchored in the plasma and vesicle membranes respectively by transmembrane domains. The loop connecting the two helical domains of Sec9p, the liner regions, and transmembrane domains have been modeled in as they were not present in the crystal structure. It is unknown how Sec9p is localized to the plasma membrane. Image rendered using Pymol (pymol.org)

Figure 1.2

Vesicle Membrane

Plasma Membrane

v-SNARE (Snc2p)   t-SNARE (Sso1p)   t-SNARE (Sec9p)
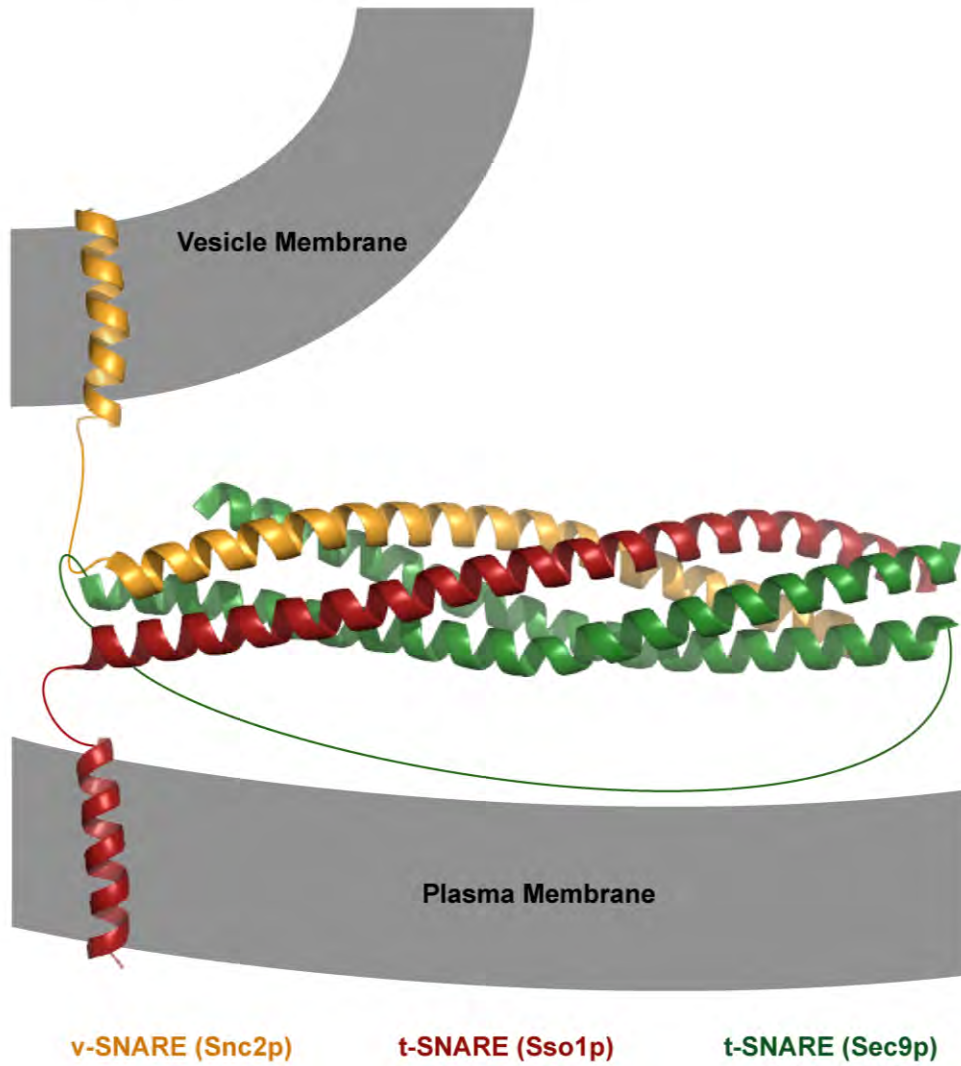
Yeast Exocytic SNARE Complex (pdb 3B51)

either transmembrane domains or reversible post-translational modifications such as palmitoylation [7,39].

     There are several classifications into which SNARE proteins can be divided. This thesis will use the locational descriptors, so-called "v-SNAREs" on the vesicle membrane and "t-SNAREs" on the target membrane [30]. The t-SNAREs can be further divided into "syntaxin t-SNAREs" that are structurally similar to the neuronal t-SNARE syntaxin1a and "non-syntaxin t-SNAREs" that have a different architecture. Syntaxin t-SNAREs contain a short N-terminal peptide and a large auto-inhibitory "H_{abc} domain" N-terminal to the SNARE motif. In some syntaxin t-SNAREs, this domain can bind the SNARE motif, forming a "closed" conformation and inhibiting formation of the SNARE complex [30,32,37,40]. For SNARE complex assembly to occur, the H_{abc} domain must be released from the SNARE motif, which then interacts with two more t-SNARE motifs, before forming a fusion-competent SNARE complex with the v-SNARE motif (Figure 1.3). In some systems, the association between the H_{abc} domain and SNARE motif is weak or almost non-existent, while in others the association is very tight. For example, in *S. cerivisiae* exocytosis to the plasma membrane the syntaxin t-SNARE Sso1p has such a tight association between the H_{abc} domain and SNARE motif that formation of SNARE complex *in vitro* happens on a scale of days [41,42]! An "opener" protein has been hypothesized to encourage Sso1p to transition from the closed to open state. Our lab is currently screening for this protein.

Figure 1.3 Schematic representation of formation of the fusion-competent SNARE complex. Syntaxin t-SNAREs may have an autoinhibited closed conformation. Sso1p has a closed conformation which provides the rate limiting step in SNARE complex formation *in vivo.* An "opener" protein has been hypothesized to facilitate transition of Sso1p from the closed to open conformation.

Figure 1.3

**Closed
Syntaxin t-SNARE**   **Open
Syntaxin t-SNARE**   **Binary
t-SNARE Complex**   **Fusion Competent
SNARE Complex**
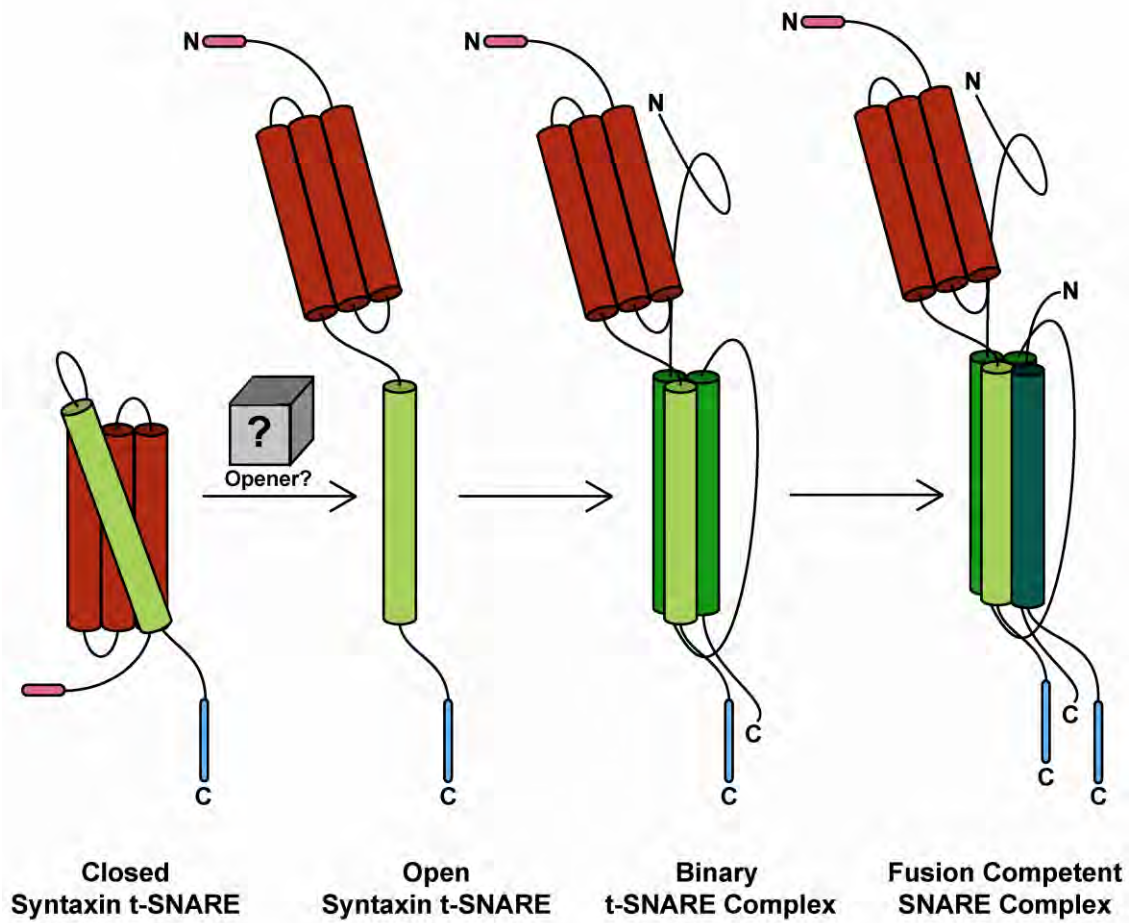
Though SNAREs are presumed to be sufficient on their own for specificity and fusion [43-45], two problems follow. First, SNARE proteins are not always localized, but may instead distribute across the entire surface of the membrane they occupy [46,47] and some SNAREs, such as Vti1p, are required for multiple trafficking steps [48]. Thus, even if SNARE proteins could ensure fidelity on their own, they cannot localize fusion to the narrow regions required for proper secretion in diverse systems including from bud formation in *S. cerevisiae* and neurite outgrowth and synaptic release in higher eukaryotes. Secondly, while *in vivo* SNARE complexes appear to maintain high fidelity, *in vitro,* SNAREs can be promiscuous in their interactions [28,40,49-51]. There are multiple examples of SNARE promiscuity [28,33,49-52]. In the context of the cell, allowing this broad localization and possible promiscuity of fusion would result in severely misdirected cargo. Prevention of misdirection requires another layer of regulatory proteins.

Tethering Factors

When the trafficking vesicle arrives at the target organelle, tethering factors are there to form a bridge between vesicle and membrane, thus providing another layer of spatial regulation (Figure 1.1 red protein/complex). Tethering factors may traffic with the vesicle or be present prior to arrival. Tethering is defined as a reversible association with the target membrane and vesicle, stabilized by these factors, prior to docking and fusion [5,53,54]. Tethering proteins can be divided into two classes, the coiled-coils and the large multi-subunit complexes [55]. This thesis will deal primarily with the exocyst, which is a member of the large multi-subunit tethering complexes, which include the COG

(conserved oligomeric Golgi), Dsl1p, GARP (Golgi-associated retrograde protein),

HOPS (homotypic fusion and vacuole protein sorting), CORVET (class C core

vacuole/endosome tethering), and TRAPP (transport protein particle) complexes.

Despite a conserved function, tethering factors have very low sequence identity,

less than 10% in most cases [55-57]. However, some structural similarity is becoming

apparent. First, there is identical bundle topology between the subunits of the exocyst and

COG complex, and, evident more recently, between subunits of the exocyst, subunits of

the Dsl1p complex, Myo2p, and recently limited domain homology with Munc13 [53,56,58-60].

A logical explanation for this overall similarity is divergent evolution. This involves

duplication and modification of relevant genes along the trafficking pathway as new

organelles evolved. It remains to be tested if these structural similarities will bear out as

predictors of subunit function. This thesis is primarily concerned with the final step of

exocytosis, trafficking from the Golgi apparatus to the plasma membrane. The tethering

factor for this process is known as the exocyst and will be discussed in more detail later.

Further, these large oligomeric complexes have multiple roles beyond simply

connecting the target membrane and vesicle. One role coming to light in recent years is

the direct regulation of docking and fusion, steps of trafficking after tethering. In these

steps, SNARE proteins form a fusion-competent SNARE complex and fusion of the

membranes occurs. For example, the HOPS complex, involved in trafficking between the

vacuole and endosomes, interacts directly with cognate SNARE proteins to "proofread"

them and ensure fidelity by restricting the formation of SNARE complexes to those

between appropriate partners [61]. In keeping with their conservation of function, but not of sequence, other complexes appear to have similar roles via different mechanisms. The GARP complex, involved in retrograde Golgi trafficking, interacts with one of the associated SNARE proteins, Tlg1p to encourage SNARE complex formation [62]. Similarly, the COG complex, involved in Golgi trafficking, interacts with the SNARE protein Sed5p to stabilize the formation of SNARE complexes [63]. Also similarly, the Dsl1 complex interacts with the SNARE protein Use1p to stabilize formation of a SNARE complex [64]. Finally, the exocyst has been shown to interact with the SNARE protein Sec9p *in vitro* [65]. This suggests that the exocyst is a member of the growing majority of large tethering complexes that interact with and regulate SNARE complex assembly. Though the exact mechanisms of these interactions are poorly understood, the similarity in their function is undeniable and strengthens the suggestion that the large oligomeric tethering complexes are indeed products of evolutionary divergence.

Sec1/Munc18 Proteins

SM (Sec1/Munc18) proteins form yet another layer of regulation via interactions directly with SNAREs and often with tethering complexes as well (Figure 1.1 yellow protein) [53,66-68]. Although the SM proteins show significant structural and sequence similarity, the elucidation of these interactions has been confusing and often contentious [8,53,67,66]. SM proteins appear to utilize diverse modes of binding to their cognate SNAREs, SNARE pairs, and SNARE complexes. SM proteins have even been shown to have both

stimulatory and inhibitory effects on SNARE complex assembly and fusion [53,66]. Recently, however, much of the prior confusion was revealed to be artifactual due to insufficiently quantitative methodologies, and a more unifying mode of binding is emerging [69]. SM proteins appear to bind primarily via two binding pockets, a small hydrophobic pocket which interacts with the N-terminal peptide of the syntaxin and a larger pocket that wraps around and binds the autoinhibited form of the target syntaxin t-SNARE [53,68,67,69].

Sec1p, the SM protein most relevant to this thesis appears to have a slightly different interaction mode than other SM proteins. First, there is no interaction between Sec1p and its cognate syntaxin t-SNARE, Sso1p [70]. Second, an interaction occurs between Sec1p and the complete ternary SNARE complex [70,71]. This interaction likely uses the same cleft that in other SM proteins binds the closed conformation of the syntaxin t-SNARE [72]. Finally, Sec1p has recently been implicated activities prior to regulation of SNARE complex formation [72].

Proteins Essential for Plasma Membrane Exocytosis

The Exocyst

The exocyst is a member of the large multi-subunit complex family of tethering factors, which also includes the COG, Dsl1p, GARP, HOPS, CORVET, and TRAPP complexes [53,58,73-76]. These factors all contain multiple subunits, assembled into a large

oligomeric complex. They have been proposed to interact reversibly with the plasma and vesicle membranes through small Rab and Rho GTPases that function as spatial landmarks before docking [22]. However, there is recent evidence that the GTPase role in tethering may be more complex than previously thought. Beyond acting as timers to associate tethering factors with membranes, GTPases may interact with proteins and even with the membrane itself to directly regulate docking, fusion, and membrane deformation [21,77].

The exocyst is the oligomeric tethering complex essential for Golgi to plasma membrane anterograde trafficking [78]. The complex is comprised of one copy each of eight subunits: Sec3p, Sec5p, Sec6p, Sec8p, Sec10p, Sec15p, Exo70p, and Exo84p and interacts with small GTPases on the vesicle and plasma membranes (Figure 1.4). The exocyst is conserved in all eukaryotes [5,53]. The "Y" shape of the exocyst in the schematic diagram derives from a similar shape displayed in high resolution rapid-freeze deep-etch EM (Electron Microscopy) images of purified bovine brain exocyst complexes fixed with glutaraldehyde [79]. Complexes that have not been fixed in glutaraldehyde appear to "bloom" into multi-armed flower shapes. It remains unclear if these structures represent *in vivo* structures or not.

The exocyst subunits, except Exo70p and Exo84p, were originally identified in a yeast mutational screen for yeast with defective secretion [15]. The complex was found to localize to active sites of polarized secretion [5,22,23,78,80]. This temporal and spatial location positions the exocyst appropriately to be an important regulatory factor in exocytosis to

Figure 1.4 The exocyst complex interacts with the vesicle and plasma membranes to tether before docking. The eight-subunit exocyst interacts with the small GTPase Sec4p on the vesicle membrane via Sec15p. Interaction with the plasma membrane occurs via an unidentified "anchor" interacting with Sec6p to localize the exocyst, and with the small GTPases Rho1p, Rho3p, and Cdc42p interacting with Sec3p and Exo70p.

Figure 1.4
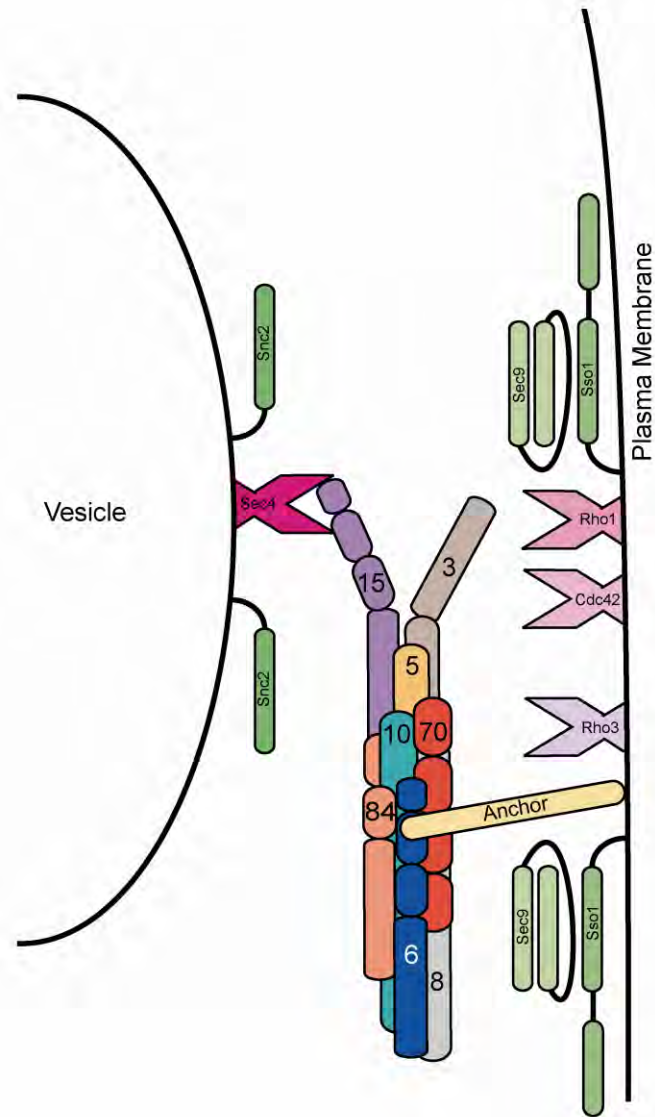
the plasma membrane. The exocyst is proposed to be the tether for Golgi-plasma membrane trafficking because of interactions with Sec4p on the vesicle and Cdc42p, Rho3p, and Rho1p on the plasma membrane [81-86]. However, it has recently been shown that these GTPases are not, in fact, sufficient for maintaining localization of the exocyst. Instead, the exocyst remains spatially localized via an interaction between the Sec6p subunit and an as-yet unidentified anchor protein [87]. Though the exocyst is considered a vesicle tether, the "tethering" function of the exocyst has yet to be directly demonstrated.

Several subunits of the exocyst or portions thereof have been crystallized, showing a conserved helical bundle structure [5,59,88,89]. Currently there are crystal structures for full-length Exo70p from yeast, mice, and humans [90-92], and C-terminal regions of yeast Sec6p [88] and Exo84p[90], and *Drosophila* Sec15p [93]. These structures display a startling similarity. The helical bundle topology of all these proteins is identical, suprising considering the lack of sequence identity. This conserved architecture has also been observed in other members of the exocyst and subunits of other large oligomeric tethering complexes as well as, non-tethering, proteins. Dsl1p, Cog4p, Myo2p, Myo4p, Sec6p, Exo70p, Exo84p, Sec15p, Tip20, and Cog2p all show an identical bundle topology and similar overall architecture by crystal structure analysis, and Sec10p displays a similar structure by threading algorithms [5,59,89,88]. These results suggest a similar origin, if not mechanism of function, possibly via the structurally similar "E domains" (named for the relevant domain of the Cog4p subunit) suggested by Richardson et. al [59]. Like the oligomeric complexes as a whole, this structural similarity suggests that a gene duplication followed by divergent evolution, was responsible for the current

exocyst consisting of eight proteins with remarkably similar structure, though little

sequence identity. Unfortunately, the new wealth of structural information has not yet led

to a directly corresponding increase in knowledge about the function of the exocyst as a

whole or of subunits individually. New research must connect the structural information

to functional knowledge of exocyst subunits and possibly to subunits of other complexes.

Though functional information is not complete, neither is it absent. We know, for

instance, that all of the exocyst subunits are essential, though *sec3Δ* strains are capable of

growing very slowly on minimal media [83]. Temperature-sensitive mutants of most strains

cease growth or die [15,5]. This makes further analysis often difficult. Disruption of the

secretory pathway often causes phenotypes that don't allow direct identification of the

disrupted mechanism, such as a backup of unfused vesicles at sites of secretion [15].

However, some insight has been acquired via the temperature-sensitive mutants, as

localization and interactions can be examined concurrently with exocytic failure.

Subunits of the Exocyst

The subunits of the exocyst play multiple roles both within and external to the

complex. Elucidation of the interactions and functions of the exocyst subunits is

challenging, however, recent *in vivo* and *in vitro* data has moved the field forward. In

addition to intra-exocyst interactions, the exocyst interacts with both the vesicle and

plasma-membrane via small Rab and Rho GTPases. Exocyst-vesicle interaction is

mediated by the exocyst subunit Sec15p via the small Rab GTPase Sec4p on the vesicle

membrane [23,94]. On the plasma-membrane, Sec3p, through interactions with the small

GTPases Cdc42 and Rho1 (Figure 1.5 a), was proposed to be the primary localizing force for the exocyst [82-84]. However, certain GFP tags on Sec3p are sufficient for stabilizing localization when it would otherwise be disrupted [87,95], though this result has been disputed [96]. Similarly, Exo70p may be a second spatial landmark for the exocyst, as it interacts with both the small GTPase Rho3p (Figure 1.5 b)[81] and with phosphatidylinositol 4,5-bisphosphate [97]. In one model of exocyst assembly wherein most subunits are trafficked with the vesicle, Exo70p and Sec3p are already present and localized at the plasma membrane presumably via interactions with their small Rho GTPases [98]. In mammals, Sec5 has an additional GTPase binding domain which interacts with RalA and RalB to aid in exocyst targeting via paxillin during cell migration [99,100]. Interestingly, Sec5-RalA/B interactions are implicated in oncogenesis and immunity responses via interaction with TBK1 [101,102].

Internal exocyst contacts are multitudinous, though far from completely described (Figure 1.5). Beginning from the vesicle, Sec15p is linked to the exocyst by interactions with the subunit Sec10p [94] (Figure 1.5 (c)) with which Sec15p forms a subcomplex, though the function of this subcomplex is unknown [23]. Sec10p in turn interacts with Sec6p [89,88], with Exo70p [90], with Sec5p and possibly Exo84p [103] (Figure 1.5 d, e, f, g). Sec6p interacts with Sec8p [88], as well as Sec5p (Figure 1.5 h,i), but Sec6p shows no interactions via a yeast two-hybrid assay [103]. The C-terminal domain of Sec6p is sufficient for interaction with Exo70p though not for Sec8p interaction [88]. Exo70p completes a loop, interacting with Sec5p (Figure 1.5 j)[103]. Sec5p interacts with Exo84p, Sec8p, and Sec3p (Figure 1.5 k, l, m) [103]. Finally, Sec3p may interact with Sec8p (Figure 1.5 n) [23].

Figure 1.5 Schematic of intra and extra exocyst interactions. Interactions between yeast exocyst components (colored, numbered proteins) have been mapped schematically as well as external interactions with known non-exocyst proteins (grey ovals). (As referenced in [5])

Figure 1.5
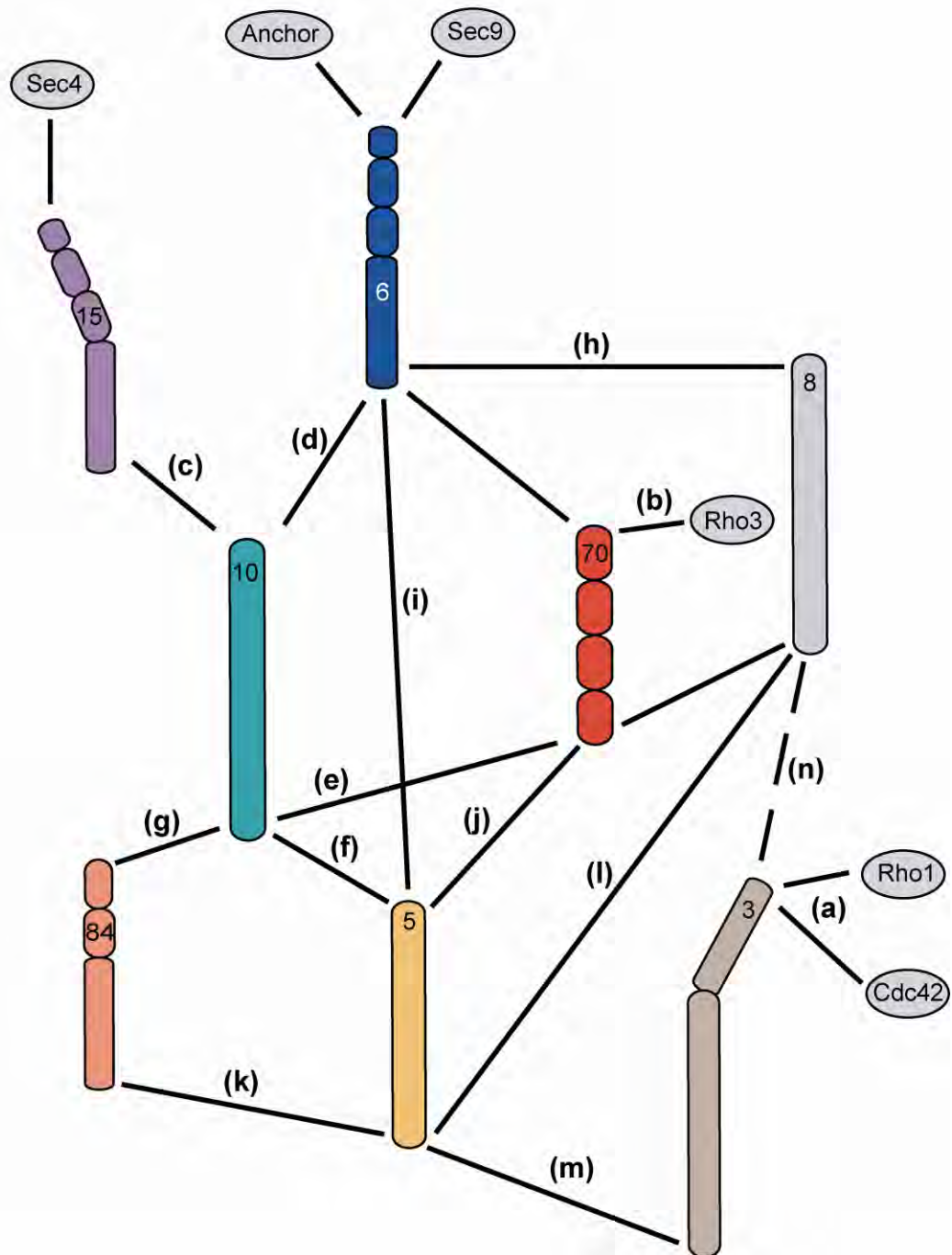
Additionally, in the mammalian exocyst, interactions have been shown by yeast two-hybrid between Exo70, Exo84, and Sec15 [104].

Subunits of the exocyst are not limited to interactions within the complex and with small GTPases. Many subunits are also involved in interactions with non-exocyst proteins. For example, Sec15 interacts with the polarity-determinant protein Bem1p [105,106] and Sec2p, the exchange factor for Sec4p [107]. Similarly, Sec10p is involved in hormone *Drosophila* oocyte [108]. Additionally in *Drosophila,* trafficking of the protein DE-Cadherin appears to be controlled by a subset of the exocyst including Sec5, Sec6, and Sec15 [109]. Exo84 is required for the foundation of apical identity of epithelial cells, via its direct interaction with the transmembrane protein Crumbs [110]. Exo84p also directly interacts with Sro7p and Sro77p which may play a role in the establishment of cell polarity and may interact with Sec9p [111].

The bulk of this thesis focuses on the role of Sec6p, and its involvement with two associated non-exocyst proteins. The C-terminal region of Sec6p has been crystallized, showing a conserved helical bundle fold, similar to other members of the exocyst and other tethering complex subunits (Figure 1.6) [5,59,89,88]. Additionally, some limited homology has been discovered between Sec6p and portions of Munc13p, which interacts with SNARE proteins [60]. Conserved residues in this C-terminal region have recently been shown to be essential for the maintenance of exocyst localization at sites of secretion via a proposed anchor protein [87]. These residues are required for localization even when wild-type Sec3p and Exo70p remain in the complex. This suggests that, while Exo70p and

Figure 1.6 The conserved architecture of the C-terminal region of Sec6p. The C-terminal region of *S. cerevisiae* Sec6p (pdb 2FJI), shows a conserved fold similar to portions of *D. melanogaster* Sec15 (pdb 2A2F), *S. cerevisiae* Exo84p (pdb 2D2S), *S. cerevisiae* Exo70p (pdb 2PFV), *S. cerevisiae* Cog2p (pdb 2JQQ), *H. sapiens* Cog4 (pdb 3HRO), *S. cerevisiae* Tip20p (pdb 3FHN), *S. cerevisiae* Dsl1p (pdb 3ETU), and *S. cerevisiae* Myo2p (pdb 2FH). Visualized using Pymol (pymol.org)

Figure 1.6

Sec6 **Exo84** **Sec15** **Exo70**

Cog2 **Cog4** **Dsl1** **Tip20**

Myo2
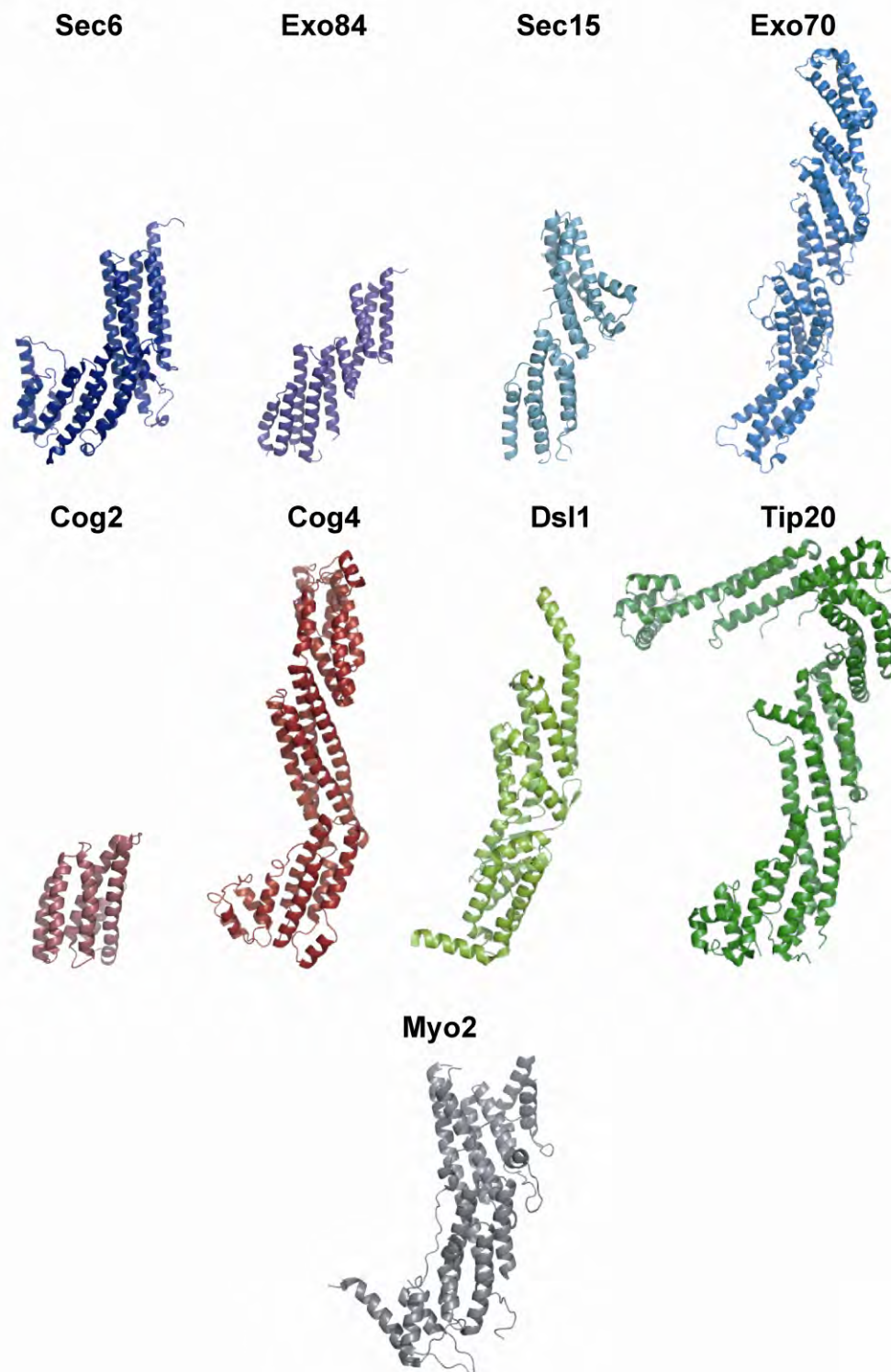
Sec3p may be required for initial localization, they are insufficient for its maintenance. Whether the exocyst can be localized via Sec6p-Anchor interaction in the absence of Exo70p and Sec3p interaction with their GTPases remains untested. The N-terminal region of Sec6p remained largely unexamined until this thesis, as it has been resistant to *in vitro* purification techniques and deletion analysis.

Sec6p tightly dimerizes *in vitro*, such that no monomer can be detected even at very low concentrations of Sec6p [65]. Though the existence of the dimer has not yet been successfully demonstrated *in vivo*, a "free pool" of Sec6p, dissociated from the exocyst, clearly exists in the cytosol of yeast. This free pool migrates similarly to purified dimer on gel filtration columns, suggesting that the free pool of Sec6p exists as a dimer in the cell [65].

Though Sec6p interacts with the t-SNARE Sec9p *in vitro*, the C-terminal region is not sufficient for that interaction [65]. I hypothesized that the interaction between Sec6p and Sec9p might relieve auto-inhibition of the syntaxin t-SNARE Sso1p, however, addition of purified Sec6p *in vitro* imposed a further threefold inhibition on formation of the binary t-SNARE complex between Sec9p and Sso1p. This suggests that *in vivo* another factor is present and the apparent slowing represents trapping of an intermediate bound state [65].

Exocytic SNAREs: Sec9p, Sso1p, Snc2p

The exocytic SNARE proteins are the v-SNARE Snc2p, the non-syntaxin t-SNARE Sec9p, and the syntaxin t-SNARE Sso1p [15,46,112-114]. Deletions of either *SSO1* or its homolog *SSO2* have no notable phenotype during normal growth, though there is evidence of sporulation-specific function of Sso1p [41,115,116]. The Ha, Hb, and 3' UTR (untranslated region) of Sso1p are required for proper sporulation [116,117]. Because Sso1p and Sso2p are functionally identical during the growth dealt with in my experiments, I will discuss only Sso1p in depth in this thesis.

Snc2p is an 115 amino acid v-SNARE homologous to synaptobrevin utilized for exocytosis to the plasma membrane with a homologous protein Snc1p with overlapping but not identical function [118,119]. For the aspects of fusion discussed in this thesis, Snc1p and Snc2p may be considered equivalent. Snc2p is anchored in the vesicle membrane by a transmembrane domain [119].

Sec9p is a 659 amino acid t-SNARE, with two SNARE motifs connected by a long linker [15,46]. When in the SNARE complex, these motifs are parallel, with the linker spanning the distance between them. However, when Sec9p is not in complex, it remains largely unstructured [27,42]. When in a heterodimeric binary t-SNARE complex with Sso1p, one Sec9p SNARE motif remains largely structured, and the other alternates between a structured and unstructured state [120]. Unlike most SNAREs, Sec9p has no transmembrane domain, and any post-translational modification that could anchor the protein to the membrane has not yet been identified [46]. Additionally, Sec9p has a large N-terminal region that appears to be non-essential in the lab environment. Deletion of this N-terminal

region has no effect on cells, and the function of this region remains unknown [46].

Additionally, Sec9p has been shown to interact with Sec6p *in vitro* [65]. As mentioned above, Sec9p forms a heterodimeric binary t-SNARE complex with its t-SNARE partner, Sso1p.

Sso1p is a prototypical syntaxin t-SNARE, with a transmembrane domain, SNARE motif, linker, and H_{abc} domain. Though the short N-terminal peptide of Sso1p is truncated and not well conserved [112,115,121]. Unlike some other syntaxin t-SNAREs, the autoinhibition of the H_{abc}:SNARE motif interaction is strong in Sso1p [42,115]. This interaction is so tight *in vitro* that the dissociation of the H_{abc} domain and formation of a binary t-SNARE complex between Sec9p and Sso1p/Sso2p requires forty-eight hours [65,112,115]! This tight association that prevents formation of the SNARE complex provides another source of regulation, as it prevents promiscuous SNARE pairing. However, the association is so tight that an "opener" protein, or proteins, appears to be required to facilitate dissociation of the H_{abc} domain and formation of the binary t-SNARE complex on a biological timescale [115]. Once the binary t-SNARE complex has been formed, *in vitro,* the ternary fusion-competent SNARE complex can form rapidly [41,42,115].

Sec1p

Sec1p is the SM protein essential for exocytosis to the plasma membrane [71]. Sec1 is the founding member of the SM proteins, though it surprisingly remains a poorly understood member of the family. Sec1p is one of the SM proteins that causes the most controversy among researchers, as its binding modes with cognate SNARES differ most

from others in the family. Unlike most other SM proteins, Sec1p interacts with the

complete ternary SNARE complex to regulate fusion with the plasma membrane [71], rather

than with the N-terminal peptide or closed, autoinhibited form of its syntaxin Sso1p

[53,68,66,67]. However, like other SM proteins, Sec1p interacts with its associate tethering

complex, the exocyst, albeit weakly [122]. The crystallized squid neuronal nSec1 displays a

common architecture shared by SM proteins [123]. Three domains are organized in a

horseshoe shape around a large central binding pocket. Additionally, there is a small N-

terminal hydrophobic binding pocket, which in some SM proteins interacts with the N-

terminal peptide of the syntaxin t-SNARE [53,68,66,67,69,124].

The State of the Field

Many questions currently loom both large and small in the field of trafficking in

general, and the exocyst specifically. Among those questions: what is the purpose of a

large, eight protein, tethering complex that is almost a megadalton in size? And why is

the exocyst a complex? What are the collective and individual functions of the subunits?

Why do the eight proteins have such similar architecture? Does the existence of a

complex suggest that these proteins function both as free and oligomeric forms? Does the

similarity of exocyst subunits to the proteins of other tethering complexes such as Dsl1p,

Cog4, etc. suggest a conserved evolutionary mechanism that evolved along with the

compartments they serve? If so, what can the study of individual protein function allow

us to predict about the function of other similarly folded proteins? Clearly, the functions

cannot be identical or all of the C-termini of the exocyst proteins would be doing the same thing; and there is enough divergence in interaction to show that this is clearly not the case. This would not be the first example of similarly structured subunits with differing functions. The proteosome provides another example of a complex likely arising from gene duplication with divergence of function between subunits [125,126].

And there are questions still more specific: Many of the uncrystallized N-termini remain largely unstudied, as was the N-terminal region of Sec6p until this thesis, due to difficulties with purification and aggregation. Are they resistant to purification for a common reason? Perhaps the difficulties indicate lengthy binding regions, buried in the core of the complex, leaving the C-termini to give rise to the mysterious flower-shaped structure revealed by the EM images [79].

And finally, on the largest, and most grandiose, scale: What can we learn about the origins of ourselves, of all eukaryotes, from this system? The elements of trafficking are clearly linked with common structures and common mechanisms separated by apparently small details that nonetheless maintain the system's fidelity, maintaining crucial life processes. What might understanding a system this ubiquitous to life reveal? In the practical realm, our understanding of trafficking as a whole is comparatively pitiful, but in recent years it is experiencing a dawning renaissance as small discoveries snowball into further, larger discoveries and conclusions. The field appears perched on a cusp of understanding, as if a few more small insights might push our collective

knowledge over a metaphorical barrier range and into a whole new realm of biological wisdom.

This sudden expansion of knowledge has vast potential in the medical realm for understanding a host of ailments. Everything, from the placement of receptors and channels on the surface of neurons to the absorption of nutrients in the stomach, is controlled by these proteins and processes [11-13,53,54,127]. The exocyst, for example, appears to be essential for the formation of functional epithelial apical membranes, which absorb nutrients, for tight junctions, which keep stomach contents in the stomach, and for growth of the neurites and dendrites, which make your brain function as a brain rather than a dissociated set of spherical cells, indeed the exocyst is required as early as the first divisions of oocyte cells [53,128,129,132,133,130,131]. Complete knowledge of these proteins and pathways could have an incalculable impact on both our understanding of and future treatment of our bodies.

On a far less grandiose, and more realistic, scale are the questions that might be answered by the study of one protein, the questions which strive to push the field over that long-sought rise. This thesis strives to answer that level of question about one such protein – Sec6p. There remains much to be discovered about the function of the exocyst and its subunits, and much that might be generalized to other trafficking pathways.

Most of the research on Sec6p, and indeed on many oligomeric complex subunits, including Exo84p, Cog2, and Sec15p has been performed on the C-terminal region due to that portion's comparative tractability [59,65,79,87,89,88,90,93,134]. Exo70p, for which the full

protein has been crystallized, displays a similar N-terminal architecture to the conserved

fold of the C-terminal region of Sec6p [5,59,89-91]. However the C-terminal region of Sec6p is

not sufficient for function in the cell [88]; so what is the function of the N-terminal region?

Our lab demonstrated that the C-terminal region is insufficient for binding of Sec9 [65].

Why might Sec6p bind to Sec9p? *in vitro* SNARE assembly assays suggest that Sec6p is

inhibiting SNARE complex assembly by more than threefold [65]. Could this apparent

inhibition actually be capturing an intermediate binding state? Is Sec6p supposed to

"hand off" Sec9p to another accessory protein *in vivo* that then enhances the formation of

the binary t-SNARE complex? What protein might this be? Sro7p has also been shown to

inhibit SNARE complex assembly through an interaction with Sec9p [135], could that be the

other regulatory factor? Could it be one of the other identified regulatory proteins?

Perhaps one of the other exocyst subunits could be the missing factor? Could the SM

protein, Sec1p, or one of the GTPases, or the putative anchor protein be involved? Or is

there another, undiscovered, step to docking? Is there a reason that Sec6p might actually

be inhibiting the formation of the binary complex?

What other functions does the N-terminal region of Sec6p perform *in vivo*? What

proteins does it interact with, and for what purpose? Does the N-terminal region form the

core of the exocyst and interact with multiple subunits thus shielding the "sticky" tail

from aggregation and non-specific interaction? What about the free pool of Sec6p that

exists as dimers in the cytosol [65]? Is the free pool a "storage tank" for Sec6p until it's

needed for exocyst assembly, or is there a separate function for Sec6p outside its function

as an exocyst subunit? Does the function of Sec6p fit with the emerging trends in

trafficking biochemistry? These are the questions I hope to explore in this thesis, and to see answered in full by the field in years to come.

Chapter II:

Disruption of N-terminal exocyst binding residues in

Sec6p results in a stable subcomplex

localized to sites of exocytosis

ABSTRACT

Trafficking of protein and lipid cargo through the secretory pathway in eukaryotic cells is mediated by membrane-bound vesicles. Secretory vesicles are targeted to sites of exocytosis on the plasma membrane in part by a conserved multi-subunit protein complex termed the exocyst. In addition to tethering vesicles to the plasma membrane, the exocyst complex may directly regulate assembly of the SNARE complex, which is required for membrane fusion. Here we show that an interaction between the plasma membrane t-SNARE Sec9p and the yeast exocyst subunit Sec6p can be observed *in vivo*. Point mutations in the N-terminal domain of Sec6p result in temperature sensitive growth and secretion defects, without loss of Sec6p-Sec9p interaction. At the non-permissive temperature, the exocyst subunits Sec5p, Sec10p and Sec15p are mislocalized and are absent from the exocyst complex. The resulting subcomplex, containing Sec6p, Sec3p, Sec8p, Exo70p and Exo84p, remains stably assembled and localized at sites of polarized secretion. Additionally, one of the *sec6* temperature sensitive mutants displays a loss of binding to the yeast regulatory protein Sec1p. *In vitro* binding studies indicate a direct interaction between Sec1p and the wild-type Sec6p protein, suggesting close interplay between Sec6p and Sec1p in the regulation of SNARE complexes.

INTRODUCTION

Exocytosis in eukaryotes requires the proper trafficking of membrane bound vesicles between functionally and chemically distinct organelles and the plasma membrane for growth, secretion, and proper cellular functioning. Trafficking is a conserved and highly regulated process, utilizing multiple classes of essential proteins to ensure that cargo is properly delivered both spatially and temporally [8] (and references therein). SNARE proteins on the target membrane (t-SNAREs) and vesicle (v-SNAREs) interact to form a four-helix-bundle, termed the SNARE complex, which bridges the vesicle and plasma membrane for fusion. The formation of fusion competent SNARE complexes is regulated by a number of proteins, including tethering factors such as the exocyst and the Sec1/Munc18 (SM) proteins [67].

Prior to SNARE complex assembly, vesicles are hypothesized to be held in a reversible association with the target membrane, a process termed tethering. Tethering is thought to be facilitated by long coiled-coil proteins and large protein complexes, including the exocyst, COG (conserved oligomeric Golgi), Dsl1p, GARP (Golgi-associated retrograde protein), HOPS (homotypic fusion and vacuole protein sorting) and TRAPP (transport protein particle) complexes [53,58,73,74]. Tethering factors have been proposed to interact with the target and vesicle membranes via small GTPases of the Ras superfamily [22], although recent data suggests that the role of the GTPases may be primarily regulatory [22,77], and membrane anchoring may be achieved through interactions with other proteins [87].

In addition to membrane tethering, multi-subunit complexes such as the exocyst may directly regulate membrane fusion through interaction with the SNARE fusion machinery. For example, a recent study revealed that the HOPS complex interacts with the vacuolar trans-SNARE complex in yeast to confer specificity [61]. Similarly, the COG, Dsl1p, and GARP complexes bind to individual SNAREs or SNARE complexes [62-64]. The exocyst is also implicated in SNARE regulation. We previously demonstrated that recombinant Sec6p, an exocyst subunit, interacts with the yeast plasma membrane t-SNARE Sec9p *in vitro*; in the absence of other factors, Sec6p inhibits the interaction between Sec9p and its SNARE partners [65].

The regulation of SNARE proteins and SNARE complex formation may also be carried out in conjunction with the Sec1/Munc18 (SM) protein family. The SM proteins interact with both individual SNARE proteins and SNARE complexes, making their function(s) challenging to elucidate, as SM proteins appear to bind their cognate SNAREs through several distinct modes of interaction (for review see [66,136]). Several of the SM proteins have also been shown to interact with tethering complexes. The interaction between the ER to Golgi SM protein Sly1p and the COG complex is required for SNARE complex assembly [137], while the HOPS complex includes the vacuolar SM protein Vps33p among its subunits [73]. Similarly, the yeast exocytic SM protein Sec1p interacts with the exocyst complex [122]. Currently, the precise functions of these interactions are unclear.

The exocyst is a large oligomeric complex implicated in tethering secretory vesicles to the plasma membrane. The exocyst is structurally and functionally related to the large multi-protein tethering complexes COG, Dsl1p, and GARP [53,58,59,138-140]. It is conserved in all eukaryotes and is essential for growth and secretion [5,22,23,78,141], playing essential roles in exocytosis, endocytosis, and cytokinesis [108,130,142,143]. The exocyst is composed of eight subunits: Sec3p, Sec5p, Sec6p, Sec8p, Sec10p, Sec15p, Exo70p, and Exo84p. Four subunits contain conserved rod-like structures composed of multiple helical bundles while the rest are predicted to contain similar structures [53,58,59,88-93]. The exocyst subunits are localized to sites of polarized growth and secretion in various eukaryotic cell types [78,144,145].

Regulation of exocyst localization was thought to be facilitated by Sec3p and Exo70p via their interactions with the small GTPases Rho1p, Rho3p and Cdc42p, and with phosphatidylinositol 4,5-bisphosphate [81,85,86,96,97]. However, localization of the exocyst by Sec3p remains controversial, as Roumanie et al. have suggested some GFP tags on Sec3p may artificially stabilize its interaction with the plasma membrane [95]. Also, the exocyst localizes in the absence of the Rho3p-Exo70p interaction [95], although these results have been disputed [96]. Additionally, we recently demonstrated that residues in the C-terminal domain of Sec6p are essential for maintenance of the exocyst complex at sites of secretion via interactions with a putative anchor protein [87].

Because previous studies demonstrated interactions between Sec6p and the SNARE Sec9p [65], between the exocyst and Sec1p [122], and between Sec1p and the SNARE

complex [70], we were interested in examining the functional relationships between these proteins *in vivo*. We used coimmunoprecipitation experiments to show that Sec6p and Sec9p interact in yeast. These coimmunoprecipitations also revealed that the interaction involves a previously identified non-exocyst-bound "free" pool of Sec6p [65]. Mutation of residues in the N-terminal region of Sec6p do not affect this interaction, rather, they destabilize intramolecular exocyst contacts responsible for exocyst assembly and/or stability. In the *sec6* mutants, the exocyst subunits Sec5p, Sec10p, and Sec15p dissociate from the complex and mislocalize at the restrictive temperature. The remaining subunits (Sec6p, Sec8p, Sec3p, Exo70p, and Exo84p) comprise a stable subcomplex localized to sites of secretion. Furthermore, one of the *sec6* mutants disrupts a direct interaction with Sec1p, suggesting a fundamental link between the exocyst and Sec1p in the regulation of SNARE-mediated exocytosis.

MATERIALS AND METHODS:

Yeast methods

Mutations (*sec6-43*: R153D, *sec6-44*: D154R, *sec6-46*: Q156K/E157K/Q158K) were introduced by PCR into the *SEC6* gene in the BamHI and NotI sites of yeast plasmid pRS315 (*LEU2 CEN*) including the endogenous regulatory regions of *SEC6* (0.5 kb of flanking genomic sequences at the 5' and 3' ends), and the presence or absence of a C-terminal triple hemagglutinin (HA$_3$) tag for immunofluorescence and immunoprecipitation studies (Table 2.1). All mutations were confirmed by sequencing.

Table 2.1. Yeast strains used in this study

Strain	Relevant genotype
MMY251	*MATα sec6Δ::KanMX-4 his3Δ1 leu2Δ0 ura3Δ0 (LEU2 CEN SEC6)*
MMY404	*MATα sec6Δ::KanMX-4 his3Δ1 leu2Δ0 ura3Δ0 (LEU2 CEN sec6-43)*
MMY405	*MATα sec6Δ::KanMX-4 his3Δ1 leu2Δ0 ura3Δ0 (LEU2 CEN sec6-44)*
MMY406	*MATα sec6Δ::KanMX-4 his3Δ1 leu2Δ0 ura3Δ0 (LEU2 CEN sec6-46)*
NY778	*MATα leu2-3,112 ura3-52 sec6-4* (Novick *et al.*, 1980)
MMY275	*MATα sec6Δ::KanMX-4 his3Δ1 leu2Δ0 ura3Δ0 (LEU2 CEN SEC6-HA$_3$)*
MMY582	*MATα sec6Δ::KanMX-4 his3Δ1 leu2Δ0 ura3Δ0 (LEU2 CEN sec6-44-HA$_3$)*
MMY583	*MATα sec6Δ::KanMX-4 his3Δ1 leu2Δ0 ura3Δ0 (LEU2 CEN sec6-56-HA$_3$)*
MMY204	*MATα sec6Δ::KanMX-4 his3Δ1 leu2Δ0 ura3Δ0 (URA3 CEN SEC6)*
MMY246	*MATa sec6Δ::KanMX-4 his3Δ1 leu2Δ 0 met15Δ0 ura3Δ0 Sec3-GFP(HIS3) (URA3 CEN SEC6)*
MMY238	*MATa sec6Δ::KanMX-4 his3Δ1 leu2Δ 0 met15Δ0 ura3Δ0 Sec5-GFP(HIS3) (URA3 CEN SEC6)*
MMY239	*MATa sec6Δ::KanMX-4 his3Δ1 leu2Δ 0 met15Δ0 ura3Δ0 Sec8-GFP(HIS3) (URA3 CEN SEC6)*
MMY247	*MATa sec6Δ::KanMX-4 his3Δ1 leu2Δ 0 met15Δ0 ura3Δ0 Sec10-GFP(HIS3) (URA3 CEN SEC6)*
MMY248	*MATa sec6Δ::KanMX-4 his3Δ1 leu2Δ 0 met15Δ0 ura3Δ0 Sec15-GFP(HIS3) (URA3 CEN SEC6)*
MMY249	*MATa sec6Δ::KanMX-4 his3Δ1 leu2Δ 0 met15Δ0 ura3Δ0 Exo70-GFP(HIS3) (URA3 CEN SEC6)*
MMY250	*MATa sec6Δ::KanMX-4 his3Δ1 leu2Δ 0 met15Δ0 ura3Δ0 Exo84-GFP(HIS3) (URA3 CEN SEC6)*
MMY532	*MATa sec6Δ::KanMX-4 his3Δ1 leu2Δ 0 met15Δ0 ura3Δ0 Sec3-GFP(HIS3) (URA3 CEN sec6-44)*
MMY534	*MATa sec6Δ::KanMX-4 his3Δ1 leu2Δ 0 met15Δ0 ura3Δ0 Sec5-GFP(HIS3) (URA3 CEN sec6-44)*
MMY537	*MATa sec6Δ::KanMX-4 his3Δ1 leu2Δ 0 met15Δ0 ura3Δ0 Sec8-GFP(HIS3) (URA3 CEN sec6-44)*
MMY540	*MATa sec6Δ::KanMX-4 his3Δ1 leu2Δ 0 met15Δ0 ura3Δ0 Sec10-GFP(HIS3) (URA3 CEN sec6-44)*
MMY543	*MATa sec6Δ::KanMX-4 his3Δ1 leu2Δ 0 met15Δ0 ura3Δ0 Sec15-GFP(HIS3) (URA3 CEN sec6-44)*
MMY546	*MATa sec6Δ::KanMX-4 his3Δ1 leu2Δ 0 met15Δ0 ura3Δ0 Exo70-GFP(HIS3) (URA3 CEN sec6-44)*
MMY549	*MATa sec6Δ::KanMX-4 his3Δ1 leu2Δ 0 met15Δ0 ura3Δ0 Exo84-GFP(HIS3) (URA3 CEN sec6-44)*
MMY532	*MATa sec6Δ::KanMX-4 his3Δ1 leu2Δ 0 met15Δ0 ura3Δ0 Sec3-GFP(HIS3) (URA3 CEN sec6-56)*
MMY535	*MATa sec6Δ::KanMX-4 his3Δ1 leu2Δ 0 met15Δ0 ura3Δ0 Sec5-GFP(HIS3) (URA3 CEN sec6-56)*
MMY538	*MATa sec6Δ::KanMX-4 his3Δ1 leu2Δ 0 met15Δ0 ura3Δ0 Sec8-GFP(HIS3) (URA3 CEN sec6-56)*
MMY541	*MATa sec6Δ::KanMX-4 his3Δ1 leu2Δ 0 met15Δ0 ura3Δ0 Sec10-GFP(HIS3) (URA3 CEN sec6-56)*
MMY544	*MATa sec6Δ::KanMX-4 his3Δ1 leu2Δ 0 met15Δ0 ura3Δ0 Sec15-GFP(HIS3) (URA3 CEN sec6-56)*
MMY547	*MATa sec6Δ::KanMX-4 his3Δ1 leu2Δ 0 met15Δ0 ura3Δ0 Exo70-GFP(HIS3) (URA3 CEN sec6-56)*
MMY550	*MATa sec6Δ::KanMX-4 his3Δ1 leu2Δ 0 met15Δ0 ura3Δ0 Exo84-GFP(HIS3) (URA3 CEN sec6-56)*
MMY441	*MATa sec6Δ::KanMX-4 hisΔ1 leu2Δ0 ura3Δ0 SEC8-MYC3(URA3) (LEU2 CEN SEC6-HA3)*
MMY651	*MATa sec6Δ::KanMX-4 hisΔ1 leu2Δ0 ura3Δ0 SEC8-MYC3(URA3) (LEU2 CEN sec6-44-HA$_3$)*
MMY652	*MATa sec6Δ::KanMX-4 hisΔ1 leu2Δ0 ura3Δ0 SEC8-MYC3(URA3) (LEU2 CEN sec6-56-HA$_3$)*
MMY138	*MATα his3Δ1 leu2Δ0 ura3Δ0 SEC6-Myc$_{13}$ (KANMX6)*
MMY584	*MATα sec9Δ::KanMX-4 his3Δ1 leu2Δ0 ura3Δ0 (URA3 CEN Sec9-HA$_3$)*

sec6 LEU2 CEN plasmids were introduced as the sole copy of *SEC6* in yeast by transformation and plating on 5-FOA (5-fluoro-orotic acid) to select for loss of the wild-type *SEC6 URA3 CEN* from MMY204 (Table 2.1). For green fluorescent protein (GFP) analyses, *sec6 LEU2 CEN* plasmids were introduced as the sole copy of *SEC6* in yeast carrying individual genomic C-terminally GFP-tagged exocyst subunits (Table 2.1) by transformation and plating on 5-FOA to select against the wild-type *SEC6 URA3 CEN*. For serial dilution testing, strains containing wild-type or mutant *sec6* were grown overnight to an O.D.$_{600}$ of 1-2 and diluted to an O.D.$_{600}$ of 1. Serial 1:10 dilutions were spotted onto SC (synthetic complete) and YPD media at various temperatures. Growth curves were acquired using strains containing wild-type or mutant s*ec6* grown overnight to an O.D.$_{600}$ of ~1 and diluted to 0.1 O.D. As growth progressed, cultures were diluted to keep the O.D.$_{600}$ below 1.0. Media, growth conditions, and yeast genetic methods were as in [115]. Yeast strains used in this study are listed in Table 2.1. Invertase secretion and Bgl2p secretion assays were performed as in [81].

Indirect Immunofluorescence

Yeast strains containing either wild-type or mutant *sec6* with a C-terminal HA$_3$ tag were grown to log phase in YPD media at 25ºC and immediately fixed or shifted to nonpermissive conditions in YPD at 37ºC for 4h and fixed in 37% formaldehyde. Cells were permeabilized in HS/SDS buffer (0.1 M HEPES, pH 7.4, 1.0 M sorbitol, and 0.5% SDS), and washed twice in HS buffer. Cells were then placed on slides (Electron Microscopy Sciences, Fort Washington, PA) that had been prepared with 0.1%

polylysine. A 1:400 dilution of α-HA-Alexa Fluor 488 (Molecular Probes, Eugene, OR)

conjugated antibody was added to visualize Sec6p-HA$_3$. Differential interference contrast

and fluorescence images were acquired at room temperature using an Axioskop2 plus

epifluorescence microscope (Zeiss, Thornwood, NY) fitted with a 100X Plan-

NEOFLUAR (Zeiss 1.30 NA oil immersion) objective lens. Images were collected using

a Diagnostic Instruments camera (Sterling Heights, MY, model 2.1.1) and 3rd Party

Interface Advanced (version 3.5.4 for MacOS) software. Immunofluorescence images

were adjusted for total contrast in Adobe Photoshop (version 7.0.1, San Jose, CA).

GFP Fluorescence

Yeast strains containing genomic C-terminally GFP-tagged exocyst subunits and

wild-type *SEC6* or mutant *sec6* were grown to log phase in YPD media at 25ºC and

shifted to non-permissive conditions for 4h or 6h. Samples were immediately centrifuged

and resuspended in PBS containing 10% glycerol, fixed with 37% formaldehyde for 10

min., and washed and resuspended in PBS plus 10% glycerol. Cells were visualized as

described above. Localization of GFP-tagged proteins was quantitated in Adobe

Photoshop by counting cells with localized or mislocalized exocyst subunits. "Localized"

was defined as a distinct patch at the bud tip or mother-daughter neck of budding cells,

and "mislocalized" as diffuse or patchy staining in the cytoplasm or mother cell. For each

strain and condition, n > 100 cells were counted for 3 replicates each.

Immunoprecipitations

Yeast were grown to an $O.D._{600}$ of 1.0 at 25ºC and samples were incubated with

shaking at 37ºC for 3 h. 50 O.D. of each sample were pelleted and resuspended in 200 μL

of lysis buffer (50 mM sodium phosphate pH 7.4, 150 mM NaCl, 0.5% IGEPAL, 1 mM

EDTA, 1 mM DTT, 1 mM PMSF, 0.7 μg/mL pepstatin, 1 μg/mL leupeptin, 1μg/mL

chymostatin, 1μg/mL antipain, 1μg/mL aprotinin) and lysed by vortexing for 3 min in a

50% slurry containing 0.5 mm zirconia/silica beads (BioSpec Products). Lysate was

centrifuged at 13,000 rpm to remove cell debris. The supernatant was pre-cleared for 1h

at 4ºC with 30 μL protein A beads (Roche), and then incubated with α-MYC (Covance),

α-HA (Roche) or α-GFP (Abcam) antibody and fresh protein A beads for 2h in lysis

buffer. Beads were washed using 3 x 1 mL of chilled lysis buffer. Beads were

resuspended in 100 μL of loading dye and proteins separated by SDS-PAGE. Proteins

were transferred to nitrocellulose and probed with appropriate antibodies. Western blots

were developed using horseradish peroxidase-conjugated α-rabbit IgG (Roche), followed

by chemiluminescent detection (ECL; Amersham) using an LAS-3000 (Fujifilm) running

Image Reader LA-3000 (Fujifilm). For binding of Sec1p to recombinant Sec6p, the

$Sec1p\text{-}V5\text{-}His_6$ immunoprecipitation was performed with 50 O.D. $_{600}$ of yeast expressing

a galactose inducible $Sec1\text{-}V5\text{-}His_6$ [70] resuspended in wash buffer (50 mM Hepes pH7.4,

20 mM NaN_3, 20 mM NaF). Cells were then pelleted, resuspended in lysis buffer, lysed,

and bound to protein A beads using α-V5 antibody, as described above. Immobilized

$Sec1\text{-}V5\text{-}His_6$ was washed with binding buffer lacking protease inhibitors, and incubated

for two hours with 1 μM purified recombinant $His_6\text{-}Sec6p$ (in 10% glycerol, 10 mM

K_2HPO_4, 100 mM KH_2PO_4, 140 mM KCl, pH 7.4, see below) and washed with binding

buffer without protease inhibitors. Samples were resuspended in 100 µL of SDS loading

dye, boiled, separated by SDS-PAGE, and analyzed by Western Blot as described above.

For ultracentrifugation experiments, 200 µL of each lysate was centrifuged for 20 min at

50,000 rpm (105,000 x g) in a Beckman Optima TL ultracentrifuge. The supernatants

were removed, and the pellet was resuspended in 200 µL of lysis buffer. The presence of

Sec1p, Sec9p and exocyst subunits in the supernatant and pellet fractions were detected

by Western blot analyses, as above. Immunoprecipitation experiments were performed as

above, using the supernatant fraction rather than complete lysate.

Crosslinking and Mass Spectrometry

Recombinant His_6-Sec6p and Sec9CT (Sec9p residues 416-651) protein

purifications were performed as described [65]. This C-terminal domain of Sec9p is

homologous to the mammalian t-SNARE SNAP-25, and is sufficient for function in yeast

[46]. Purified Sec6p and Sec9CT were incubated at 3 mg/mL for 1h at 4ºC in 100 mM MES

buffer to pre-form the Sec6p-Sec9CT complex. The "zero-length" cross-linking agent

EDC (1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride), was dissolved to

0.1 M in deionized water, and added in combination with *N*-hydroxysulfosuccinimide

(0.1 M) as per [146]. Crosslinking proceeded at room temperature for 1h, and the reaction

was quenched with an excess of 2-mercaptoethanol. After separation by SDS-PAGE and

Coomassie staining, the cross-linked and non-cross-linked bands were excised. Western

blot analysis was used to verify that both proteins were present in the cross-linked bands.

Samples of Sec6p and Sec9CT were then subjected to chymotryptic in-gel digestion (50

ng/µL) in the presence or absence of heavy water (^{18}O) for 16h at 30ºC as described in [147].

Heavy water (^{18}O) was used in order to label peptides and cross-links. Peptides digested

in the presence of ^{18}O water will be 4 mass units larger than those digested in the

presence of ^{16}O water, while cross-linked peptides will be 8 mass units larger. Digested

samples were purified using an Omix C18 ZipTip reverse-phase cleanup tip. The ZipTip

was prewashed with 50% acetonitrile and equilibrated with 0.1% trifluoroacetic acid.

Peptides were bound to the ZipTip by aspirating 5 times. The ZipTip was then washed by

repeated aspiration with 10 µL of 0.1% TFA, and eluted by aspiration with 50%

acetonitrile. Eluant was spotted (1 µL) onto a Matrix-assisted laser desorption/ionization

time-of-flight mass spectrometry (MALDI-TOFMS) target plate with 1µL of α-cyno-4-

hydroxycinnaminic acid matrix at 10 mg/mL in 50% acetonitrile, 0.1% TFA, and then

allowed to air dry. MALDI-TOFMS analysis was performed on Waters MALDI L/R

MALDI-TOF mass spectrometer in reflectron mode to acquire spectra from *m/z* 400-

6000. The instrument was calibrated by lock mass calibration using a pre-defined

mixture of peptides in the lock mass well. Between 15 and 20 spectra were taken for each

measurement. Each experiment was repeated in triplicate. Peptides were identified from

their molecular weight and analyzed using GPMAW (General Protein Mass Analysis for

Windows) and MassLynx software [148]. GPMAW was used to match sequences of normal

and cross-linked peptides observed in MALDI-TOF MS analysis. Mass identity

assignment of cross-linked peptides was made if they met the following criteria: 1) the

peptide was present in the cross-linked sample and absent in the non-cross-linked sample.

2) the peptide was present in at least two of three replicates. 3) the peptide resulted from a

chymotrypsin cleavage (Y, W, F, L, M). 4) In the heavy water samples, two ^{18}O atoms were incorporated into the C-terminus of each peptide. This results in a +4 Da shift in linear peptides and a +8 Da shift in cross-linked peptides; cross-linked peptides were assigned only if they showed a +8 Da shift.

RESULTS

Sec6p interacts with Sec9p *in vivo*

Sec6p is an essential exocyst subunit that localizes to sites of polarized secretion and interacts with several other exocyst subunits (reviewed in [5]). Previously, we defined a robust *in vitro* interaction between recombinant Sec6p and the plasma membrane t-SNARE Sec9p; in the absence of additional factors, this interaction appears to negatively regulate SNARE complex assembly *in vitro* [65]. These results suggest a direct role for a subunit of the exocyst complex in regulating SNARE complex assembly. In order to determine if the Sec6p-Sec9p interaction occurs *in vivo,* we constructed yeast strains in which the wild-type copies of *SEC6* or *SEC9* were replaced with low copy *CEN* plasmids containing *SEC6-MYC$_{13}$* or *SEC9-HA$_3$* (Table 2.1). Both strains expressed the tagged proteins at levels comparable to endogenous, as detected by Western blot analyses. To test the Sec6p-Sec9p interaction in yeast, Sec6p-Myc was immunoprecipitated with α-Myc antibody, and the presence of Sec9p was assayed using α-Sec9p antibody. We found that Sec9p coimmunoprecipitated specifically with Sec6p-Myc (Figure 2.1A).

Figure 2.1 Sec6p interacts with Sec9p *in vivo*. (A) Immunoprecipitations from yeast lysate carrying Sec6p-Myc as the sole copy of Sec6p. Bound proteins were detected by Western blot analyses with α-Sec6p and α-Sec9p antibodies. Sec9p runs as a doublet; both bands are detected by α-Sec9p. (B) Sec9p-HA was immunoprecipitated, and the bound fraction was probed for the presence of Sec6p. Untagged *SEC6* and *SEC9* strains were used as negative controls. For all immunoprecipitation experiments, 1% of the total cell lysate, and 10% of the bound material were run on SDS-PAGE and the presence of indicated proteins were detected by Western blot analyses.

Figure 2.1

A

	1% Input	10% Bound

IP: αMyc

SEC6/SEC9   *SEC6-Myc/SEC9*   *SEC6-WT/SEC9*   *SEC6-Myc/SEC9*

αSec6p   Sec6p-Myc / Sec6p

αSec9p   Sec9p

B

	1% Input	10% Bound

IP: αHA

SEC6/SEC9   *SEC6/SEC9-HA*   *SEC6/SEC9*   *SEC6/SEC9-HA*

αSec9p   Sec9-HA / Sec9p
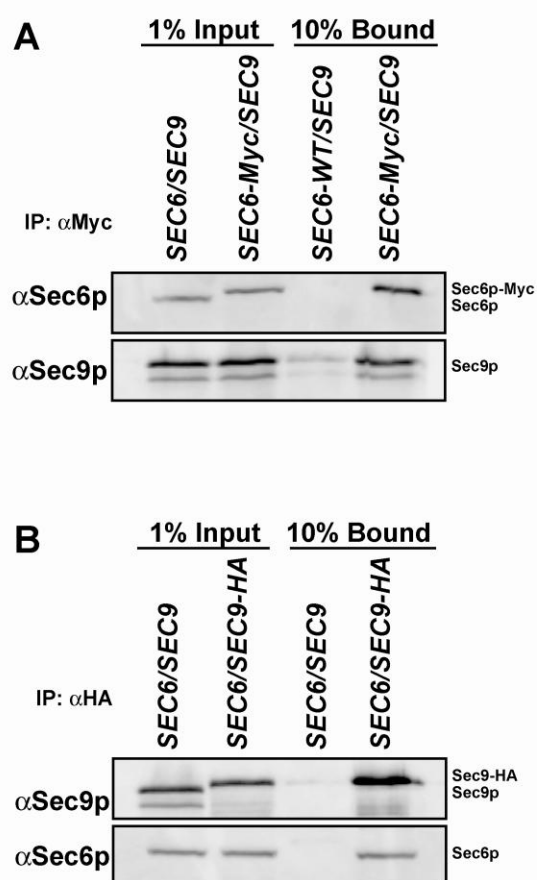
αSec6p   Sec6p

Conversely, when we immunoprecipitated Sec9p-HA with α-HA antibody, we found that

it coimmunoprecipitated Sec6p (Figure 2.1B), demonstrating that the Sec6p-Sec9p

interaction exists *in vivo.*

Mutations in the N-terminal domain of Sec6p Lead to Growth and Secretion Defects

To explore the Sec6p-Sec9p interaction further, we performed cross-linking

experiments, combined with mass spectroscopy, to isolate interacting regions. We

incubated recombinant His_6-Sec6p and Sec9CT (residues 416-651, which are

homologous to SNAP-25) together at 4ºC for 1h before cross-linking the proteins using

the "zero-length" crosslinker EDC (1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide

hydrochloride, Pierce) [146]. Samples were proteolyzed to completion with chymotrypsin in

normal water or heavy water (^{18}O) before analysis by reflectron MALDI-TOF mass spec.

Cross-linked fragments were identified as described in Materials and Methods. Our

analysis revealed a single putative pair of cross-linked peptides: Sec6p TSARDFQE

(MW 951.96 Da, a.a. 150-157) and Sec9p QRKNVLEKAKRY (MW 1531.78 Da, a.a.

568-579). This residue is not a typical chymotryptic peptide, but results from a non-

specific cleavage. These residues in Sec6p lie in the N-terminal region, consistent with

previous data showing that the C-terminal domain alone is not sufficient for Sec9p

binding *in vitro* [65]. The cross-linked peptide of Sec9p lies centered in the long loop region

between the two SNARE motifs [121]. There are currently no identified mutations in this

region, though there are conserved residues both in the region and downstream, as

mutational analysis has focused largely on the SNARE motifs of this protein [29,46]. It remains unclear if there is a function to the loop beyond linking the SNARE motifs.

To elucidate the function of the exocyst and the Sec6p-Sec9p interaction in yeast, we designed mutants in Sec6p that we deemed likely to disrupt this interaction. The Sec6p peptide contains several highly conserved charged residues: R153, D154, and E157. We reasoned that charge reversal mutations in this region might result in significant disruption of Sec6p-Sec9p interaction. Therefore, we made the following mutations in the context of full length Sec6p: R153D (*sec6-43*), D154R (*sec6-44*), and the triple mutant Q156K/E157K/Q158K (*sec6-46*). These mutations are in the N-terminal region of the protein, whereas prior temperature sensitive mutations were in the C-terminal region (Figure 2.2A). We then substituted the mutant *sec6* alleles for wild-type in yeast and tested for growth and secretion phenotypes. All three mutant strains displayed wild-type growth rates at 25ºC on both SC and YPD media. However, at 37ºC the growth of the mutant strains was impaired (Figure 2.2B). Growth curves of mutants in liquid YPD at 37ºC revealed that this growth defect did not become apparent until approximately 4h after the temperature shift (Figure 2.2C). Because preliminary results indicated that the growth, secretion, and localization phenotypes of *sec6-43* and *sec6-44* were similar, further analyses were only carried out with the *sec6-44* and *sec6-46* alleles.

To elucidate the growth defects of the mutant *sec6* strains, we assayed the secretion of a typical secretory marker protein, the periplasmic sucrase enzyme invertase. Invertase is transported in high density secretory vesicles, a class of vesicles that can be separated

Figure 2.2 *sec6* **mutant strains have altered growth and secretion phenotypes at**

37°C. (A) Location of *sec6* temperature sensitive mutations in the Sec6p protein.

Indicated are the new *sec6* alleles in the N-terminal domain: *sec6-43* (R153D), *sec6-44*

(D154R), and *sec6-46* (Q156K/E157K/Q158K). Other previously characterized *sec6*

alleles are *sec6-4* [15] (L633P), and the patch mutants *sec6-49* (L418A, Y422A, W433A,

Q470A, Q474A) and *sec6-54* [87] (D607A, T632A, E635R, Y636A, D639R), which are

located in the Sec6p C-terminal domain whose structure was previously determined [88].

(B) Serial dilutions of wild-type and *sec6* mutants were spotted onto YPD media and

incubated at 25°C and 37°C. (C) Growth curves for the wild-type *SEC6* strain vs. the

sec6 mutants in YPD at 37°C. Before 4h, growth of the mutants is similar to the wild-

type. One representative growth curve is shown for each strain. (D) *sec6* mutants show

only a minor defect in invertase secretion at 4h. Invertase secretion was assayed at

permissive and non-permissive temperatures at the time points listed. *sec6-4* is only

shown at the early time point, as a positive control for the block in secretion of invertase.

(E) Analysis of internal vs. external Bgl2 levels reveals a substantial secretion defect after

4h at 37ºC. Quantitation of the internal bands is shown below the blots. Each experiment

was performed in triplicate.

Figure 2.2

A

B

C

D

E
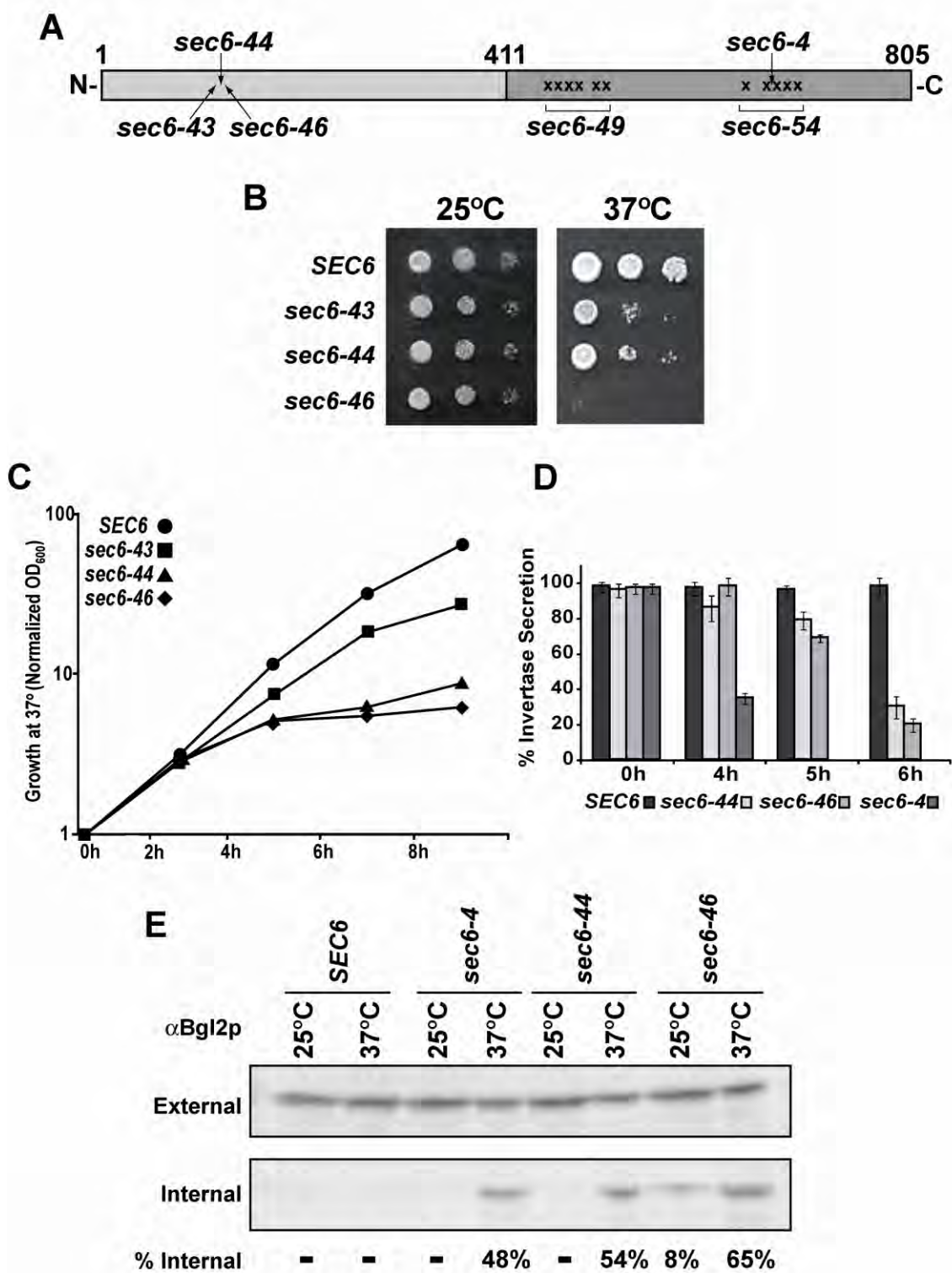
from a low density population via Nycodenz gradient fractionation [149]. We detected no significant defect in invertase secretion in *sec6-44* or *sec6-46* yeast after 4h at the non-permissive temperature (Figure 2.2D). However, after 6h, an ~75% drop in invertase secretion was observed (Figure 2.2D). This is in stark contrast with other temperature sensitive exocyst mutations, such as *sec6-4* (Figure 2.2D), or *sec10-2*, which disrupt invertase secretion within minutes after transfer to the restrictive temperatures [150,15], suggesting that the 6h defect in invertase secretion might be a secondary effect.

We also assayed secretion of the endo-β-1,3-glucanase cell wall component Bgl2p, which is trafficked in the more abundant, lower density population of secretory vesicles, which are distinct from the invertase-containing vesicle population [149]. In wild-type yeast, 100% of the Bgl2p is secreted [150] (Figure 2.2E). However, both *sec6-44* and *sec6-46* display a significant defect in Bgl2p after 4h at the non-permissive temperature (Figure 2.2E). The reduction in Bgl2p secretion (~54%) by the *sec6-44* mutant after 4h was similar to the disruption by *sec6-4* after 1h under restrictive condition. The *sec6-46* mutant displays a more severe (~65%) defect after 4h at 37°C, with a slight (~8%) defect observed even at the permissive temperature. Secretion of Bgl2p at 3h was similar to wild-type, indicating that the onset of the Bgl2p secretion defect is rapid and concurrent with the observed growth defect. Similar results were demonstrated with the *exo70-35* and *exo70-38* temperature sensitive alleles, which were defective for secretion of Bgl2p-containing vesicles and not invertase-containing vesicles [150]. The difference between Bgl2p and invertase secretion suggests that the *sec6-44* and *sec6-46* mutants have a

specific block in the trafficking of the Bgl2p-containing vesicles at the restrictive temperature.

One explanation for the detection of growth and secretion defects only after a 4 h temperature shift, is that the mutant proteins are destabilized and fail to fold, or are degraded prematurely. To test these possibilities, we blocked protein synthesis in wild-type and mutant yeast using cyclohexamide, and measured Sec6p protein levels at 37°C at several time points by Western blot. No significant loss of either wild-type or mutant Sec6p was observed within 2h (data not shown), indicating that wild-type and mutant Sec6p have half-lives greater than two hours. Further, in later immunoprecipitation experiments, the levels of wild-type and mutant proteins were similar, indicating that the growth defects were not caused by degradation of destabilized mutant proteins.

Because the *sec6-44* and *sec6-46* mutations were designed to disrupt a putative interaction between Sec6p and Sec9p, we predicted that immunoprecipitation of Sec6p-HA from cells grown at the restrictive conditions would fail to coimmunoprecipitate Sec9p. However, no abrogation of the Sec6p-Sec9p interaction was observed after a shift to the non-permissive conditions (4h at 37ºC in YPD; Figure 2.3A). One explanation is that the Sec6p-Sec9p complex is disrupted at the restrictive temperature *in vivo,* but reassembles at 4ºC during the immunoprecipitation experiment. To rule out this possibility, lysate from cells expressing tagged Sec6p-Myc and untagged Sec9p was mixed with lysate from cells expressing tagged Sec9p-HA and untagged Sec6p. Half of the mixed lysate was subjected to an α-HA coimmunoprecipitation, while the other half

Figure 2.3 Sec6p mutant proteins interact with Sec9p at the restrictive conditions.

(A) Sec6p and the *sec6* mutants were HA_3-tagged and immunoprecipitated from cells grown at the permissive and restrictive conditions (4h at 37°C). No significant difference in the amount of Sec9p was observed in the mutant vs. wild-type strain. (B) Sec6p-Sec9p complexes do not form during the immunoprecipitation experiments. Lysates from Sec6p-Myc/Sec9p and Sec9p-HA/Sec6p yeast strains were lysed and either immunoprecipitated separately, or mixed and immunoprecipitated with α-HA (lanes 3 and 5) or α-Myc (lanes 4 and 6) antibodies. The experiments were performed at least 3 times and one representative experiment is shown.

Figure 2.3
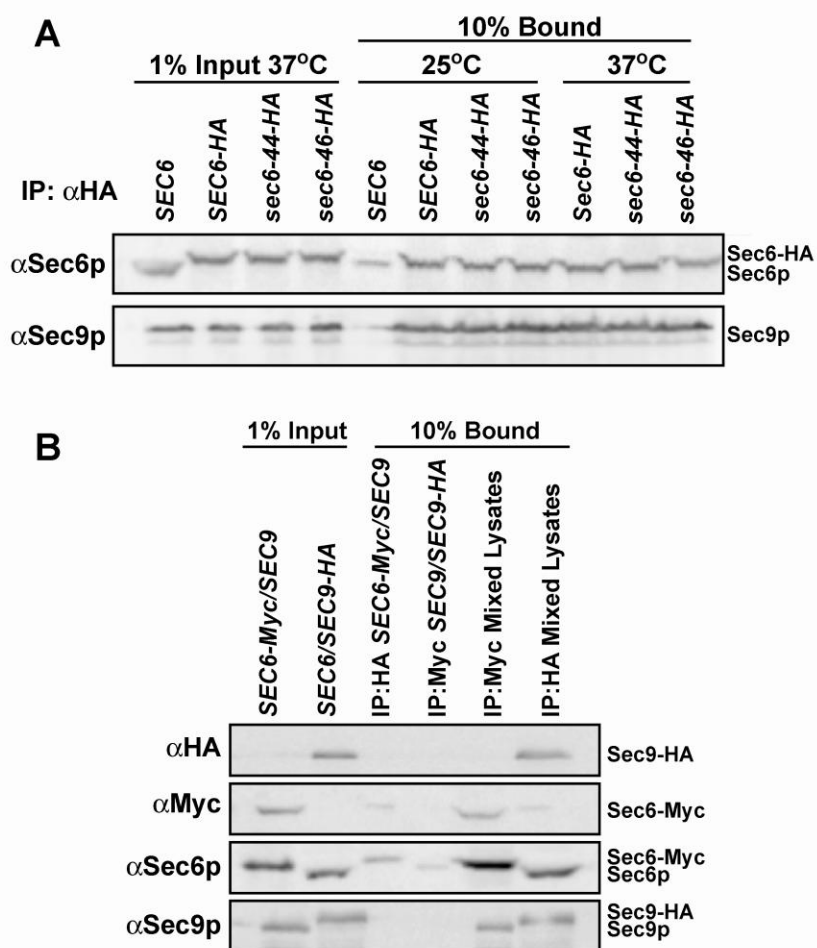
was immunoprecipitated with α-Myc. If the complex between Sec6p and Sec9p reforms

during the course of the experiment, then the Sec6p-Myc protein would

coimmunoprecipitate the Sec9p-HA in the mixed lysate sample, and vice versa. Our

results show, however, that Sec6p-Myc only coimmunoprecipitated wild-type Sec9p and

that Sec9p-HA only coimmunoprecipitated the wild-type Sec6p (Figure 2.3B). These

results indicate that the Sec6p-Sec9p complex assembles *in vivo* and not during the

course of the experiment. Thus, interactions between the mutant Sec6p proteins and

Sec9p appear not to be disrupted *in vivo.* It is likely that the peptides identified by the

cross-linking experiment do not constitute the entire Sec6p-Sec9p binding interface, and

that these specific point mutations are not sufficient to significantly disrupt the interaction

in vivo. Attempts to verify this finding with purified recombinant proteins *in vitro* were

unsuccessful, as the mutant Sec6p proteins were mostly insoluble when expressed in *E.*

coli (data not shown).

Mutant Sec6p Remains Localized while Sec5p, Sec10p, Sec15p are Mislocalized

If the Sec6p mutants are not deficient in Sec9p binding, what is causing the growth

and secretion phenotypes? We previously demonstrated that residues in the C-terminal

region of Sec6p are essential to maintain proper localization of the exocyst complex via

interactions with an unidentified putative anchoring protein [87]. One explanation for the

growth and secretion defects observed for the *sec6-44* and *sec6-46* alleles are that they

are caused by disruption of interactions with the anchor similar to the *sec6-49* and *sec6-*

54 mutants [87], resulting in mislocalization of the mutant Sec6p proteins. To test their localization, C-terminally HA$_3$-tagged constructs of the wild-type *SEC6*, *sec6-44* and *sec6-46* were constructed and expressed as the sole copy of Sec6p. The presence of the HA tag did not alter the growth rates of the wild-type and mutant Sec6p strains at either permissive or restrictive temperatures. Immunofluorescence experiments revealed that both the Sec6-44-HA and Sec6-46-HA proteins were properly localized to the bud tips and mother-daughter necks under permissive conditions (Figure 2.4). Surprisingly, after 4h at the non-permissive temperature (YPD at 37°C), the mutant proteins were still properly localized. In contrast, the *sec6-4* (Figure 2.4), *sec6-49* and *sec6-54* alleles become rapidly mislocalized after only 1h at the non-permissive temperature [87,134]. Given that the Sec6p proteins in *sec6-44* and *sec6-46* are still localized at restrictive temperatures, in contrast to other Sec6p mutants, we reasoned that the mutant proteins still interact with the anchor protein. Additionally, these results indicate that the mutant Sec6p proteins are not globally destabilized.

We hypothesized that the *sec6-44* and *sec6-46* mutations are disrupting other interaction sites, possibly by attenuating intra-exocyst subunit interactions. To explore this possibility, we tested whether the other exocyst subunits remained properly localized. We used strains with genomic GFP-tagged exocyst subunits (Table 2.1) [151] carrying either wild-type or mutant Sec6p as the sole source of Sec6p protein. We observed that many of the exocyst subunits remained properly localized (Figure 2.5A and 2.5B). However, Sec5-GFP (~80%), Sec10-GFP (~55%), and Sec15p-GFP (~60%) were mislocalized after 4h at

Figure 2.4 Sec6p mutants are properly localized at the restrictive conditions. *sec6* mutants remain localized to sites of polarized secretion (bud tips and mother-daughter necks) after 4h at 37°C, as assayed by immunofluorescence using α-HA antibodies. In contrast, localization of *sec6-4* is disrupted after 1h at 37ºC. Panels are representative samples.

Figure 2.4
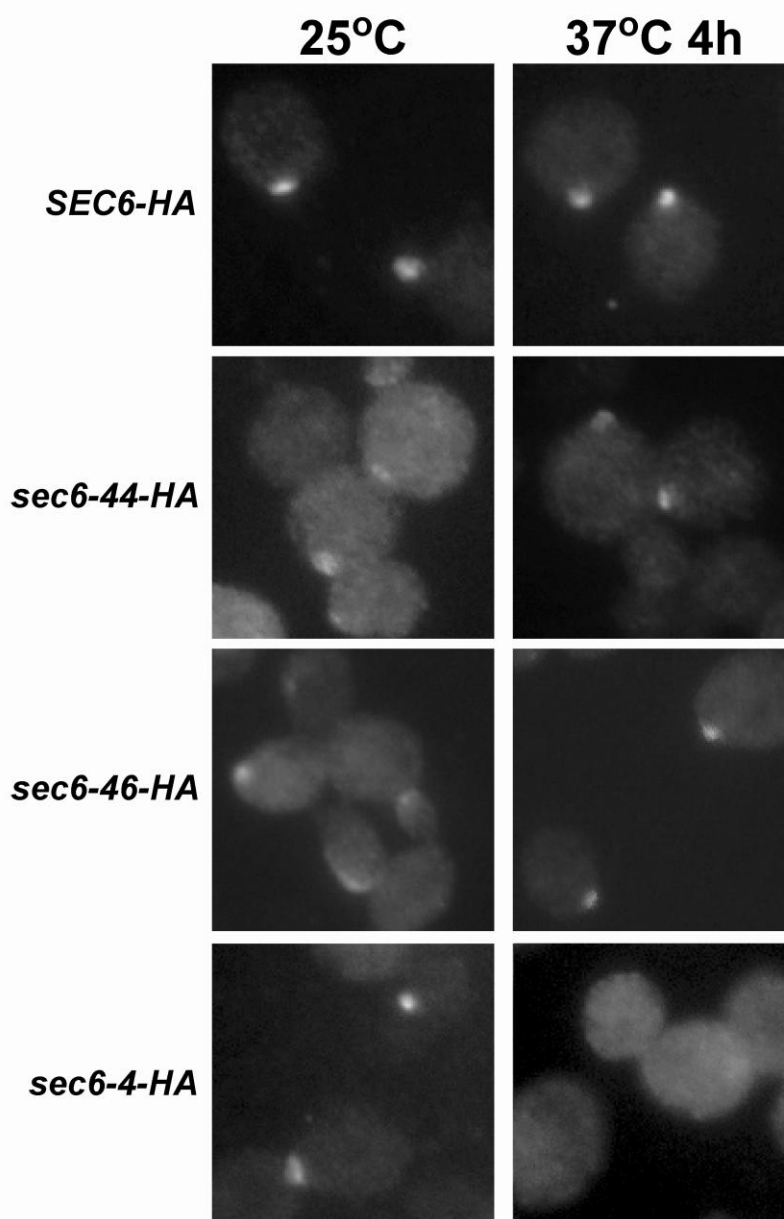
Figure 2.5 The exocyst subunits Sec5p, Sec10p, and Sec15p are mislocalized after 4h at 37ºC. (A) Strains contained GFP-tagged exocyst subunits, and either wild-type Sec6p or the *sec6* mutants as the sole copy of Sec6p. Sec3p, Sec8p, Exo70p, and Exo84p remain localized in Sec6p mutants, while GFP tagged Sec5p, Sec10p, and Sec15p are mislocalized after 4h at 37ºC. (B) Quantification of the percent of yeast cells with mislocalized exocyst-GFP fluorescence. At least three samples of >300 yeast were analyzed for each strain.

Figure 2.5

A
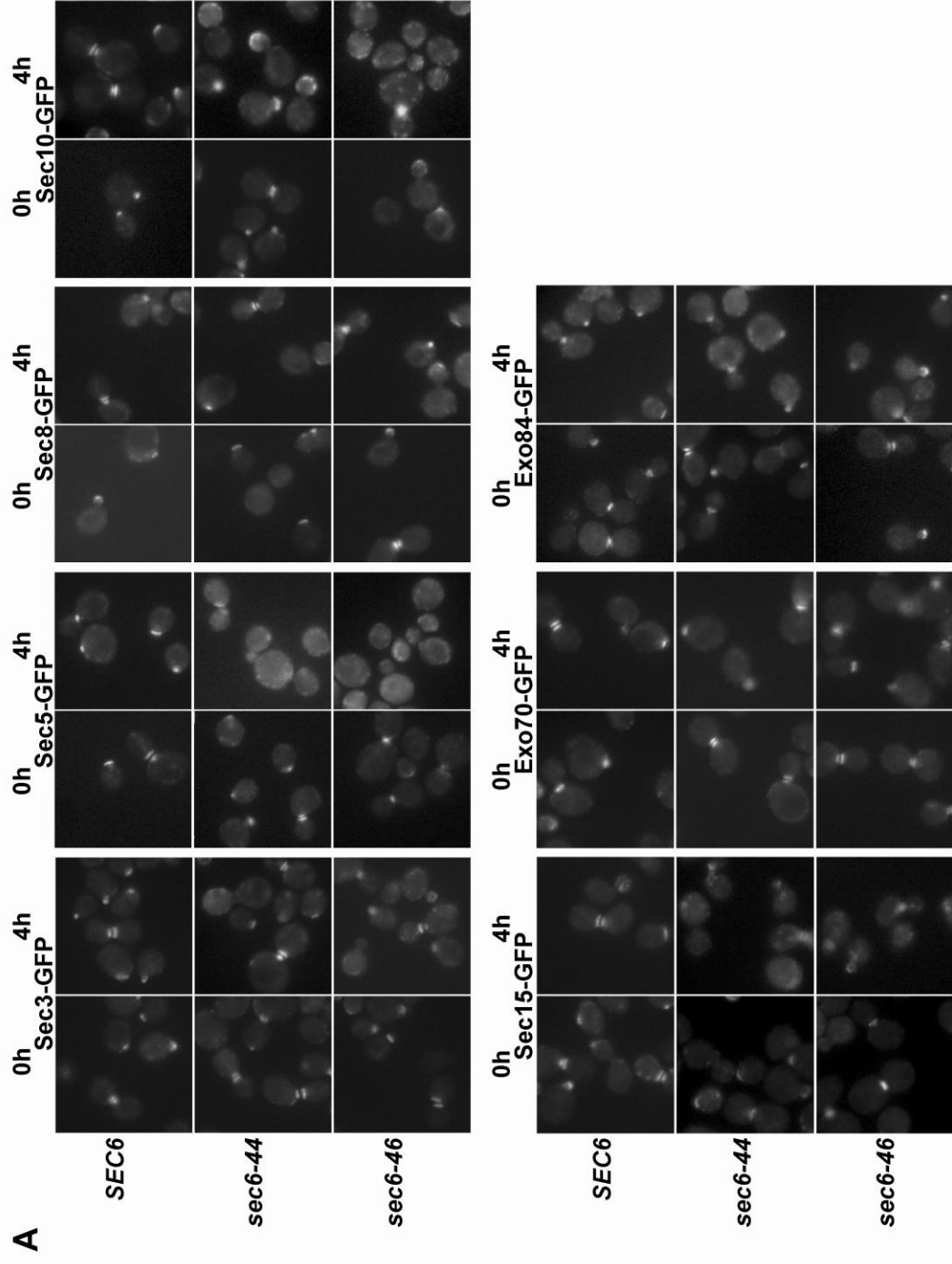
Figure 2.5
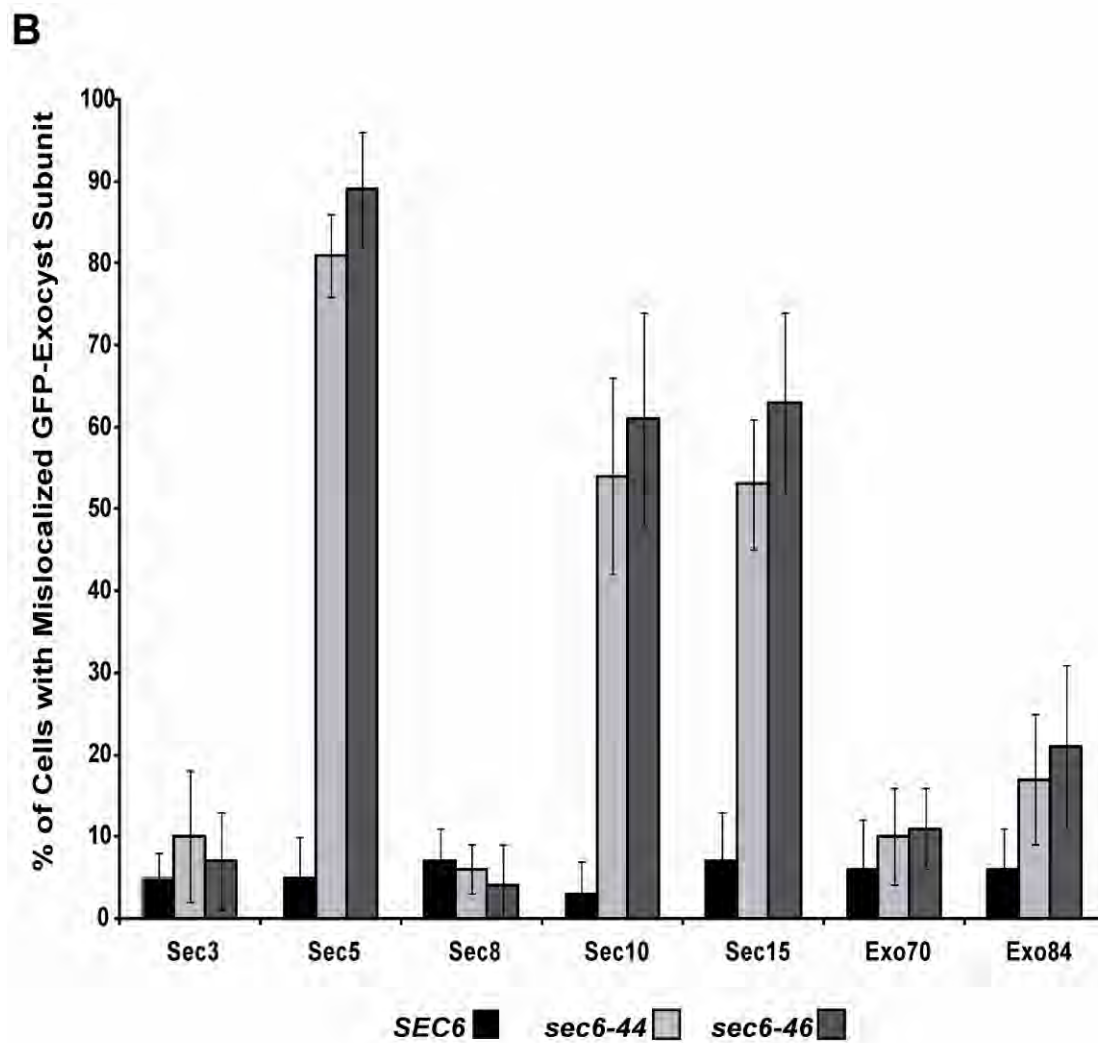
the restrictive temperature. Exo84-GFP also showed a modest (~20%) mislocalization in the *sec6-46* allele. This mislocalization of only a subset of the exocyst complex differs from previous experiments involving *sec6-4* and other temperature sensitive exocyst mutants, which result in mislocalization of all exocyst subunits [84,95,97]. In contrast to those findings, our results using the *sec6-44* and *sec6-46* alleles demonstrate that Sec6p, Sec8p, Sec3p, Exo70p and Exo84p remain localized to sites of secretion at the non-permissive conditions.

Mutant Sec6p Subcomplex Contains Sec6p, Sec3p, Sec8p, Exo70p and Exo84p

We reasoned that the *sec6-44* and *sec6-46* mutations might cause destabilization and/or disassembly of Sec5p, Sec10p and Sec15p from the exocyst complex. These mutant alleles are located in the N-terminal region of Sec6p (Figure 2.2A), which is required for interaction with the exocyst subunit Sec8p and the t-SNARE Sec9p [65,88]. Additionally, the full-length Sec6p interacts with other exocyst subunits, including Sec5p, Sec10p and Exo70p (reviewed in Munson and Novick, 2006). In order to examine the assembled state of the exocyst complex, we performed several different coimmunoprecipitation experiments. First, using α-HA antibody to immunoprecipitate wild-type or mutant Sec6p-HA, we determined which exocyst proteins remain bound to Sec6p at permissive vs. non-permissive temperature. Secondly, using Sec8-Myc$_6$ coexpressed with either mutant or wild-type Sec6p, we monitored the status of the assembled exocyst complex independently from those that remained bound to the Sec6-HA. Coimmunoprecipitation assays corroborated the GFP localization experiments.

While most of the exocyst subunits coimmunoprecipitated with either Sec8p-Myc or Sec6p-HA after 4h at the non-permissive temperature, there was a selective loss of Sec5p, Sec10p, and Sec15p from the complex (Figure 2.6A and 2.6B). The remaining subunits, Sec6p, Sec3p, Sec8p, Exo70p and Exo84p reside as an intact subcomplex. To investigate whether Sec5p, Sec10p and Sec15p might form a second stable subcomplex, we performed similar immunoprecipitation experiments using strains containing Sec5p-GFP and either wild-type Sec6p or the mutant Sec6p alleles. Immunoprecipitation of Sec5-GFP from mutant cells grown at the non-permissive temperature for 4h coimmunoprecipitated a small amount of Sec10p and Sec15p, but not Sec6p or Exo70p (Figure 2.6C). Thus, there is only a minor Sec5p-Sec10p-Sec15p population at restrictive conditions, which suggests that the interactions between these proteins are not very stable in the absence of the other exocyst subunits.

A trivial explanation for the selective disruption of Sec5p, Sec10p, and Sec15p from the exocyst is that disassembly of the subcomplex is a slow phenomenon, and that the remaining subunits might disassemble at later time points. To test this possibility, coimmunoprecipitation experiments with both Sec6p-HA and Sec8-Myc were repeated after 6h at 37ºC in YPD, with identical results (data not shown), suggesting that the *sec-44* and *sec6-46* alleles result in a stable exocyst subcomplex at the restrictive temperature, rather than complete disassembly. To further confirm this, we monitored the localization of the GFP-tagged exocyst subunits (as above) at both 6h and 8h at the restrictive temperatures; we observed no additional change in localization.

Figure 2.6 Mutant Sec6p proteins form stable exocyst subcomplexes. (A) α-HA

immunoprecipitations of HA$_3$-tagged Sec6p and Sec6p mutants were performed using

cells grown at 25ºC and 37ºC for 4h. Binding of the exocyst subunits Sec5p, Sec10p and

Sec15p, as well as the regulatory protein Sec1p, is reduced in the mutant strains at the

restrictive conditions. However, binding of Sec3p, Exo70p and Exo84p is similar to wild-

type. (B) α-Myc immunoprecipitation of the exocyst complex with Sec8-Myc shows

similar results to the Sec6p immunoprecipitations in A, except that Sec1p is not bound.

Sec9p also shows no significant amount of co-immunoprecipitation with Sec8-Myc. (C)

Immunoprecipitation of Sec5-GFP with α-GFP indicates that Sec5p is only weakly

assembled with Sec10p and Sec15p at the restrictive conditions. (D) Suppression of the

sec6-46 growth phenotype by overexpression of exocytic SNAREs and exocyst subunits.

2µ *URA3* plasmids were transformed into *sec6-46*; 10-fold serial dilutions were plated

onto SC-ura and incubated at the indicated temperatures.

Figure 2.6

Figure 2.6

C
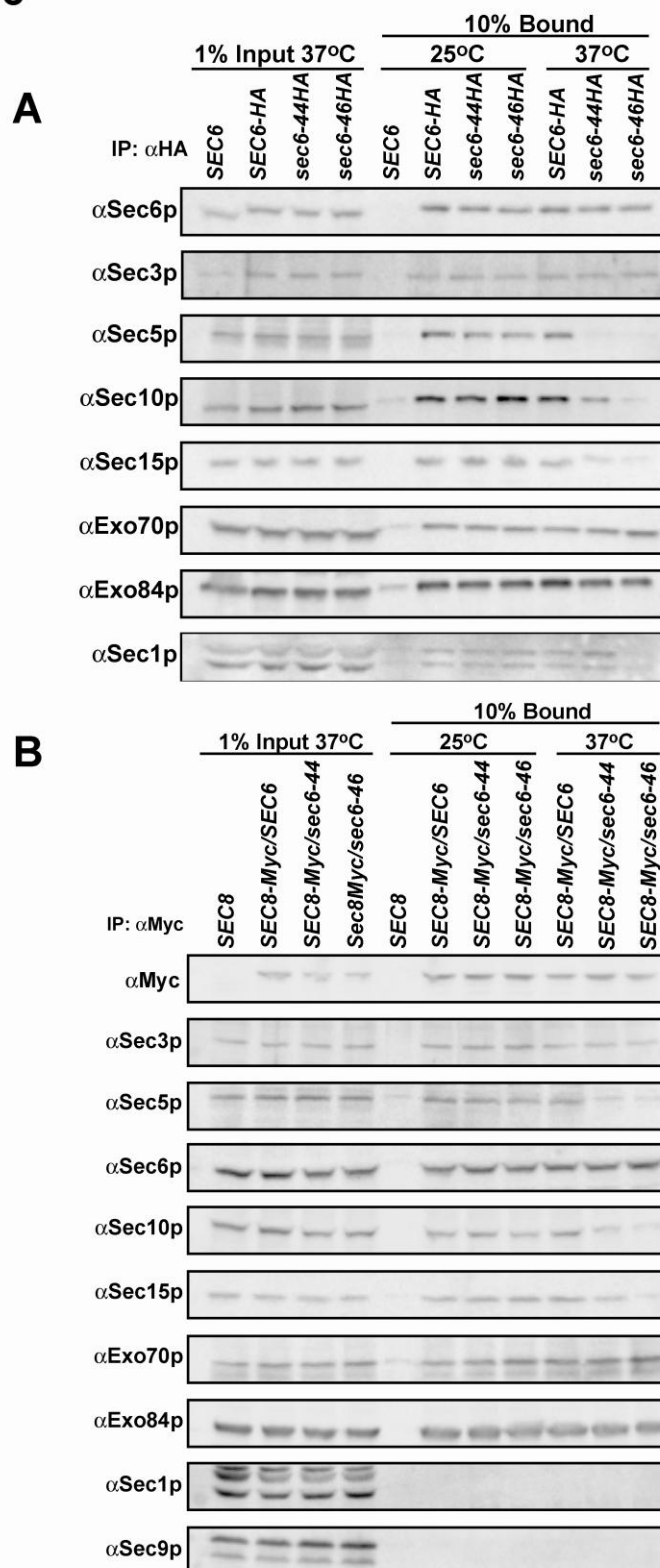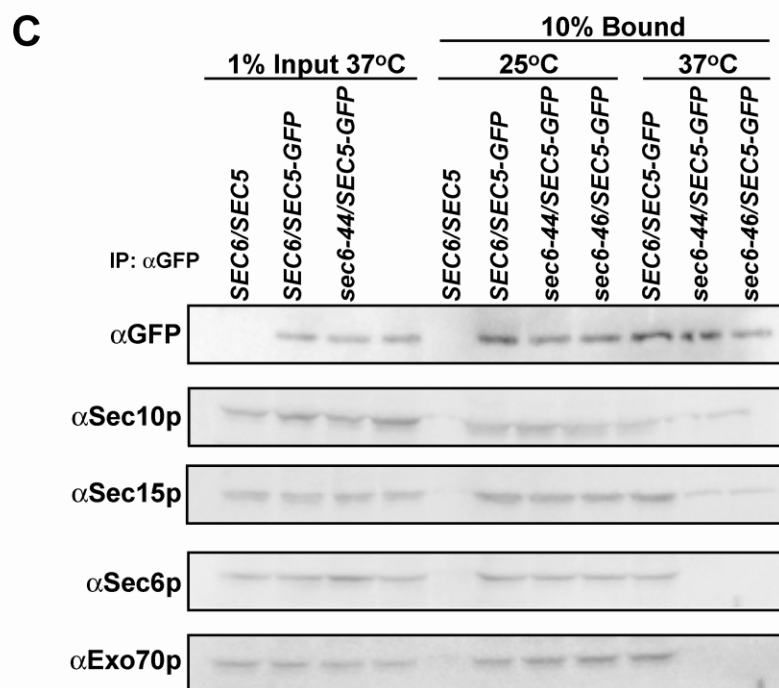
D
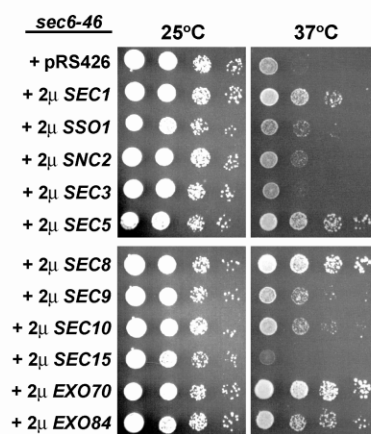
To identify candidates for the direct binding partner for these Sec6p residues, we examined genetic interactions between these mutants and various late-acting *sec* mutants. Combining these *sec6* alleles with other exocyst temperature sensitive mutations resulted in synthetic growth defects, but none were significantly more severe than others (data not shown). However, the *sec6-44* and *sec6-46* alleles could be suppressed by overexpression of a subset of *sec* proteins (Figure 2.6D; *sec6-46* is shown). 2µ plasmids containing the exocytic SNAREs *SSO1*, *SNC2* or *SEC9* or the exocyst subunit *SEC10* were able to weakly suppress the temperature sensitive defect. Overexpression of *SEC5*, *SEC8*, *EXO70* and *EXO84* strongly suppressed the growth phenotype. Conversely, overexpression of *SEC15* had a negative effect on the growth of the *sec6* alleles, consistent with previously observed detrimental effects upon overexpression of *SEC15* on wild-type cells [152]. Interestingly, the 2µ plasmid expressing the regulator *SEC1* also suppressed the *sec6-46* phenotype, suggesting a possible interaction between Sec6p and Sec1p. Because Sec5p is one of the exocyst subunits lost at the non-permissive temperature, and is a strong suppressor of the temperature sensitive alleles, we propose that Sec5p is the direct binding partner for this region of Sec6p. Currently, the lack of soluble recombinant proteins has prevented this from being directly tested; however, this conclusion is supported by the presence of a similar exocyst subcomplex found in the *sec5-24* temperature sensitive strain [80].

The Free Pool of Sec6p Interacts with the t-SNARE Sec9p

We previously showed that Sec6p is present in both exocyst-bound and free pools *in vivo* [65]; our current results suggest that it is this free pool of Sec6p that interacts with Sec9p. First, no significant amount of Sec9p was coimmunoprecipitated with Sec8-Myc and the exocyst complex (Figure 2.6B), in contrast to the ~10% that coimmunoprecipitated with Sec6-HA (Figure 2.3A). Secondly, when Sec9-HA was immunoprecipitated from the wild-type *SEC6* strain, the only exocyst subunit to coimmunoprecipitate was Sec6p (Figure 2.7A). To test this more directly, we performed ultracentrifugation experiments to separate the free pool of Sec6p from the exocyst-bound pool. Yeast lysates were centrifuged at 50,000 rpm (105,000 x g) for 20 min, and the supernatant and pellet fractions were tested for the presence of exocyst proteins, as well as Sec9p (Figure 2.7B). Sec6p was present in both supernatant and pellet fractions, as expected from prior gel filtration results [65], although Sec6p was previously found only in the pellet fraction after a 100,000 rpm spin [23]. Sec10p and Sec15 were present in both the supernatant and pellet fractions as well, as previously shown [23]. Sec9p was located only in the supernatant fraction, suggesting that it was unlikely to be stably interacting with the exocyst. To confirm that Sec9p interacts with the non-exocyst-bound Sec6p, immunoprecipitation of Sec6-HA from the supernatant fraction co-immunoprecipitated Sec9p but failed to immunoprecipitate Sec10p and Sec15p (Figure 2.7C). We conclude from these data that Sec9p interacts with Sec6p when it is not assembled into the exocyst complex.

Figure 2.7 Sec9p and Sec1p interact primarily with the non-exocyst-bound free pool of Sec6p. (A) α-HA immunoprecipitations of HA_3-tagged Sec9p and were performed. Immunoprecipitation of Sec9-HA only coimmunoprecipitates Sec6p. (B) Cells were centrifuged for 20 min at 50,000 rpm and the input lysate, pellet and supernatant fractions were each tested for the presence of exocyst, Sec1p and Sec9p proteins via western blot analyses. Sec9p and Sec1p appear predominantly in the supernatant, separate from the exocyst, which appears in the pellet fraction. Sec10p and Sec15p are found in both the supernatant and pellet fractions, as shown previously [23]. (C) The non-exocyst-bound Sec6-HA was immunoprecipitated from the supernatant fraction of A and bound proteins were analyzed by western blot. Sec6-HA co-immunoprecipitated Sec1p and Sec9p, but not the Sec10p and Sec15p proteins. Experiments were repeated three times.

Figure 2.7
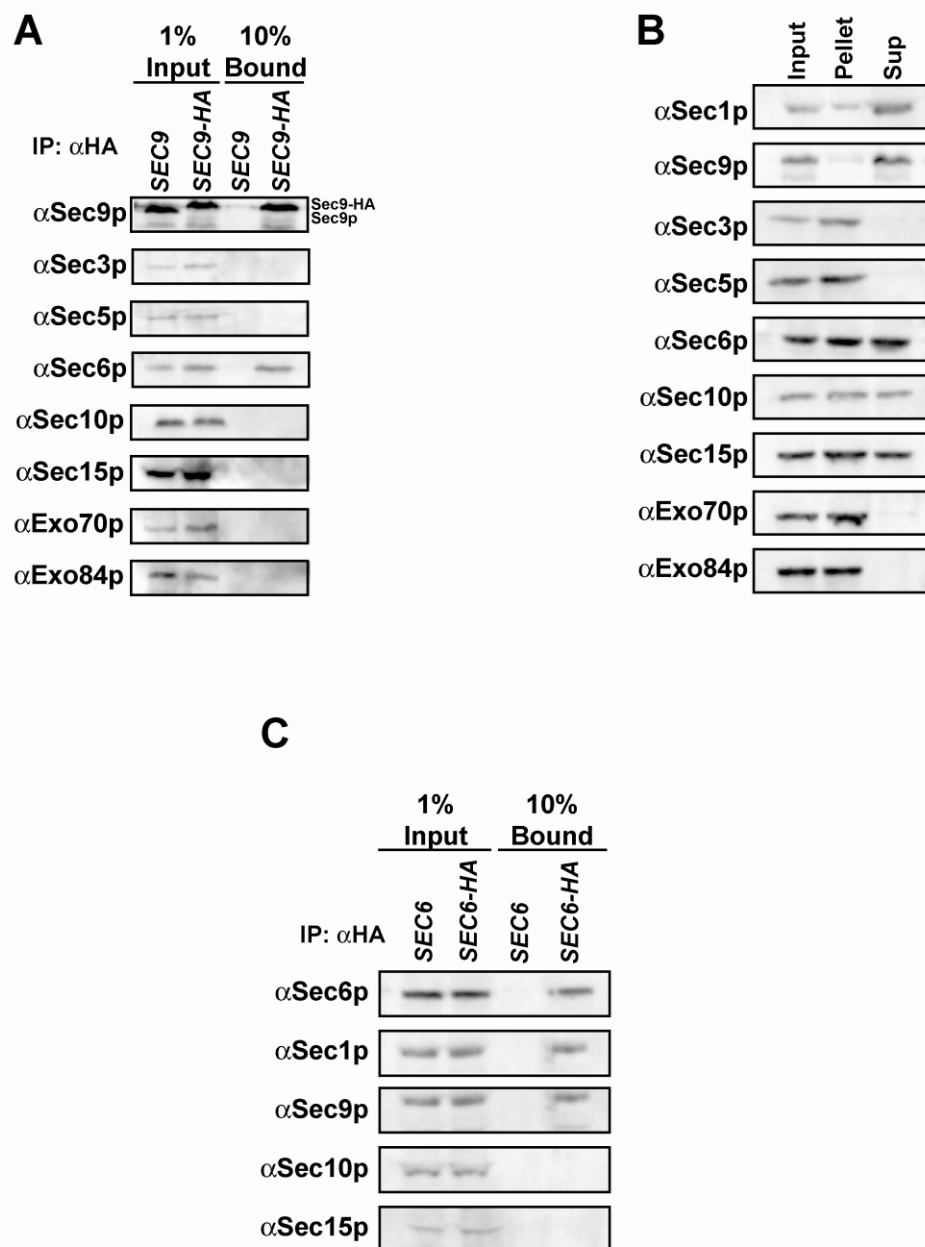
Sec6p Directly Interacts with the SNARE Regulator Sec1p

Previously, a weak interaction was observed between the exocytic SM protein

Sec1p and the exocyst complex [122]. Other tethering complexes interact with their partner

SM proteins: the vacuolar SM protein Vps33p is a member of the Class C/HOPS

tethering complex [73,153] and Sly1p, the SM protein that regulates traffic between the ER

and Golgi, was recently shown to interact with the assembled COG complex [137]. When

we assayed for Sec1p in our immunoprecipitation experiments, Sec6-HA$_3$

coimmunoprecipitated ~10% of the total Sec1p (Figure 2.6A). Interestingly, the Sec8-

Myc immunoprecipitation from wild-type Sec6p cells did not coimmunoprecipitate any

significant amount of Sec1p (Figure 2.6B). This differs from previous results in which a

minor amount (~0.2 - 0.4%) of Sec1p was bound to the exocyst complex (unless Sec1p

was overexpressed), as monitored by coimmunoprecipitation with Sec8-Myc or Sec10-

Myc [122]. However, our experiments used approximately one-sixth of the total amount of

yeast cells compared to the prior experiments. We repeated our coimmunoprecipitation

experiments using Sec8-Myc cells at the higher cell concentration and obtained similar

results to the previously published data. These data suggest that Sec6p directly interacts

with Sec1p, but Sec1p may also have weak interactions with other exocyst subunits. Data

from the *sec6-46-HA$_3$* mutant supports the idea of a direct Sec6p-Sec1p interaction; at the

non-permissive temperature, Sec1p does not coimmunoprecipitate with *sec6-46* (Figure

2.6A). In addition, the *sec6-46* phenotype was strongly suppressed by overexpression of

Sec1p (Figure 2.6D). Furthermore, when Sec6-HA was immunoprecipitated from the

ultracentrifuged lysates (as performed above with Sec9p), Sec6-HA co-

immunoprecipitated Sec1p from the supernatant fraction (Figure 2.7C). Together, these data reveal that the free, non-exocyst-bound pool of Sec6p interacts with Sec1p.

To determine if the interaction between Sec1p and the exocyst is directly mediated through Sec6p, we immunoprecipitated yeast Sec1-V5-His$_6$ [70] using an α-V5 antibody, and subsequently tested binding of the immobilized Sec1p to recombinant purified His$_6$-Sec6p (Figure 2.8). In the V5 immunoprecipitation, a fraction of the endogenous Sec6p is coprecipitated in the absence of additional Sec6p (+buffer lane). The bound Sec1p was washed thoroughly with binding buffer and then incubated with excess recombinant His$_6$-Sec6p; a substantial amount of the recombinant Sec6p bound to the Sec1p. As a negative control, we incubated purified Sso1p with the immobilized Sec1p and did not detect any Sso1p binding. Sso1p is the other exocytic t-SNARE; in the absence of the Sec9p and Snc2p SNARE proteins, it does not interact with Sec1p [70]. Together, these results support the hypothesis that Sec1p binds directly to Sec6p, and, like the Sec6p-Sec9p interaction, the majority of the Sec6p-Sec1p interaction involves the non-exocyst-bound pool of Sec6p.

Figure 2.8 Purified recombinant Sec6p binds to immobilized Sec1p-V5-His$_6$. Sec1p-V5-His$_6$ was immunoprecipitated with α-V5 antibody and washed 3 times with binding buffer. *In vitro* purified His$_6$-Sec6p was then incubated with the immobilized Sec1p, unbound protein was washed off, and bound proteins detected by Western blot analysis. An untagged *SEC1* strains was used as a negative control (lane 3). Lane 4 is a buffer control in which no His$_6$-Sec6p was added, to show the amount of endogenous yeast Sec6p bound to the immobilized Sec1p after washing. The t-SNARE Sso1p, which does not bind to Sec1p in the absence of the other SNAREs [70], is shown as a negative control.

Figure 2.8

	1% Input		10% Bound		
IP: αV5	*SEC1*	*SEC1-V5*	*SEC1*	*SEC1-V5*	*SEC1-V5*

αSec1p

	SEC1+His-Sec6	*SEC1-V5*+His-Sec6	*SEC1*+His-Sec6	*SEC1-V5*+Buffer	*SEC1-V5*+His-Sec6

αSec6p

	SEC1+Sso1	*SEC1-V5*+Sso1	*SEC1*+Sso1	*SEC1-V5*+Sso1

αSso1p
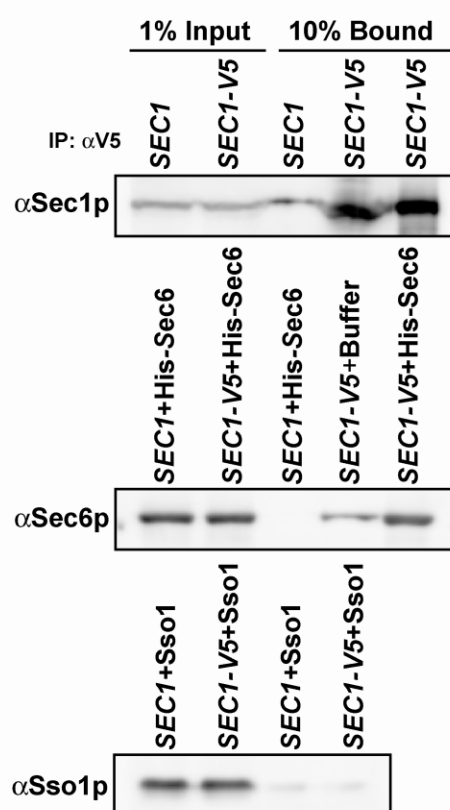
DISCUSSION

We identified a novel class of temperature sensitive *sec6* alleles in the N-terminal region of Sec6p (Figure 2.2A) that specifically disrupt exocyst complex assembly and/or stability without disrupting polarized localization. Immunoprecipitation experiments indicated that Sec9p interacts with a non-exocyst bound pool of Sec6p *in vivo*, and not with exocyst-bound Sec6p (Figure 2.1, Figure 2.7A and C), and that the *sec6* mutations by themselves are not sufficient to disrupt the Sec6p-Sec9p interaction (Figure 2.3A). Instead, these mutants disrupt an interaction within the exocyst complex, leaving a stable subcomplex consisting of Sec6p, Sec3p, Sec8p, Exo70p and Exo84p localized to sites of growth and secretion.

The mutations in Sec6p result in a temperature sensitive growth phenotype (Figure 2.2B and C), and show a block in Bgl2p secretion concurrent with the visible growth defect, while exhibiting a delayed defect in invertase secretion (Figure 2.2D and E). Bgl2p is an endo-B-1,3-glucanase and major component of the yeast cell wall [154]. Bgl2p is transported in a population of lower density secretory vesicles, while invertase is found in a higher density population [149]. In many different trafficking mutants that lead to an accumulation of secretory vesicles, the cargo of higher density vesicles continues to be secreted [155]. This suggests that these higher density vesicles (as marked by invertase) may not be as sensitive to the state of the regulatory machinery as the lower density vesicles, which carry material specifically for membrane and cell wall expansion. Similar results were observed for the *exo70-35* and *exo70-38* mutants [150], which had a selective defect in Bgl2p secretion although they continued to secrete invertase-containing vesicles.

Under non-permissive conditions, the mutant Sec6p proteins remained properly localized (Figure 2.4), consistent with our previous identification of residues in the C-terminal domain of Sec6p that are critical for anchoring the complex at sites of secretion [87]. Most of the other subunits of the exocyst were also localized. Only Sec5p, Sec10p, and Sec15p showed significant mislocalization (Figure 2.5). Coimmunoprecipitation of the exocyst complexes using tagged Sec6p or Sec8p showed similar results; Sec5p, Sec10p and Sec15p did not remain bound to the Sec6p subcomplex at the non-permissive temperature (Figure 2.6). This Sec6p-Sec3p-Sec8p-Exo70p-Exo84p subcomplex may provide a direct link between sites of secretion on the plasma membrane and the Sec15p-Sec10p subcomplex on secretory vesicles [23].

Which subunit of the exocyst interacts with these N-terminal Sec6p residues? The strongest candidate to bridge the plasma membrane and vesicle-bound components of the exocyst is Sec5p. This conclusion is supported by the previously observed *in vitro* interaction between Sec5p and Sec6p [23], and the strong suppression of the *sec6-44* and *sec6-46* mutant phenotypes observed upon overexpression of *SEC5* (Figure 2.6D). Sec10p is also a possibility; however, we previously showed that the C-terminal region of Sec6p alone is sufficient for binding to Sec10p *in vitro* [88]. It is likely that Sec10p is lost from the complex indirectly due to the loss of Sec5p, which also interacts with Sec10p [23]. Sec15p has not been shown to interact directly with Sec6p or Sec5p, but is likely disrupted from the complex through its interaction with Sec10p [23]. Overexpression of other subunits that remain bound to the mutant Sec6p proteins (*SEC8*, *EXO70*, and *EXO84*) also suppresses the mutants, likely by stabilizing the mutant proteins and

interactions, and thus stabilizing the interaction of Sec6p with Sec5p. Together, these results indicate that while the C-terminal region of Sec6p functions to regulate localization of the complex [87], the N-terminal region of Sec6p functions as an interaction core for exocyst complex assembly and maintenance. Thus, Sec6p would anchor the core exocyst subunits at the plasma membrane, and with Sec5p, would bridge to the vesicle-associated Sec15p-Sec10p proteins, to directly tether vesicles to the plasma membrane before fusion. Consistent with these dual functions, we observed that a Sec6p protein containing the combined mutations from the N-terminal *sec6-46*, and C-terminal *sec6-54* alleles, was completely non-functional as the sole copy of Sec6p in yeast (data not shown).

We also identified a direct interaction between Sec6p and the exocytic SM protein Sec1p (Figure 2.8). The *sec6-46* mutation disrupts interactions between Sec6p and Sec1p at the non-permissive temperature (Figure 2.6A), and its temperature sensitive growth defect is suppressed by overexpression of *SEC1* (Figure 2.6D). SM proteins are regulators of SNARE complex assembly and fusion [66], and Sec1p specifically interacts with the assembled exocytic SNARE complex [70]. The Sec6p-Sec1p interaction may serve to recruit Sec1p to sites of secretion, as this function cannot be provided by its cognate syntaxin Sso1p [70]. Also, the interaction may add further regulation and specificity to the fusion process. In other systems, interactions between tethering complexes and SM proteins are required for proofreading SNARE complexes or for SNARE complex formation [53,61,137]. We previously showed that the Sec6p-Sec9p interaction slowed formation of binary SNARE complexes *in vitro* and hypothesized that missing cofactors

would be necessary to stimulate assembly (Sivaram *et al.*, 2005). Sec1p could be one of those cofactors, functioning similarly to Sly1p in its interactions with the COG complex to stimulate formation of the SNARE complex [137]. A functional interaction between Sec1p and Sec6p is consistent with the recent finding that Sec1p is required upstream of SNARE complex assembly [72].

The interactions of Sec6p with the t-SNARE Sec9p and the SNARE regulatory protein Sec1p take place predominantly in the absence of the assembled exocyst complex (Figure 2.6, Figure 2.7, Figure 2.8). Prior to vesicle arrival and exocyst complex assembly, Sec6p could function to prevent premature SNARE complex assembly. In the presence of the other exocyst subunits and Sec1p, Sec6p would function to anchor the assembled exocyst complex and may, in collaboration with Sec1p, facilitate proper SNARE complex assembly. This could explain the disruption of exocyst assembly when residues that crosslink between Sec6p and Sec9p are mutated. If binding to Sec6p is a competitive process between Sec5p and Sec9p then assembly of the exocyst would disengage Sec6p from Sec9p releasing any inhibition caused by Sec6p. The free pool of Sec6p may interact with Sec1p in order to recruit a small amount of that protein to the assembling exocyst. Testing of these models requires further biochemical and genetic studies to explore whether the Sec1p interaction with Sec6p occurs concurrently with the Sec6p-Sec9p interaction, and what effect Sec1p and Sec6p have on exocyst and SNARE complex assembly *in vitro* and *in vivo*.

Chapter III:

Conclusions, Future Directions,

and the Future of the Field

Discussion

In all eukaryotes, the basic functioning of cells, growth and survival, requires the proper trafficking of cargo between membrane-bound organelles and the plasma membrane. Exocytosis and endocytosis require multiple classes of proteins, including tethering factors, SNARE proteins, and SM proteins to control and regulate these processes both temporally and spatially [1,156]. Most of these proteins are essential, defects in any protein class leads to disease or death, and every new discovery reveals further reaching effects of trafficking into less obviously trafficking linked systems [127,12,13,59]. Understanding trafficking systems – their defects, the diseases they cause, and discovering treatments – requires elucidation of the basic interactions between the proteins involved, both *in vitro* and *in vivo*.

The field of trafficking is fast progressing; the huge body of collective knowledge is growing to the point where smaller and smaller answers lead us to larger and larger conclusions. Each individual discovery reveals multiple missing pieces of the puzzle. Characterization of individual interactions between proteins allows the construction of larger models that lead toward a paradigm of trafficking systems biology, a complete system-wide model. To build an effective paradigm for trafficking, we must understand the interactions and regulation networks between the SNARE proteins, SM proteins, and tethering factors. The more individual trafficking interactions we characterize both *in vivo* and *in vitro* the clearer it becomes that many of the mechanisms, structures, and functions of the proteins involved are conserved.

In this thesis I have focused on the *in vivo* interactions between three proteins in one step of trafficking, exocytosis to the plasma membrane. I have clarified the interactions between the SM protein, Sec1p, the SNARE protein Sec9p and the tethering complex subunit Sec6p creating a picture of conservation of function if not the finer points of mechanism by demonstrating SM:tethering complex:SNARE interactions. Tethering complexes and their subunits interact with both SNARE and SM proteins along with other factors to form a regulatory network that ensures fidelity of fusion. The conclusions reached in the previous chapter of my thesis shine light on questions both large and small, while also raising many new questions and possibilities.

The purpose of large tethering complexes has been a considerable and vexing question challenging the field. As more individual interactions are elucidated, these large complexes are revealed both as members and as focal points of complex, dynamic interaction networks. These networks form a multilayer system that regulates the last steps of trafficking and fusion. Subunits function both as members of and external to their complexes. And now, Sec6p joins this paradigm, through its interactions both within the exocyst, and with external proteins such as Sec9 (Figure 2.1), Sec1p (Figure 2.8), and the anchor [87].

The identification of a stable subcomplex of the exocyst is a novel discovery. Prior mutations that cause complex disruption have displayed semi-stable or unstable subcomplexes and have not been explored further [5,23]. Further, the *sec6-44* and *sec6-46* mutants appear to selectively block only one branch of the secretory pathway, the Bgl2p

pathway, while leaving the invertase pathway unaffected until six hours (Figure 2.3D and

E). This selective block is similar to that shown in certain Exo70p mutants [150]. The

possibilities this raises are intriguing. The interaction between Sec6p and Sec5p is

disrupted (Figure 3.1 i) in the *sec6-44* and *sec6-46* mutants, which leads to loss of Sec5p,

Sec10p, and Sec15p from the complex (Figure 3.2). Though the loss of Sec5p, Sec10p,

and Sec15p causes an immediate block in Bgl2p secretion (Figure 2.3D) invertase

secretion continues as normal for another several hours (Figure 2.3E). This is surprising

given that the loss of Sec15p and Sec10p via the disruption of Sec5p:Sec6p interaction

(Figure 3.2) apparently denies vesicles a direct connection to the target membrane. This

result suggests that at least some vesicles can continue to fuse using only a subcomplex

of the exocyst that is not directly interacting with the vesicle. One possibility for the

delay in complete cessation of secretion is that the lack of fusion of the Bgl2p-carrying

vesicles causes slow depletion of factors on the membrane required for fusion of the

vesicles that otherwise can fuse utilizing only a subcomplex of the exocyst. Another

possibility is that the Bgl2p pathway is simply more sensitive to perturbations in exocytic

function. This could be the case if the Bgl2p pathway requires more properly assembled

exocyst, for instance.

Previously, we knew that Sec6p interacted with the t-SNARE Sec9p. However,

the Sec6p:Sec9p interaction appeared to inhibit the formation of the binary t-SNARE

complex, which is essential for formation of a fusion-competent SNARE complex [42,65].

The identification of a hypothesized factor required for *in vivo* function has been elusive.

An assembled exocyst complex could be required, but has not been reconstituted

Figure 3.1 Schematic of disrupted Sec6p:Sec5p interaction in *sec6-44* and *sec6-66* mutants and Sec6p:Sec1p interaction in the *sec6-66* mutant. Sec5p interaction (i) with Sec6p is abrogated in mutants, disrupting exocyst assembly/maintenance. Sec1p:Sec6p interaction may also be disrupted in the *sec6-46* mutant (o)
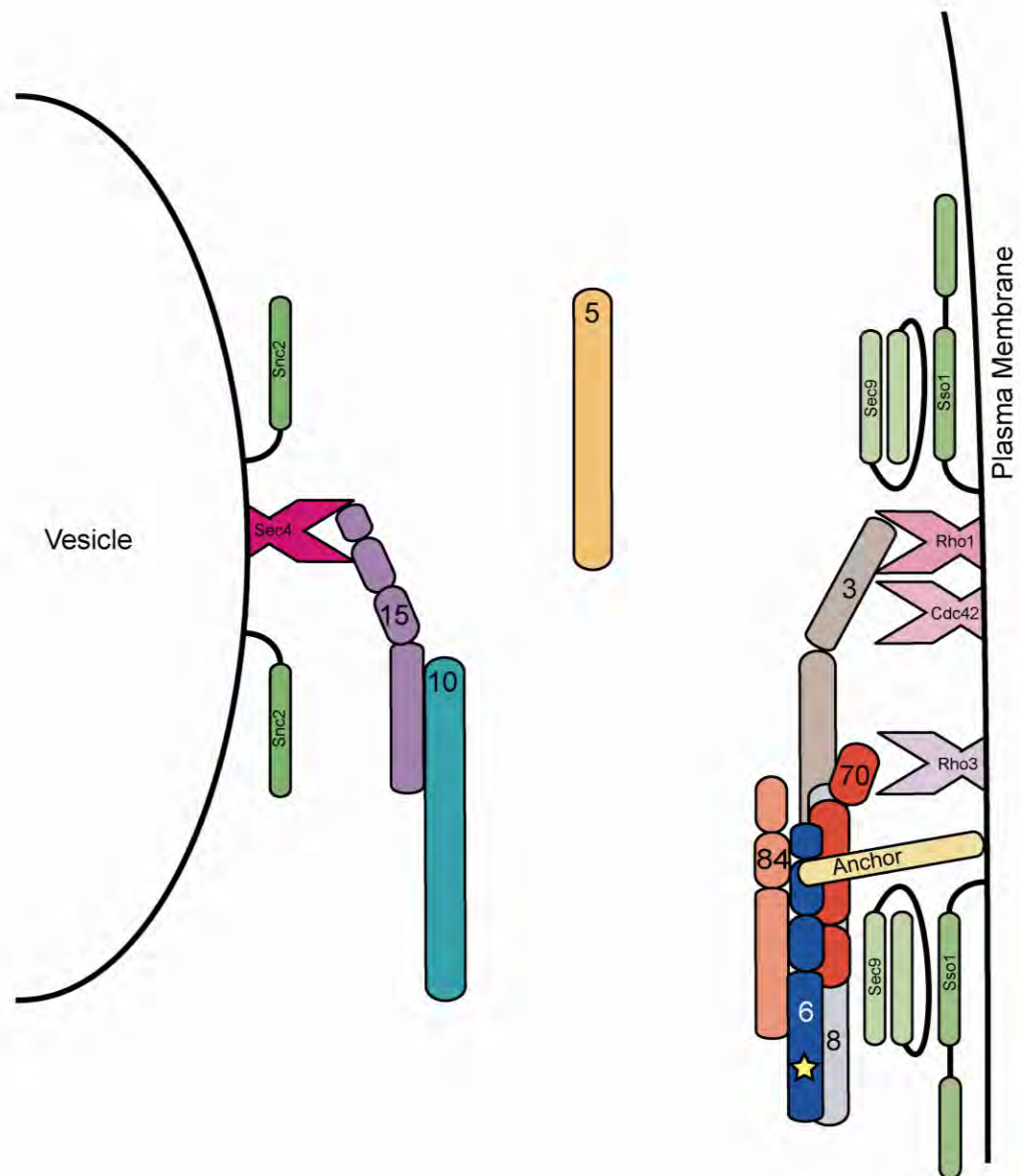
Figure 3.1
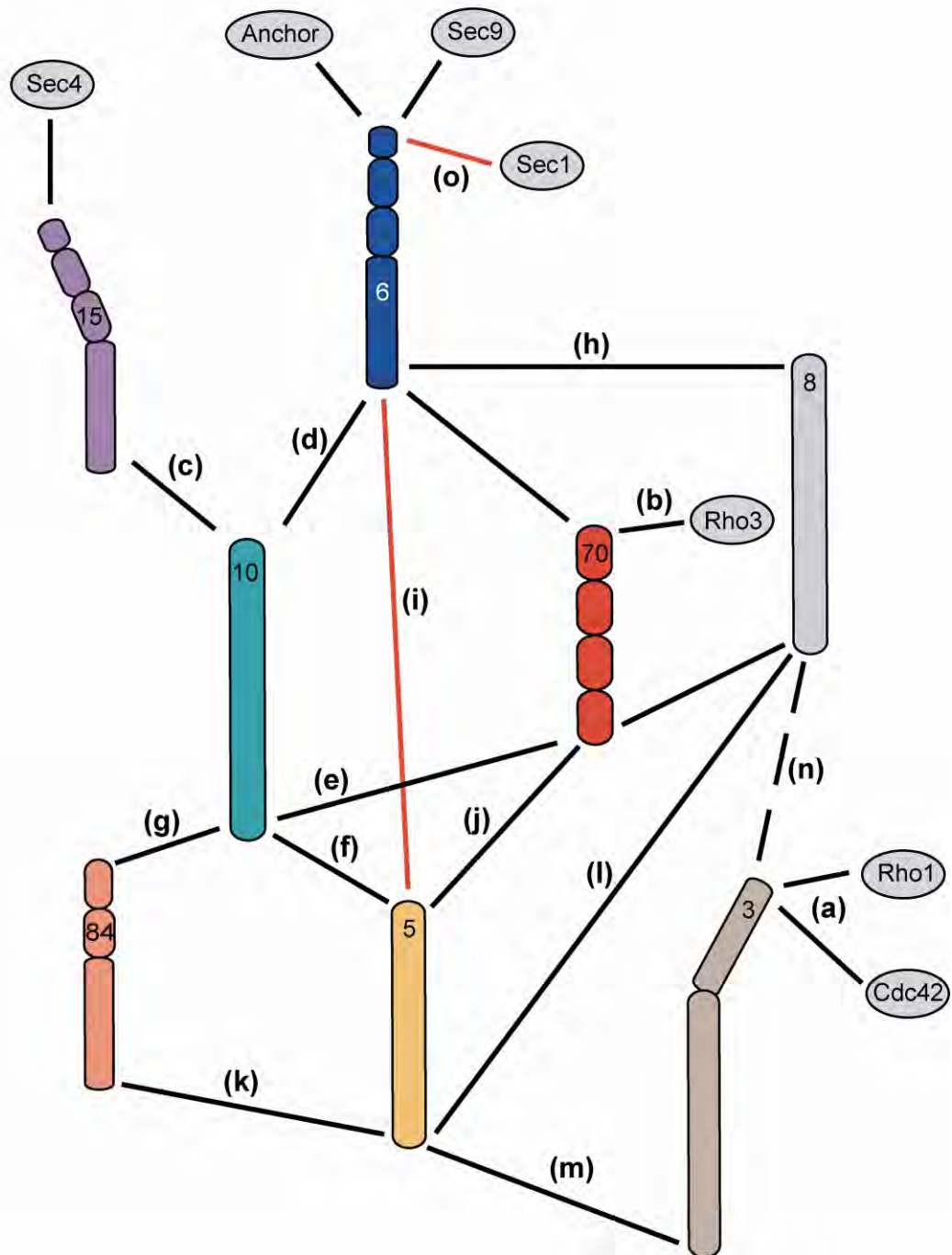
Figure 3.2 Schematic of loss of fusion in *sec6-44* and *sec6-66* mutants. Sec6p:Sec5p interaction is disrupted, causing the loss of Sec5p, Sec10p, and Sec15p from the complex. The connection between the vesicle and the plasma membrane is lost, and any tethering function is disrupted. The mutations in Sec6p are represented by a yellow star.

Figure 3.2

in vitro yet. Until my recent experiments, it was possible that the Sec6p:Sec9p

interaction might be an artifact of the *in vitro* assay rather than an interaction that occurs

in vivo. That is now clearly not the case, as I have demonstrated a robust *in vivo*

interaction between Sec6p and Sec9p via *in vivo* immunoprecipitations of each protein

(Figure 2.1). Though I expected to find Sec9p interacting with the exocyst via Sec6p, I

instead found that the interaction between Sec6p and Sec9p involves the previously

identified "free pool" of Sec6p [65]. The absence of Sec9p bound to exocyst

immunoprecipitated via Myc-Sec8 (Figure 2.6B) and ultracentrifugation which shows

Sec9p present only in the supernatant fraction (Figure 2.7) demonstrate this. The function

of this interaction, however, remains unclear. Sec6p may be sequestering Sec9p from

interaction with Sso1p, preventing premature formation of the binary SNARE complex

before the arrival and tethering of vesicles. Or perhaps, the Sec6p:Sec9p interaction

involves a third protein, required for proper SNARE complex assembly. SNARE

assembly assays conducted in the presence of assembling exocyst and other factors will

help to shed light on this possibility. Additionally, mutants in Sec6p that disrupt the Sec9

interaction will help elucidate the function of this interaction *in vivo*.

A growing theme in the field has become the interaction between tethering

complexes and SM proteins. Some SM proteins are even included among the subunits of

their relevant tethering complex [53,66-68]. Similarly the SM protein Sec1p interacts with the

exocyst complex, though only weakly unless Sec1p is overexpressed [122]. On closer

inspection, it now appears that Sec1p interaction with the exocyst complex occurs

through Sec6p, which interacts directly with Sec1p (Figure 2.8). And that this interaction

preferentially utilizes the free pool of Sec6p (Figure 2.7). Interaction between the exocyst

complex and Sec1p appears to be minimal when compared to that with Sec6p. The

function of this interaction requires further study. The well defined functions of Sec1p

and other SM proteins involve SNARE complex assembly and fusion, but my Sec6p

coimmunoprecipitation of Sec1p does not simultaneously coimmunoprecipitate Snc2p or

Sso1p. Additionally, Sec1p only interacts with the complete SNARE complex [70,71]. These

data suggest that the Sec6p:Sec1p interaction occurs prior to SNARE complex assembly.

This conclusion is supported by recent genetic data suggesting that Sec1p has an as yet

unidentified function prior to SNARE assembly [72]. SNARE assembly assays in the

presence of both Sec1p and Sec6p will determine whether this interaction is required for

proper SNARE assembly. Further mutants that disrupt Sec6p:Sec1p interaction may also

be useful.

The direct *in vivo* interactions described here raise many further questions even as

they answer others. Sec6p interacts directly with Sec1p (Figure 2.8), and the t-SNARE

Sec9p (Figure 2.1), and with the unidentified anchor protein [87].Could these interactions

occur simultaneously? Perhaps Sec1p is required for proper SNARE assembly, the third

factor suggested to work with Sec6p to enhance SNARE complex assembly. In

conjunction with Sec6p, Sec1 may enhance SNARE assembly to *in vivo* levels (Figure

3.3A). This possibility is compelling, but Sec1p does not interact with Sec9p or Sso1p

except as a member of the complete SNARE complex [71,118]. However, if Sec6p interacts

simultaneously with both Sec1p and Sec9p, then a direct interaction between Sec1p and

Sec9p may not be required. This model would, however, require yet a third factor to

Figure 3.3 Models for Sec1p:Sec6p:Sec9p interaction. A) Sec6p interacts

simultaneously with Sec9p and Sec1p and a third factor, possibly the opener, to control

SNARE complex assembly. B) SNARE complex assembly is a concerted action, rather

than stepwise as occurs *in vitro*. C) Sec6p interaction prevents Sec9p from forming

SNARE complex with Sso1p opened by a localized and promiscuous opener. Exocyst

assembly dislocates Sec6p from Sec9p allowing SNARE assembly to proceed.

Figure 3.3

A
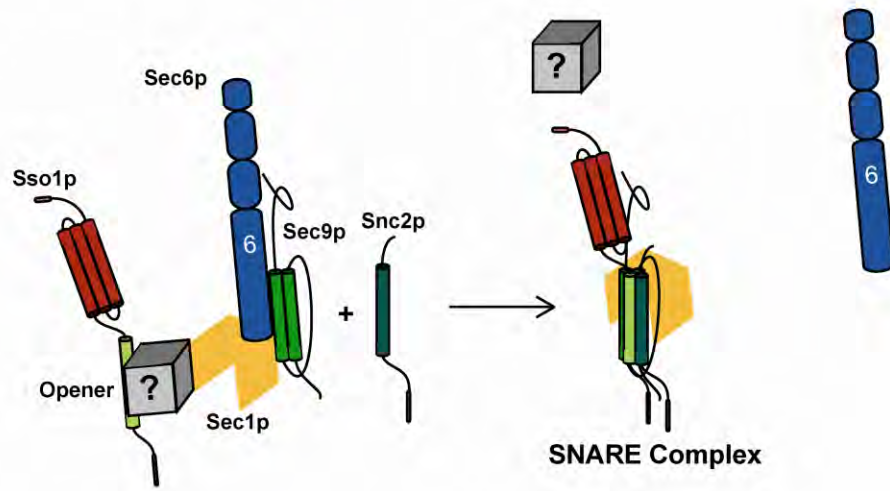
B
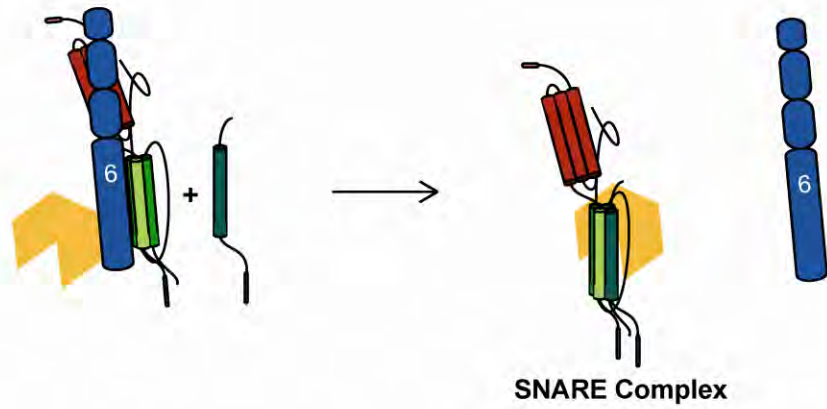
C
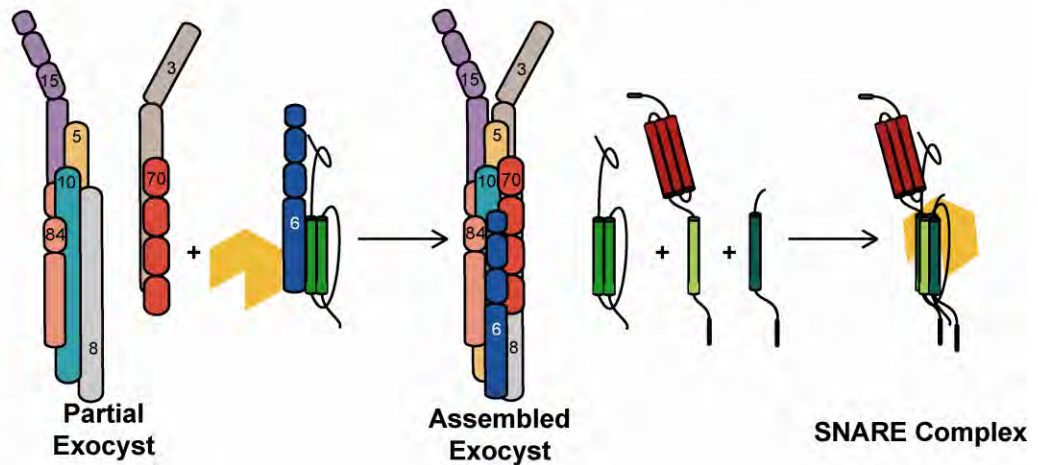
interact with Sso1p (Figure 3.3A). Perhaps it is the hypothesized" opener" protein that releases Sso1p autoinhibition [41,42].

There is a remote possibility that the *in vivo* formation of the SNARE complex is a concerted reaction and that the apparent order of *in vitro* assembly is an artifact of the assay. That is, *in vitro* the syntaxin t-SNARE Sso1p opens, binds to its partner t-SNARE Sec9p, and then the complex binds the final motif from the v-SNARE Snc2p, initiating fusion. *in vivo* this sequence may occur rapidly. In this case, all the players are waiting just offstage, possibly bound to other factors, for the maestro Sec1p to start the play. When Sec1p raises its baton and signals, the proteins all swoop in at once from their factors to bind Sec1p as a complete SNARE complex (Figure 3.1B). However, in this model, Sec6p would be expected to coimmunoprecipitate Sso1p, but no coimmunoprecipitation is displayed. Additionally, the closed formation of Sso1p and requirement for an opener protein [115] makes this possibility seem unlikely. To examine these possibilities, SNARE assembly assays with the SNARE proteins, as well as Sec1p and Sec6p must be performed. Ideally, other potential factors such as the complexed exocyst could be added. However, many of the exocyst subunits still defy purification. Previously, SNARE assembly assays have been performed largely with the t-SNARES because opening of the syntaxin t-SNARE Sso1p is the rate-limiting step [41,42]. However, given that Sec1p interacts with the complete SNARE complex, assays involving Sec1p would likely require a complete set of SNARE proteins – Sec9p, Sso1p, and Snc2p.

A final hypothesis, and the one currently best supported by the evidence, is that Sec6p inhibition of SNARE complex assembly is not an artifact, despite my original hypothesis that it was. In this model, Sec6p binds to Sec9p before the arrival of the vesicle, thus preventing premature formation of the binary t-SNARE complex. With the arrival of the vesicle, and newly synthesized exocyst components [98], the exocyst assembles from a mix of new and disassembled subunits. Exocyst assembly causes Sec6p to dissociate from Sec9p to enter the complex, freeing Sec9p. Sec9p, Sso1p, and Snc2 then form the SNARE complex, uninhibited (Figure 3.1C). This hypothesis is consistent with the current data both *in vitro* and *in vivo.* The overlap of the putative Sec6p:Sec9p interaction region revealed by mass spectrometry and the Sec6p:Sec5p interaction region revealed by the *sec6-44* and *sec6-46* mutations supports a competitive model where Sec5p interaction with Sec6p, exocyst assembly, releases Sec9p from Sec6p. However, Sec6p appears localized to sites of secretion, not the entire membrane, as is Sec9p. The "opener" for Sso1 may be localized to sites of secretion and is promiscuous, opening Sso1p prematurely. This open Sso1p would create a requirement for inhibition of SNARE complex formation prior to arrival of the vesicle, possibly by Sec6p.The free pool of Sec6p may also be distributed across the plasma membrane, without sufficient density to compare to the foci of polarized exocytosis. As Sec6p:Sec9p interaction utilizes the free Sec6p pool, FRET between Sec6p and Sec9p may be an effective way to test the localization of the free pool of Sec6p. One further detail that requires some explanation is that Boyd et al. identified Sec6p as being trafficked with the vesicle [98], implying that final exocyst assembly at the membrane would not cause free Sec6p to

dissociate from Sec9p. However, Boyd et al. did not see any retrograde traffic of exocyst components. Given the extended halflife of Sec6p noted in this thesis, we assume that significant exocyst assembly and disassembly occurs at sites of secretion if there is no significant retrograde trafficking of subunits. The remaining question in this model, then, is the function of Sec6p:Sec1p interaction (and possible exocyst interaction). One possibility is that Sec6p is recruiting Sec1p for the upstream function suggested by Hashizume et al (2009). Additionally, interaction with Sec6p may protect Sec9p from proteolysis, though Sec9p is not localized to sites of secretion as Sec6p appears to be.

Ideally, SNARE assembly experiments could be performed with both wild type and the *sec6-46* mutant protein that disrupts the Sec1p:Sec6p interaction. However, the triple mutation in the N-terminal region of Sec6p appears to destabilize the already "sticky" N-terminal region sufficiently to make the mutant resistant to *in vitro* expression and purification. The disruption of the interaction between Sec6p and Sec1p by the *sec6-46* mutation does raise further questions. Where does the Sec6p:Sec1p interaction occur on Sec1p? What is the structure of the N-terminal region of Sec6p that interacts with Sec1p? Could Sec6p be utilizing the large binding cleft of Sec1p that is the main binding site on other SM proteins for SNARE interaction with the SM protein? Alternatively, perhaps the N-terminal region of Sec6p binds to Sec1p on the opposite face of Sec1p in order to change the conformation of Sec1p and encourage SNARE complex assembly. Also, given that Sec1p overexpression rescues the phenotypes of *sec6-44* and *sec6-46* mutants, does that overexpression rescue exocyst assembly? If so, why? Interaction assays using *in vitro* expressed proteins with mutations in conserved regions of Sec1p

might allow greater elucidation of the location of Sec1p:Sec6p interaction surfaces. However, Sec1p has proven somewhat resistant to large-scale purification, though our lab has promising results with Sec1p purified from yeast. The possibility of a crystal structure of Sec6p interacting with Sec1p is titillating, but unlikely due to difficulties with purification of the N-terminal region of Sec6p and Sec1p.

Though SM proteins were thought to differ extensively with respect to binding mode, as more quantitative *in vitro* methods are used we see that many of those differences are disappearing [69]. And despite their differences all SM proteins interact with cognate SNAREs [66,67]. The apparent differences between SM proteins may be small and mechanistic encouraging fidelity while the proteins conserve the same overall function. The emerging function of SM proteins is that of a master regulator of SNARE complex assembly, regulating fusion via multiple interactions with tethers and SNAREs, conserved across multiple trafficking steps.

To be sure, some differences between trafficking steps will remain. Differences in fine mechanistic detail between various tethering complexes, SNAREs, and SM proteins are essential. If interactions between all tethers and their cognate SNARES were identical, the fidelity and specificity necessary for cell survival would be lost. The individual demands of certain steps will call for slight variations in mechanism. For example, synaptic release – a highly specialized, and temporal and volumetric specific function of secretion and exocytosis – requires far more specific and layered regulation in

the docking step of the pathway than does the constitutive process of growth and budding in yeast.

Interestingly, as I demonstrate in this thesis, the portion of Sec6p that appears to be responsible for Sec9p interaction [65] is also a region critical to maintenance of a stable, complete exocyst complex. This likely ensures that Sec9p cannot interact with Sec6p while Sec6p is functioning as an exocyst subunit. The interaction region will be buried in the exocyst, perhaps with the sticky, intractable N-terminal regions of other subunits. This suggests that the exocyst complex is probably not directly involved in any regulation that Sec6p:Sec9p interaction is responsible for, though assembly regulates the Sec6p:Sec9p interaction. The fact that a free pool of Sec6p is required to interact with Sec1p and Sec9p also raises an interesting question about similarly structured proteins: Are there free pools of other proteins with similar folds? Clearly fold is not the only determinant, as only Sec6p, Sec10p, and Sec15p are found in free pools of exocyst subunits (Figure 2.7B). Size-exclusion fractionation of yeast lysate followed by western blots would allow exploration of this possibility. Some tethering complexes clearly interact directly with their SM proteins, rather than via a free pool of a subunit [61,137]; but the question is, to what purpose? Is this difference simply an evolutionary accident, or a result of a difference in mechanism or function?

The recent discovery of a putative anchoring protein that interacts with Sec6p [87] raises some engaging questions that dovetail with those raised by this thesis: What is the anchor? How does its role fit into that of Sec6p? The anchor may simply hold the exocyst

at sites of polarized secretion. However, the anchor could also act to secure/localize the free pool of Sec6p, which leads to yet another question: Is Sec6p anchored when interaction with Sec1p occurs? When Sec6p:Sec9p interaction occurs? My hypothesis is that Sec1p:Sec6p:Sec9p interaction occurs simultaneously. The free pool of Sec6p would then inhibit premature SNARE complex formation, while recruiting Sec1p to sites of secretion for its as yet unknown pre-docking function. This seems a likely hypothesis as the mechanism of Sec1p recruiting to sites of secretion is unknown, and the suggestion of a function prior to docking only enhances that question. Elucidation of the order or simultaneity of Sec6p, Sec9p, Sec1p, and anchor interactions is an essential next step in understanding the final steps of exocytosis, and possibly of trafficking in general. Studies will involve the immunoprecipitation and *in vivo* assays with yeast that combine anchor mutants such as *sec6-54* with Sec1p interaction mutants such as *sec6-46*. Successful disruption of the Sec6:Sec9p interaction via mutant proteins will also be essential to fully elucidate the function of that interaction.

Further, the role that the Sec6p dimer plays in the cell is still elusive. It has been demonstrated *in vitro* and suggested in the *in vivo* free pool [65] but the function remains unknown. Does the dimer truly exist *in vivo*? Perhaps dimerization is required to stabilize the N-terminal region of the protein when Sec6p is not interacting with multiple other exocyst subunits. Perhaps despite prior evidence [78] Sec6p is a dimer in the exocyst complex. Coexpression of truncations of Sec6p in the presence of wild-type Sec6p may allow for better elucidation of the dimer, and other intra and extra exocyst interactions.

<u>Final Conclusions</u>

The work presented in my thesis has contributed significantly to the understanding of the role of the exocyst subunit Sec6p in exocytosis, as well as elucidating further the roles of Sec9p and Sec1p. Though prior studies had suggested that Sec5p was the structural core of the exocyst, I identified point mutations in Sec6p that result in the formation of a previously unidentified stable subcomplex. Surprisingly, this subcomplex can exist in the absence of Sec5p, Sec10p, and Sec15p. This subcomplex is the first demonstrated persisting subcomplex of the exocyst. I also showed that the growth defect caused by exocyst disruption displays a delayed onset, and, prior to a disruption in invertase secretion, blocks Bgl2p secretion. The delay in growth-defect onset suggests an intriguing division of function between portions of the exocyst, similar to that previously identified in mutants in Exo70p [150].

Further, I showed that the previously demonstrated *in vitro* interaction between Sec6p and Sec9p occurs *in vivo* as well, but that it involves the free pool of Sec6p, rather than exocyst-bound Sec6p. Similarly, this free pool of Sec6p interacts directly with the SM protein Sec1p. These results raise compelling possibilities for the regulation of SNARE complex assembly and membrane fusion, and, to elucidate this system, suggest further experiments. The direct interaction between the free pool of Sec6p and Sec9p as well as Sec1ps suggests further lines of inquiry in mammalian systems, especially the neuronal system where the extent of exocyst involvement has remained unclear. Does Sec6p serve to regulate SNARE complex assembly via SNAP25 in mammalian cells as

well? Is the inhibitory effect of Sec6p perhaps even more critical in mammals where syntaxin maintains a more open state than Sso1p? My results and conclusions expand the knowledge of the field and serve to further the formation of a unified paradigm of trafficking function.

Appendix I:

Sec8 Methods

Sec8p is the essential, 121kD subunit of the exocyst complex [78]. Sec8p has been implicated in both morphological differentiation of oligodendrocytes and Schwann cell differentiation [157,131]. Sec8 deletions are embryonic lethal in mice, and fail to develop past gastrulation [158].

Within to the exocyst, I showed that Sec8p interacts directly with Sec6p and that the C-terminal region is insufficient for this interaction [88]. Based on the interaction between Sec6p and Sec8p, I hypothesized that Sec8p might play a role in regulating interaction between Sec6p and Sec9p, thus regulation of SNARE complex assembly. This hypothesis was formed prior to the data in this thesis demonstrating that Sec6p:Sec9p interaction utilized the free pool of Sec6p.

In order to explore this hypothesis and elucidate the role of Sec8p in the exocyst, I designed multiple constructs of Sec8 for expression in *E. coli* based on secondary structure predictions (Figure A1.1). Expression was attempted under multiple conditions (Table A1.1) but was largely unsuccessful, most proteins failed to express or aggregated on purification columns. A small quantity of full length, Sec8-His$_6$ construct could be purified in order to perform qualitative interaction studies [88]. However this construct resisted further purification and scaling efforts. More recently, a His$_6$ form of one of the constructs, Sec8NT4 (a.a. 19-459) has been purified using a denaturing procedure (QiaExpressionist). Cells are lysed at RT in denaturing lysis buffer (100mM NaH$_2$PO$_4$, 10mM Tris, 6M guanidine hydrochloride, pH 8.0) and pelleted. The supernatant is

Figure A1.1 Constructs of Sec8p for *in vitro* protein expression. Various constructs of

Sec8p comprising various regions of the protein, based on predicted secondary structure

were designed.

Figure A1.1
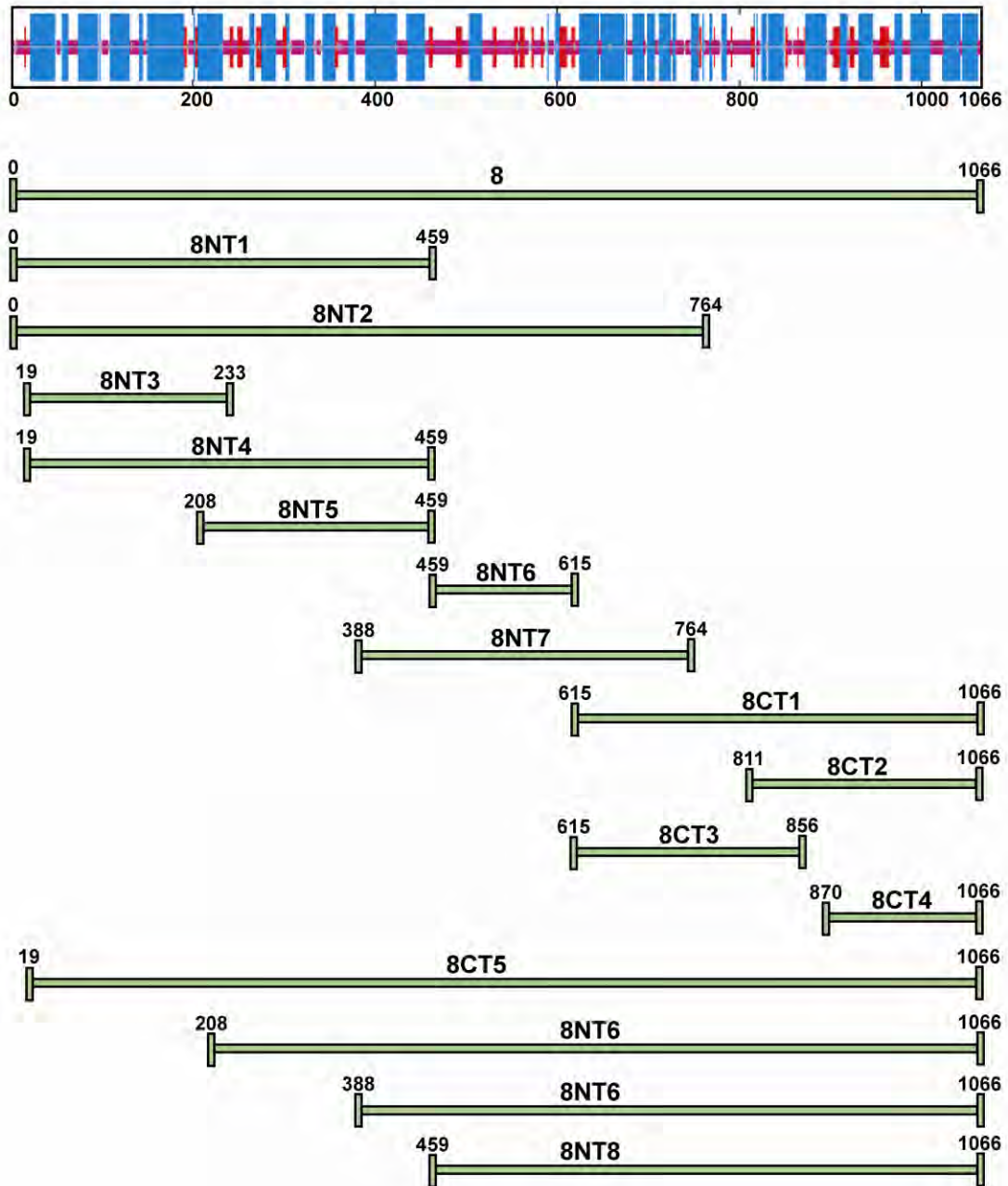
Table A1.2 Sec8 Constructs for *in vitro* purification

Construct	Final Column	Buffer	Notes
8N	Ni Beads	His Wash Buffer	1, 2, 7, 8
MBP8	Ni Beads	MBP Lysis Buffer	3
GST8	Ni Beads	GST Lysis Buffer	3
Yeast 8N	Ni Beads	His Wash Buffer	3
8NT1N	Ni Beads	His Wash Buffer	3
8NT2N	Ni Beads	His Wash Buffer	3
8CT1N	Ni Beads	His Wash Buffer	3
8CT2N	Ni Beads	His Wash Buffer	1, 4,5, 6, 7, 8
8CT2N	Sup75	His Wash Buffer	2
8NT3N	MonoQ	200mM HEPES, pH 7.0	9
8NT4N	MonoQ	200mM HEPES, pH 7.0	9
8NT5N	NA	NA	12
8NT6N	Ni Beads/Sup75	His Wash/ Tris pH 7.5	3,13
8NT7N	NA	NA	12
8CT3N	Ni Beads	His Wash Buffer	3
8CT4N	Ni Beads	His Wash Buffer	3, 14
8CT5N	Sup200	Tris pH 7.5	2, 3, 11
8CT6N	Ni Beads	His Wash Buffer	3
8CT7N	NA	NA	12
8CT8N	NA	NA	12
Baculovirus	NA	NA	10

1 – Enough for bead binding
2 – Copurifies with heat shock proteins
3 – Insufficient protein to continue purification
4 – Binds to MonoQ column
5 – Binds to Sup6 column
6 – Binds to Hydrophobic column
7 – Buffers at pH 6.5, 7.5, 8.0
8 – Coexpression with MBP6 does not improve purification
9 – Binds to Sup200
10 – Cloning incorrect
11 – Protein in void fraction
12 – Insufficient protein expression
13 – Degrades during purification
14 – Denaturing prep successful (performed by Ashleigh Wood)

nutated for one hour with Ni beads and washed with denaturing wash buffer with successively lower pH (100mM NaH$_2$PO$_4$, 10mM Tris, 8M urea, pH 6.3, pH 5.9, pH 4.5) before being dialyzed into potassium phosphate buffer. There is currently no assay for folding, so we can't use this protein for functional studies, it is being used to create what will hopefully be the first αSec8p antibody in the field.

In addition to constructs designed for *E. coli,* I designed full length constructs for *S. cerevisiae* and for baculovirus expression in insect SF9 cells. A His6-tagged Sec8 construct was cloned into 2μ URA3 PGAL1 and CEN URA3 PGAL1 expression vectors (pPP639 and pPP1247, respectively), and transformed into wild type yeast in addition to the genomic copy of Sec8p. The plasmid was maintained by growth on selective media. Colonies were suspended in liquid synthetic complete media lacking leucine or uracil and grown at 25ºC and 30ºC. I determined that Sec8-His$_6$ can be expressed in yeast under the galactose inducible promoter without causing growth defects in liquid culture, but expression remains low and expressed protein is not amenable to purification.

Baculovirus expression in insect cells was attempted using the Bac-to-Bac system by Invitrogen, utilizing the HisBIVT and 3ATR plasmids. SF9 cells were cultured in SF900 II Serum Free Media (Invitrogen). Cells were cultured without difficulty as per standard directions; however, success with the baculovirus vector was prevented by difficulties with infection and vector shuffling. No significant expression was displayed; however I was unable to demonstrate viral infection and thus that the virus contained the Sec8 clone. I suspect that the problem was in the cloning process. This was not a

methodology familiar to the lab. Though unsuccessful, baculovirus expression may yet

prove an effective means of expressing Sec8 and other exocyst proteins in more

experienced hands.

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