

# **Exploring the Interactions of Yeast Exocyst Complex Proteins Using Immunoprecipitation Experiments**

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1. Exocyst
2. Immunoprecipitation
3. *Saccharomyces Cerevisiae*

## ABSTRACT

Vesicle trafficking is vital for the growth and life of a cell. The exocyst is an eight protein complex involved in vesicular trafficking. A model of the interactions among the exocyst proteins have been demonstrated through yeast 2-hybrid and *in vitro* translation experiments, but not by more stringent methods. To support these findings *in vivo* and to further map the binding domains, a series of immunoprecipitation experiments was performed. Green fluorescent protein (GFP) genomically tagged proteins Sec5p and Sec8p were pulled down from whole yeast lysates, and the samples were probed with anti-Sec6 antibody. If Sec6p binds Sec5-GFP or Sec8-GFP a band will appear in the western blot in the bound lane. Likewise, a similar experiment was carried out using Sec8-myc to determine its interaction with Sec6p. Results from both experiments show an *in vivo* interaction between Sec6p and Sec5-GFP and Sec8-GFP.

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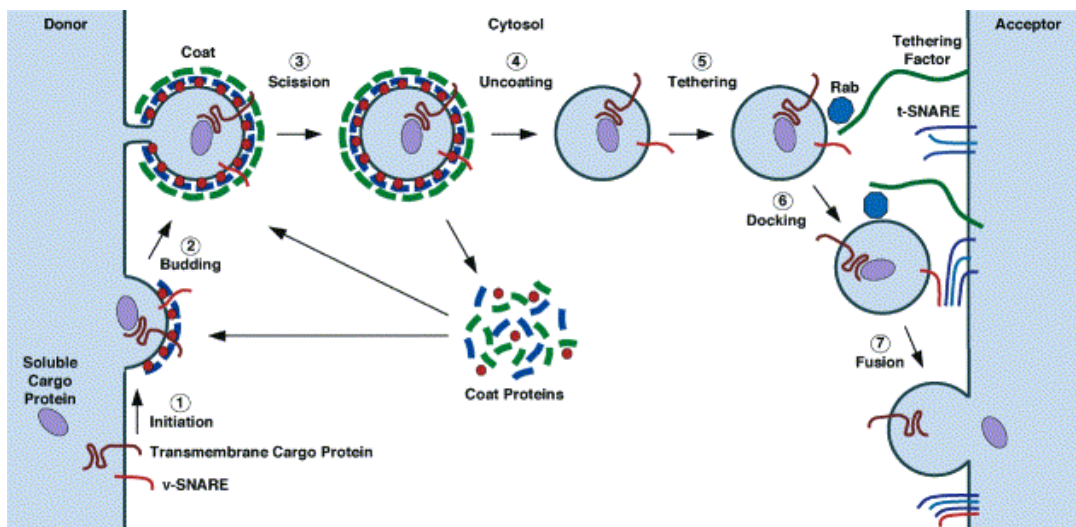
## **ACKNOWLEDGEMENTS**

First and foremost, a special thank you and gratitude to Dr. Mary Munson at the University of Massachusetts Medical School in Worcester, who not only opened her lab doors, but has provided guidance and inspiration. To the members of the Munson Lab, I sincerely thank you for your patience, help, and wisdom. Thank you, as well, to Open Biosystems for the GFP constructs and to Dr. Pat Brennwald for donating the Sec8-myc plasmid. Finally, thank you to Dr. Dave Adams at WPI for initiating this MQP and sharing your passion for biology.

# INTRODUCTION

## Vesicle Trafficking

Vesicle trafficking, or the molecular movement of cellular materials in enclosed vesicles, is vital for the growth and continued existence of an eukaryotic cell (Bonifacino and Glick, 2004). Trafficking is essential in the organization of a cell because the activity allows for materials to be transported between various membrane-bound organelles (Cooper, 2000). The movement of a vesicle throughout the cell begins when the vesicle buds off the donor membrane (see Figure 1). The vesicle is then transported through the cytoplasm to the acceptor membrane. Once the vesicle is within the vicinity of the target membrane, the vesicle is tethered, docked and fused to the accepting membrane (Bonifacino and Glick, 2004). Two types of vesicle trafficking exist in a cell, namely, endocytosis and exocytosis. Endocytosis refers to the movement of vesicles within a cell, or intracellular trafficking, whereas exocytosis is the transportation of a vesicle from inside to outside of the cell (Whyte and Munro, 2002).



**Figure 1. Vesicle trafficking.** Vesicle transport is an essential function in eukaryotic cell development and growth. The movement of the vesicle from one membrane to another is initiated by vesicle “budding (2).” The vesicle is transported through the cytosol where it is then “tethered” (5), “docked” (6), and “fused” (7) with the accepting membrane (Bonifacino and Glick, 2004).

The process of tethering is a complex mechanism with various components. The target membrane recognizes a specific vesicle based on “the selective packaging of the intended cargo” (Cooper, 2000). Rab GTPases are specific tethering proteins that regulate intracellular trafficking (Waters and Pfeffer, 1999). A molecule of GTP is required to activate this molecular switch. Various other proteins include GAPs, which helps to hydrolyze GTP; GEF (GTP exchange factors), which aid in removing GDP and replacing with GTP; and GDI, which helps bind GDP. These proteins are all involved in this step of tethering. Once GTP is exchanged to GDP, the mechanism halts. Thus, GTP is required as an initial tethering step (Waters and Pfeffer, 1999).

Other proteins involved in tethering are EEA or Early Endosome Associated Protein, which is required for fusion of endocytic vesicles. There are also large complexes engaged in tethering such as COG which tether vesicles to the Golgi, as well as the exocyst which tether post-Golgi vesicles to the plasma membrane (Waters and Pfeffer, 1999).

Docking is regulated by the v- and t- SNAREs. SNAREs, soluble NSF attachment receptors, are present on both the vesicle (v) and the target (t) membranes. SNAREs are also responsible, although not entirely sufficient, for the fusion of the vesicle to the membrane. Fusion occurs once the SNARE complex is formed on the vesicle and the target membrane. Finally, NSF/ SNAP proteins break down the SNARE complex once the vesicle is fused to the membrane. This allows for the SNARE complex to be recycled for future transport activity (Cooper, 2000).

## **The Exocyst**

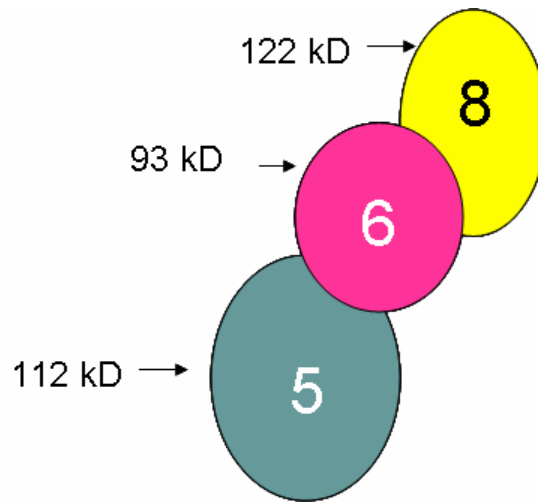
### *Function*

The exocyst is responsible for directing the post-Golgi or secretory vesicles to the sites of active secretion (TerBush et al., 1996). More specifically, the exocyst tethers and regulates vesicle fusion post-Golgi to the plasma membrane. This large multi-subunit complex is made up of eight proteins found in the cytosol and on the edges of the plasma membrane. While the specific function of each of these eight proteins is still being investigated, it recently was hypothesized that the exocyst regulates the SNARE complex. This was shown by a direct interaction between Sec6p and Sec9p (a SNARE) (Sivaram et al., 2005). The exocyst is upstream of SNARE complex formation. This was demonstrated using temperature sensitive yeast exocyst mutants. When the exocyst complex was mutated, the SNAREs failed to form (Grote et al., 2000).

### *The exocyst proteins*

As previously stated, the exocyst is a large (1-2 mega Dalton) eight protein complex. The proteins appear to be in a 1:1:1:1:1:1:1:1 ratio, indicating that there is one of each of the eight proteins present in the complex (TerBush et al., 1996). Sec4p is a Rab GTPase, and although it is not part of the exocyst, it binds to Sec15p in the complex. Once GTP is bound to Sec4p (see Figure 3), the protein binds to the vesicle. The functions of a few members have been explored. Sec3p and Exo70p are specifically involved with the target membrane. This project investigates the interaction between Sec5p, Sec6p and Sec8p (see Figure 2). Sec5p (about 112 kD) is the “structural core” of the complex. It interacts with 5 of the 8 proteins, according to yeast-two-hybrid and *in vitro* translation experiments (Guo et al., 1999). Sec8p is roughly 122 kD and appears to have a leucine-zipper like motif. This motif could indicate a binding domain to other

leucine-zipper proteins. All of these proteins are also found at high concentrations at the bud tip (TerBush et al., 1996).



**Figure 2. Three of the eight exocyst proteins.** Sec5p, Sec6p, and Sec8p are all members of the exocyst complex. These proteins slightly vary in size. Their function is not yet understood. The tentative interactions shown are based on yeast two-hybrid data.

Sec6p is about 93 kD and is extensively studied in Dr. Mary Munson's lab at University of Massachusetts Medical School in Worcester. Recently, it has shown to be a well folded, moderately stable, helical, dimer *in vivo*. It was discovered using circular dichroism spectra that the protein is about 60% helical with a melting temperature of about 41°C (Sivaram et al., 2005). Sec6p was determined to be a dimer using gel filtration chromatography (Sivaram et al., 2005). A crystal structure of the C-terminus end of Sec6p was recently discovered as being an independently folded monomer (Sivaram et al., submitted). Finally, Sec6p N-terminus binds to the t-SNARE Sec9p. This 2:2 molecular ratio (because Sec6p is a dimer) is thought to inhibit the t-SNARE binding of Sso1p (Sivaram et al 2005). The function of other exocyst proteins is still unknown.



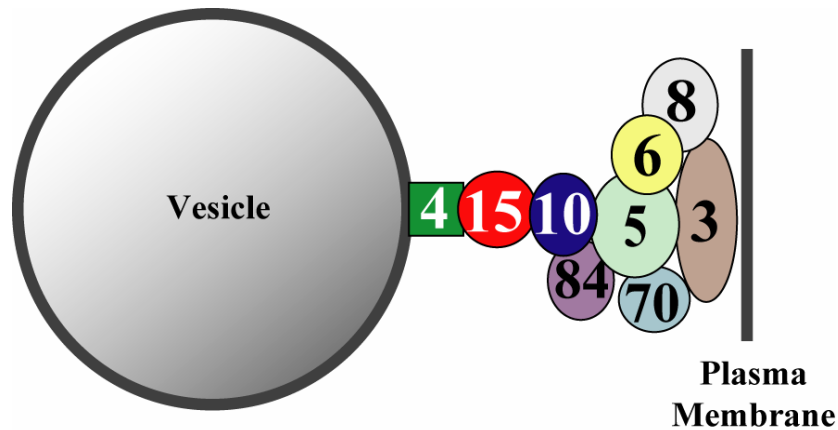
### *Conservation*

The exocyst is conserved from yeast to mammals. The exocyst can be found in *Drosophila* in neurite extensions and creates the polarity in *Drosophila* oocytes (Sommer et al., 2005). Finally, the exocyst has been observed in hippocampal neurons and muscle cells (Cooper, 2000).

### *The exocyst model*

A model of the protein interactions have been proposed using yeast-2-hybrid and *in vitro* translation experiments (Figure 3). The exocyst is more commonly investigated in *Saccharomyces cerevisiae* due to its easy genetic manipulation. However, experiments have been performed in *Drosophila* as well. One experiment involved integrating two exocytic proteins into two different vectors (a yeast two-hybrid experiment). Samples were then transformed into wild type yeast and probed via western blots using a specific antibody (Guo et al., 1999). Although previous yeast two- hybrid experiments have been successful there is always the problem of false negatives which indicate a problem integrating the sample DNA into the nucleus of the cell.

In Guo et al (1999), they demonstrated exocytic interactions between the eight proteins using *in vitro* translation and immunoprecipitation assays, developing the tentative diagram in Figure 3 (Guo et al., 1999).



**Figure 3. The Exocyst Complex.** A proposed model of the complex derived from previous data from *in vitro* translation and yeast two hybrid experiments. The focus of this MQP was on Sec6p and its interactions with Sec5p and/or Sec 8p (Guo et al., 1999).

An excerpt of their experiment is as follows: Exocyst proteins tagged with FLAG were synthesized using cDNA (synthetically reverse transcribed). Samples were cloned into a vector and translated in rabbit reticulocyte cells which were then co-immunoprecipitated. This experiment was attempted with other exocytic proteins as well. The anti-FLAG antibody pulled down Sec8p along with FLAG-Sec6. Sec8p without FLAG-Sec6 was not pulled down with Anti-FLAG. This indicates a “direct” interaction *in vitro*. Although this experiment was ground breaking in introducing the blueprints of the exocyst, *in vitro* translation can be faulty due to the misfolding of proteins and often leads to both false negatives and false positives (Guo et al., 1999). Likewise, their experiment explores the *in vitro* relationships between the exocyst protein and not what happens inside of a cell.

### Project Purpose

The goals of this experiment are to:

- ❖ Examine the *in vivo* interactions between Sec6 with Sec5-GFP and Sec8-GFP.

- ❖ Further explore the *in vivo* interactions between Sec8-myc with Sec6p.
- ❖ Develop an immunoprecipitation protocol to demonstrate the *in vivo* interactions of the exocyst proteins

## METHODOLOGY

### **Sec5-GFP, Sec6-GFP, and Sec8-GFP constructs**

#### *Growing GFP constructs*

*S. cerevisiae* strains containing Sec6p, Sec8p, and Sec5p that were genomically tagged with green fluorescence protein (GFP) were purchased through Open Biosystems (a stock culture was made inoculating fresh YPD and incubating at 30°C). Samples were grown to log phase in YPD overnight at 30°C. The optical densities of the samples were taken at 600nm until an absorbance around 0.6-1.0 was obtained. Samples were then analyzed under a fluorescence microscope.

#### *Bead beating and western blot*

The following technique was used for all western blots (lysis buffers may vary). The lysis buffer contained the following reagents: 50 mM HEPES pH 7.4, 150 mM KCl, 1-2 mM EDTA, 1 mM DTT (add fresh), 0.5% NP-40 (IPGAL), 500x PICS (antipain, aprotinin, leupeptin, chymostatin, and pepstatin), and 1mM PMSF. The 5 OD cell pellet from 25 ml of media was mixed with 200 µL of lysis buffer. Cells were vortexed vigorously for one minute and placed on ice for one minute (repeated 4 times). Whole cell lysate samples (0.5 OD worth of protein per lane) were run on a gel and transferred overnight onto a nitrocellulose membrane. Antibodies used for the GFP Western Blot include primary antibody mouse anti-GFP at a 1:4,000 dilution (purchased from BD Biosciences). The secondary antibody used was goat anti-mouse at a 1:10,000 dilution (purchased from Jackson Immuno Research). ECL (purchased from Amersham Biosciences) was added to the membrane and the sample was exposed to Kodak film for 5-10 minutes.

### *Immunoprecipitation (IP)*

An immunoprecipitation is an experiment in which an antibody precipitates a protein out of cellular extract based on the specificity of the epitope. Likewise, other proteins that are bound to the one with the epitope can be "pulled down" in a complex (Wikipedia, 2002). Once the protein is removed from the extract, it can be analyzed using the western blotting method. The explanation that follows is the protocol used for this experiment.

Cells were grown to log phase in 100 ml of YPD. 50 OD samples were frozen and stored at -80°C until use. Samples were lysed with 200 µL western blot lysis buffer [HEPES pH 7.4 with no DTT and 0.05% NP-40] and vortexed with zirconia beads for 5 minutes. The lysate was then rocked (or nutated) for one hour with protein G-sepharose beads (purchased from Amersham Biosciences) at 4°C. A sample was taken and labeled as 'lysate before'. The sample was centrifuged and 200 µL of supernatant was removed and added to 1:60 dilution of anti-GFP antibody. The sample was rocked for an additional one hour at 4°C. The supernatant and antibody were added to fresh protein G-sepharose beads and nutated for another hour at 4°C. The sample was then centrifuged and a sample of supernatant was saved labeled as 'lysate after' or the unbound sample. The beads were washed three times with 40 µL lysate buffer. 2x loading dye was added to supernatant samples, and 1x loading dye was added to beads. Samples were boiled for 5 minutes and run (0.5 OD worth of protein per lane) in duplicate on a 10% SDS page gel. A western blot was then performed according to the western blot protocol (see above). One blot used a 1:4,000 dilution of mouse anti-GFP as primary antibody with a 1:10,000 dilution of goat anti mouse as secondary antibody. The other blot used 1:5,000 dilution of anti-Sec6 antibody (Sivaram et al., 2005) with a 1:10,000 dilution of goat anti

rabbit as secondary antibody (purchased from Jackson Immuno Research). A negative control for both the western blot and IP was non-transformed parental strain BY4741 (*α* strain).

### **Sec8-Myc Construct**

#### *Restriction digest of Sec8-Myc*

The Sec8-myc plasmid construct was donated by Dr. Pat Brennwald's lab at the University of North Carolina. A transformed *E. coli* culture was grown overnight at 37°C in 5 mL LB and 1000x Ampicillin and run on a 0.9% agarose gel. The plasmid sample was then digested for 3 hours with restriction enzyme XbaI, then purified using a Qiagen Qiaspin kit. Sample was then run on a 0.9% agarose gel. Positive samples were then transformed into yeast [parental strain BY4742 (*α* strain)] and grown overnight in 20 mL Synthetic Complete (SC) –URA plus 2% glucose. The sample was then lysed according to the western blot procedure on a 12% SDS page gel (see above) with lysis buffer containing 0.05% NP-40 and DTT. The primary antibody for this sample was a 1:1,000 dilution of anti-c-myc primary (Covance Research Products), and a 1:10,000 dilution of secondary antibody goat anti mouse.

#### *IP using Anti-c-myc Kit*

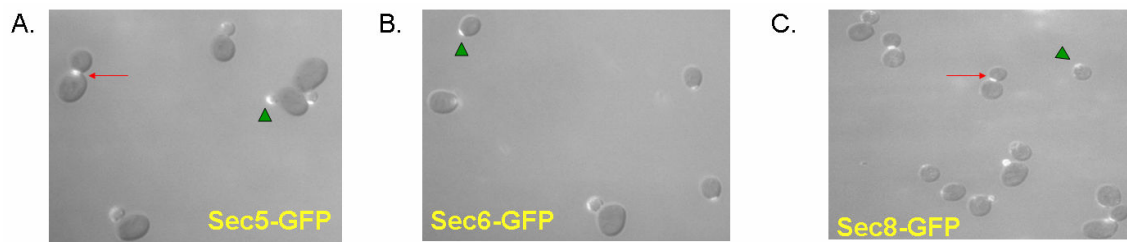
A 50 OD pellet sample made in 100 mL SC –URA plus 2% glucose was lysed using 200 µl lysis buffer with 0.05% NP-40 and DTT (see above). The sample was lysed using zirconia beads and a sample of the supernatant was saved as 'lysate before.' The following protocol was developed following the protocol from the Sigma-Aldrich anti-c-myc IP kit. In an empty kit column, 200 µl (range between 100 µl - 600 µl) of cell lysate, 20 µl of anti-c-myc agarose suspension, and 380 µl lysis buffer (to bring the total volume

to 600  $\mu$ l) were combined. The sample was nutated for one hour at 4°C. The tip of the column was then removed and the column was placed in a collection tube and centrifuged. A sample was collected and labeled 'lysate after' or unbound sample. Beads were then washed with lysis buffer and 1x loading dye was added to column. Beads were boiled for 5 minutes at 95°C and centrifuged to collect bead sample. 2x loading dye was added to supernatant samples. Samples were then loaded in duplicate (0.5 OD worth of protein per lane) on a 12% SDS page gel and transferred overnight onto a nitrocellulose membrane. The western blot procedure from above was followed using a 1:1,000 dilution of mouse anti-c-myc as primary antibody (purchased from Covance Research Products) and a 1:10,000 dilution of goat anti-mouse as secondary antibody. The other blot used 1:5,000 dilution of rabbit anti-Sec6 antibody with a 1:10,000 dilution of goat anti rabbit as secondary antibody. A negative control for both the western blot and IP was non-transformed parental strain BY4742 ( $\alpha$  strain).

## RESULTS

### Sec5-GFP, Sec6-GFP, and Sec8-GFP constructs

To determine if Sec6p interacts *in vivo* with Sec5p and/or Sec8p, genomically tagged GFP Sec5, Sec6, and Sec8 constructs were purchased. The constructs were grown to log phase and analyzed under a fluorescence microscope to observe where the proteins localize. Un-tagged Sec6p, Sec5p, and Sec8p localize at sites of secretion near the plasma membrane (TerBush et al., 1996). This is also the site of active cell growth which is at the bud tip of the daughter cell and/or at the mother-daughter neck (TerBush et al., 1996). Figure 4 is an image of the constructs viewed under the fluorescence microscope.



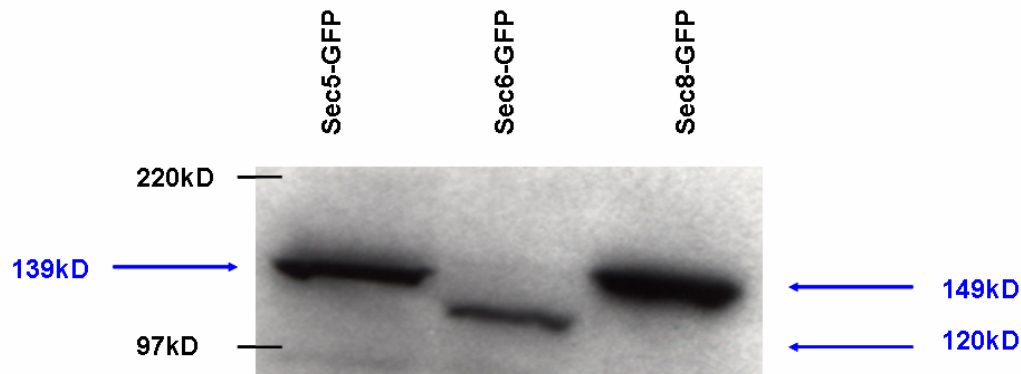
**Figure 4. All constructs localize properly to sites of active secretion.** Genomically tagged (A) Sec5-GFP, (B) Sec6-GFP, and (C) Sec8-GFP were purchased from Open Biosystems. The yeast were grown to log phase and protein localization examined by fluorescence microscopy. The bud tip and bud neck are represented by the arrowhead and arrow, respectively.

Under the fluorescence microscope, the illuminated area of the cell is the exocyst. Sec5-GFP correctly locates at the sites of secretion as denoted by the arrows at the bud tip and the mother-daughter neck (Figure 4A). Sec6-GFP (Figure 4B) also localized properly at the bud tip and is not shown at the mother-daughter neck site. Finally, Sec8-GFP (Figure 4C) localizes correctly at both sites of secretion. Thus, all constructs



localize properly at the site of active cell growth where the exocyst is found (TerBush et al., 1996).

Although the constructs appeared to localize properly, it was necessary to determine if the constructs express correctly. Again, the constructs were grown to log phase and lysed using the bead beating technique. Samples were loaded on a 10% SDS page gel and transferred overnight onto a nitrocellulose membrane. The membrane was then blotted with anti-GFP antibody to determine if the sample expressed the proper tagged-protein. Figure 5 is the western blot of the samples.



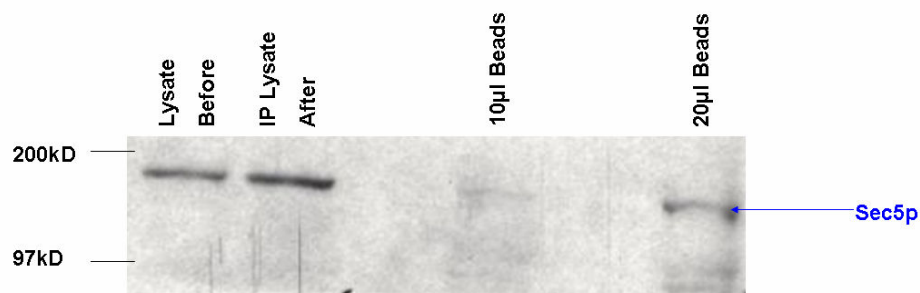
**Figure 5. All constructs properly express.** Sec5-GFP, Sec6-GFP, and Sec8-GFP were tested to determine if the constructs properly express and that the GFP antibody and goat anti-mouse secondary antibodies were working properly. The samples were grown to log phase, lysed by bead beating, and run on a 10% SDS PAGE gel. The lysates were transferred onto a nitrocellulose membrane and probed with anti-GFP antibody followed by goat anti-mouse antibody. The blue arrows denote the expected Sec-GFP fusion protein sizes.

The GFP tag added 27kD onto the original molecular weight of each protein. Thus, Sec5p-GFP, Sec6p-GFP, and Sec8p-GFP are about 139 kD, 120 kD, and 149 kD, respectively. Although it is difficult to determine the exact size of the samples present in Figure 5, it can be estimated that the constructs are the correct size. The Sec constructs, therefore, appear to express properly.

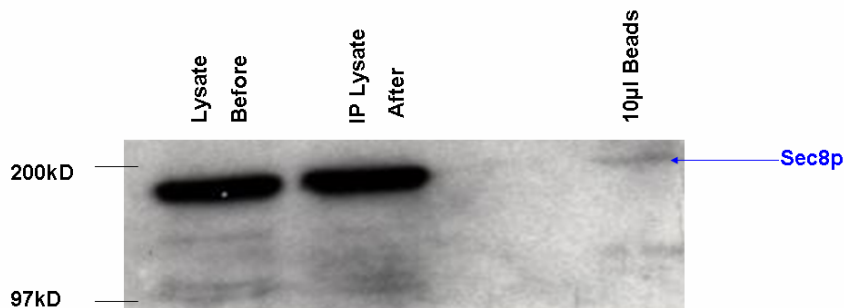
The next objective was to determine if Sec6p was pulled down with any of the GFP constructs in an immunoprecipitation (IP) experiment. First, the IP protocol had to be optimized for the GFP constructs. Samples were subjected to various lysing techniques such as bead beating (as described in the methodology) versus spheroplasting. Spheroplasting is a gentler way of breaking apart a cell using the enzyme zymolyase. The spheroplast protocol is time consuming compared to the traditional bead beating with zirconia beads. There was no significant difference between the protein yields obtained using the spheroplast protocol versus bead beating lysis procedure. Thus, bead beating technique was used to save time.

For optimal IP results, it was determined that samples needed to shake on the nutator with the antibody on the beads for at least one hour at 4°C. Samples were also nutated overnight at 4°C, but there was no significant increase in the amount of protein that bound to the beads. Thus, all nutations were performed for one hour at 4°C.

### A. Sec5-GFP

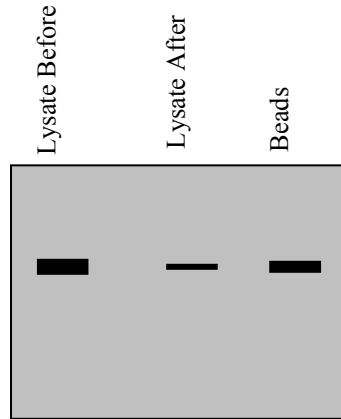


### B. Sec8-GFP



**Figure 6. Immunoprecipitation of Sec-5 and Sec-8 with anti-GFP antibody.** 40 OD pellets of cell lysate containing (A) Sec5-GFP and (B) Sec8-GFP were immunoprecipitated by bead beating to lyse the cells and adding a 1:50 dilution of anti-GFP antibody. The samples were then nutated with Protein G sepharose beads for 1 hour at 4°C and run on a 10% SDS PAGE gel. After transferring overnight to a nitrocellulose membrane, the samples were probed with anti-GFP antibody followed by goat anti-mouse antibody.

Figure 6 depicts the immunoblots of GFP-immunoprecipitated Sec5-GFP and Sec8p-GFP. The first lane sample ('lysate before') in both Figure 6B and Figure 6C is only the yeast lysate. The second lane is the sample that is not bound to the protein G-sepharose beads. The last lane is the sample bound to the beads. Sec5-GFP required 20 µl of beads to see a substantial Sec5-GFP band, whereas Sec8-GFP required only 10 µl of beads. Ideally, the western blot should have revealed a strong band in the 'lysate before' lane because of the abundance of the GFP construct in the lysate, but a slight reduction in band intensity in the supernatant following IP. The hope is that the majority of the GFP construct is on the beads. Finally, the 'beads' band should be very strong, because it contains plenty of the GFP protein (see figure 7).

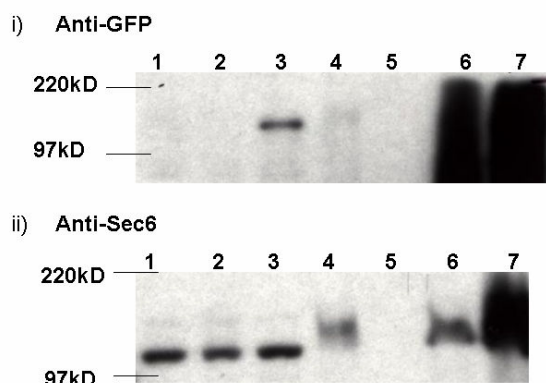


**Figure 7. Ideal SDS page immunoblot shows a high yield of desired protein in the ‘lysate before’ and ‘beads’ lanes.** There should be a significantly weaker band in the ‘lysate after’ lane due to most of the protein being bound to the beads.

Although the results were not as optimal as expected, the immunoprecipitation protocol can be used with the anti-GFP antibody as it does show both of the GFP constructs binding to the beads (Figure 6). Why are the results not the best? An explanation for this could be that the GFP tag is not sticking to the beads as well as it should. There also could be something else bound to the Sec6/Sec5 or Sec8 complex that is inhibiting the sample from binding to the beads. However, there is enough of the sample on the beads to continue the experiment.

The same protocol was then run in duplicate. However, one blot was probed with anti-GFP antibody and the other blot was probed with anti-Sec6 antibody. Anti-Sec6 antibody is used to detect bound Sec6p to either Sec5-GFP or Sec8p-GFP. When the GFP construct is caught by the beads is there something else attached? If Sec6p does pull down with either construct, this could indicate a protein:protein interaction. Blotting with anti-GFP is a positive control to determine if the GFP epitope is present in the sample. The parental strain BY4741 was used as a negative control demonstrating that in non-transformed wild type yeast, Sec6p is present and GFP is not.

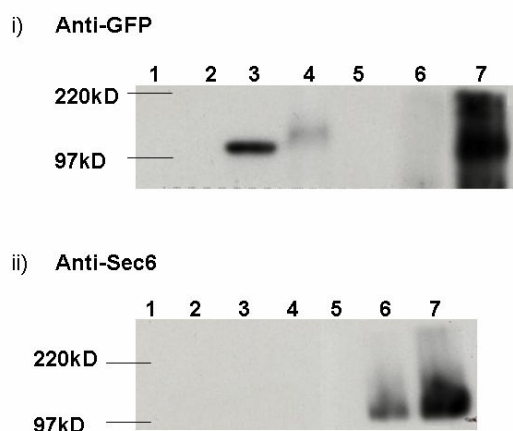
## A. Sec5-GFP



## C.

- 1 Negative control lysate before IP
- 2 Negative control lysate after IP
- 3 Sample lysate before IP
- 4 Sample lysate after IP
- 5 Blank
- 6 Negative control bound
- 7 Sample bound

## B. Sec8-GFP



**Figure 8. Immunoblots suggest interaction of Sec6p with Sec5-GFP and Sec8-GFP.** (A) Sec5-GFP was immunoprecipitated with anti-GFP to determine if Sec6p binds to Sec5-GFP. (B) Similarly, Sec8-GFP was immunoprecipitated with anti-GFP to determine if Sec6p binds to Sec8-GFP. The samples were grown to log phase, lysed by bead beading, immunoprecipitated with anti-GFP, then run in duplicate on a SDS PAGE gel. The lysates were transferred to a nitrocellulose membrane and probed with (i) anti-GFP antibody or (ii) anti-Sec6 antibody, followed by goat anti-mouse and goat anti-rabbit antibodies respectively. Figure 5C indicates the lane assignments for all gels with the lysate and WT parent strain BY4741.

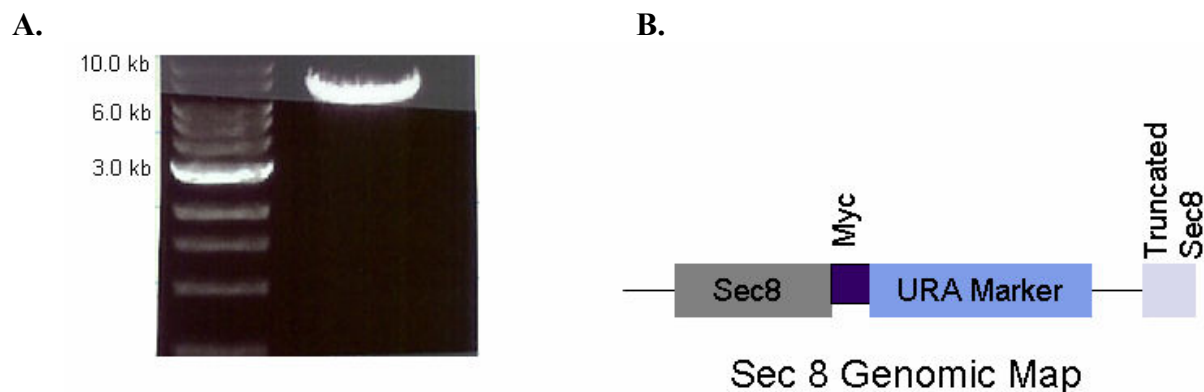
In Figure 8A, lanes 3, 4, and 7 are of interest. Sec5-GFP is found in the ‘lysate before’ lane. Lane 4, ‘lysate after,’ is significantly weaker. In lane 7, there is a strong over exposed lane of Sec5-GFP indicating that too much of the sample was loaded in this lane. Figure 8Ai also indicates that the construct Sec5-GFP is present in the cell. When the same blot was probed with anti-Sec6, Sec6 is found in all negative control lanes, indicating that Sec6 is present in wild type as well as the sample lysate. The band is

significantly weaker in lane 4, indicating that very little is not bound to the beads. Finally, when the beads are probed with anti-Sec6, Sec6p appears in the same lane 7 where the Sec5-GFP was found. The same data appears in Figure 8B which is the Sec8-GFP construct. Again, when probed with anti-Sec6, Sec6p appears in the same lane 7 as the Sec8-GFP. It is interesting to also notice a difference in the 'lysate before' lane to the 'lysate after' lane. There appears to be an increase in molecular weight of the proteins in the 'lysate after' lane that continues in the beads lane of both the Sec5-GFP (Figure 8A) and Sec8-GFP (figure 8B) blots.

It can be concluded from these results that anti-Sec6 antibody detected Sec6p in a complex with Sec5-GFP and Sec8-GFP. However, the immunoprecipitation protocol used did not provide efficient results. It was hypothesized that using an immunoprecipitation kit with antibody plus the agarose beads would increase the sample's affinity to the beads. Since Sigma-Aldrich does not make an anti-GFP IP kit, Sec8-myc was chosen because of the availability of the construct and anti-c-myc IP kit.

### **Sec8-myc**

Sec8p was hypothesized to interact *in vitro* with Sec6p (Guo et al., 1999). Using a myc-tagged Sec8 protein, the interaction could be analyzed *in vivo* in the same way as the GFP constructs. Sec8-myc plasmid was obtained from Dr. Pat Brennwald's lab and run on a 0.9% agarose gel. The uncut plasmid ran as two bands (data not shown) presumed to be supercoiled and relaxed circular plasmid DNA. The Sec8-myc was then digested with restriction enzyme XbaI and run on a 0.9% agarose gel (Figure 9). Although the band appears linear, the sequence of the construct was unknown. Therefore, the exact size was unable to be determined on this percent gel.



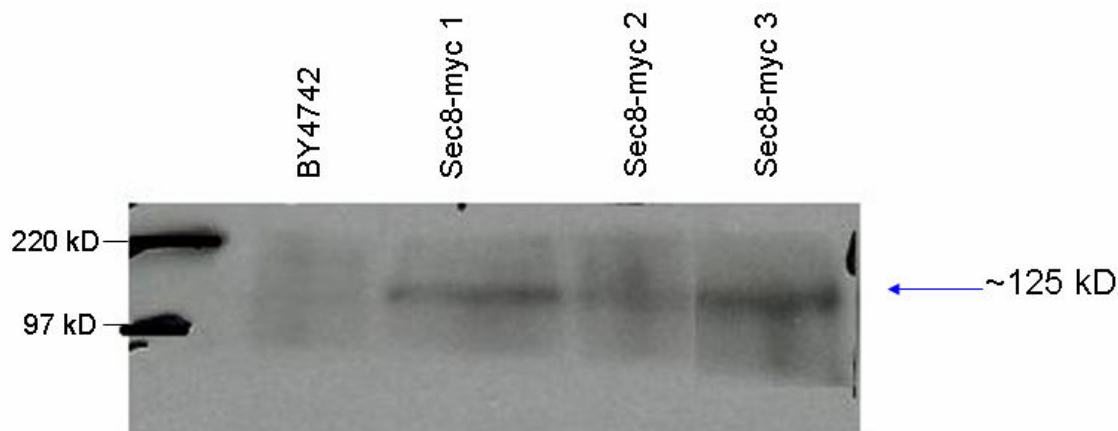
**Figure 9. Sec8-myc is linear.** Sec8-myc plasmid was digested with XbaI (A). The sample was run on a 0.9% agarose gel. The single band present indicates linear DNA. Restriction enzyme XbaI truncated the Sec8 gene to allow integration of the Myc-tag (B).

Sec8-myc DNA was then purified and transformed into the parental wild type strain BY4742 ( $\alpha$  strain). The specific marker (either LEU2 or URA3) was unknown. The sample was plated on both synthetic complete –LEU and synthetic complete –URA plates. Colonies appeared on –URA.

A myc-tagged protein has amino acids EQKLISEEDL (Smith et al., 2004) added to its sequence. Depending on the protein, there can be multiple copies of the tag. How many tags present in Sec-8myc was unknown, although estimated to be between 1-3 tags. The myc-tag adds only one kD onto the molecular weight of the protein. Thus the estimated molecular weight of Sec8-myc is about 125 kD.

It was necessary to determine if the Sec8-myc construct expressed properly. The construct was grown to log phase in synthetic complete –URA, and lysed using the bead beating technique. Samples were loaded on a 10% SDS page gel and transferred overnight onto a nitrocellulose membrane. The membrane was then blotted with anti-c-myc antibody to determine if the sample expressed the proper myc-tagged protein.

Figure 10 is the western blot of several different colonies of Sec8-myc.



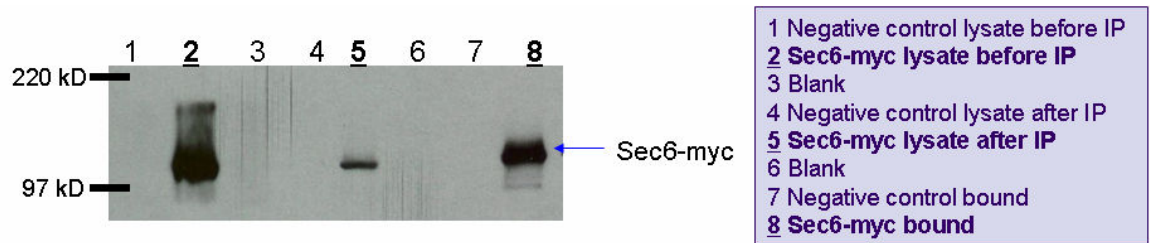
**Figure 10. Sec8-myc constructs properly express.** Multiple colonies of Sec8-myc were tested to determine if the constructs properly express and that the anti-c-myc antibody worked properly. The samples were grown to log phase in SC -URA, lysed by bead beating, and run on a 10% SDS PAGE gel. The lysates were transferred onto a nitrocellulose membrane and probed with mouse anti-c-myc antibody followed by goat anti-mouse antibody.

As stated before, Sec8-myc appeared to be slightly bigger in molecular weight than the untagged protein. The negative control in this experiment was the parental strain BY4742. This data indicates that all three colonies of Sec 8-myc expressed properly.

Like the previous GFP experiment, it was hypothesized that anti-Sec6 antibody would detect Sec6p in a complex with Sec8-myc in an immunoprecipitation experiment. In order to optimize the IP, an anti-c-myc IP kit was purchased. The kit shortens the duration of the immunoprecipitation experiment by providing anti-c-myc antibody attached to agarose beads (see methodology). The kit was tested first on a previously tested myc-tag protein, Sec6-myc in which the correct size and expression is known. The sample of Sec6-myc used contained 13 myc-tags, increasing the molecular weight (120 kD) by about 13 kD. Sec6-myc lysate was added to the anti-c-myc-beads and nutated at 4°C. The ‘before lysate,’ ‘after lysate,’ and ‘beads’ were run on a 12% SDS page gel. Samples were transferred to a nitrocellulose membrane and probed with mouse anti-c-

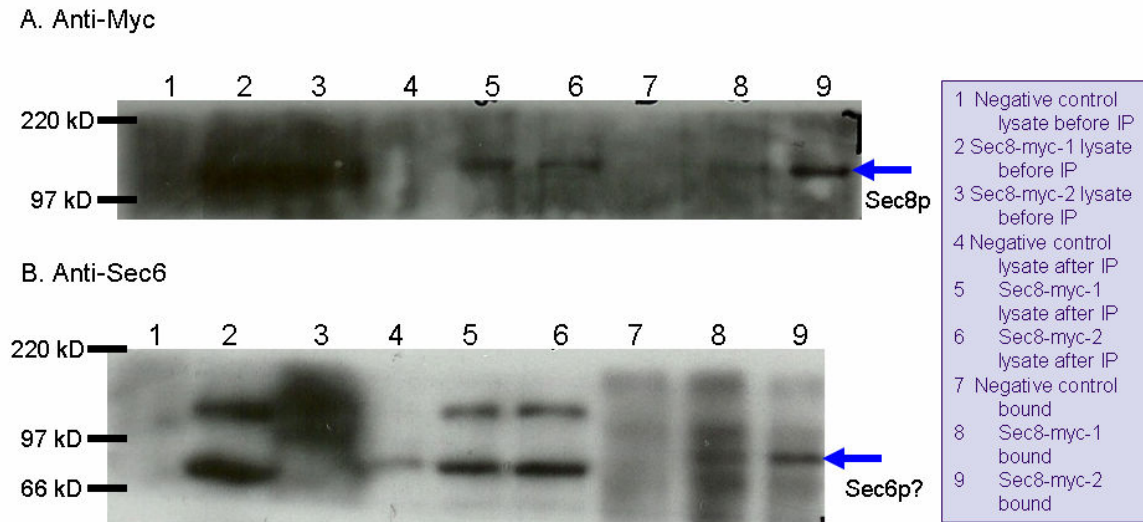


myc antibody and goat anti-mouse antibody. As demonstrated in figure 11, the kit worked exceptionally well and would be used with the Sec8-myc constructs.



**Figure 11. Anti-c-myc kit can be used with myc-tag constructs.** A 5OD pellet of Sec6-myc was immunoprecipitated by bead beating to lyse the cells and added to anti-c-myc-beads for 1 hour at 4°C and run on a 12% SDS PAGE gel. After transferring overnight to a nitrocellulose membrane, the samples were probed with mouse anti-c-myc antibody followed by goat anti-mouse antibody.

The same protocol was then run in duplicate with two Sec8-myc constructs. However, one blot was probed with anti-c-myc antibody and the other blot was probed with anti-Sec6 antibody (Figure 12A and B). The hypothesis is Sec6p pulls down with the Sec8-myc using anti-c-myc antibody. In the same way as the GFP experiment, does the Sec8-myc have anything else attached to it when it is bound in the beads? Again, if Sec6p does pull down with Sec8-myc, this would be further evidence to indicate a protein:protein interaction between Sec6p and Sec8p. Blotting with anti-c-myc is a positive control to determine if the c-myc epitope is present in the sample. The parental strain BY4742 was used as a negative control demonstrating that in wild type yeast, Sec6p is present whereas the myc-tag is not.



**Figure 12. Immunoblots suggest interaction of Sec6p with Sec8-myc.** Sec8-myc was immunoprecipitated to determine if Sec6p binds to Sec8-myc. The samples were grown to log phase in SC -URA, lysed by bead beating, immunoprecipitated with anti-myc, then run in duplicate on a 12% SDS PAGE gel. The lysates were transferred to a nitrocellulose membrane and probed with (A) anti-c-myc antibody or (B) anti-Sec6 antibody, followed by goat anti-mouse and goat anti-rabbit antibodies respectively. The background noise in this experiment could be due to excess ECL and/or leakage.

In Figure 12A, lanes 3, 6, and 9 are of interest. Sec8-Myc is found in the ‘lysate before’ lane 3. Lane 6, ‘lysate after,’ is significantly weaker. In lane 9, the band present is the correct size for Sec8-myc. When the same blot was probed with anti-Sec6 (Figure 12B), Sec6 is found in all negative control lanes (BY4742), indicating that Sec6 is present in wild type as well as the sample lysate. The band is significantly weaker in lane 5, indicating that very little Sec6 is not bound to the beads. Finally, when the beads are probed with anti-Sec6, Sec6p appears in the same lane 8 and 9 where the Sec8-myc was found. It is interesting again to notice the shift in the ‘lysate before’ lane to the ‘lysate after’ lane. There appears to be an increase in molecular weight in the ‘lysate after’ lane that continues in the ‘beads’ lane of both Sec8-myc constructs. This same shift occurred in the GFP experiments. It can be speculated that the cause could be due to a buffer or salt difference as well as a different amount of dye. The issue will be further

addressed in the discussion section. Nevertheless, this data indicated a possible *in vivo* interaction between Sec6p and Sec8-myc.

## DISCUSSION

### Sec5-GFP, Sec6-GFP, and Sec8-GFP experiments

A model of the interactions among the exocyst proteins have been demonstrated through yeast 2-hybrid and *in vitro* translation experiments, but not by more stringent methods (Guo et al., 1999). To support these findings *in vivo* and to further map the binding domains, a series of immunoprecipitation experiments were performed. Green fluorescence protein (GFP) constructs of Sec5, Sec6, and Sec8 were purchased. These constructs proved to localize properly to the sites of secretion at the bud tip and mother-daughter neck. The GFP constructs were shown by western blots to properly express proteins of the expected sizes with no degradation.

The GFP constructs were immunoprecipitated with anti-GFP antibody and anti-Sec6 antibody (for western blot analysis). The goal of the IP was to develop a protocol that could efficiently pull down either Sec5-GFP or Sec8-GFP along with Sec6p. Sec6p did indeed pull down with the GFP constructs indicating a possible *in vivo* interaction, although it cannot be determined if the interaction is direct or indirect. Is there another factor that is being pulled down with the Sec8/Sec5-GFP –Sec6p complex? Is it another exocyst member or perhaps other cellular components? Answers to these questions cannot be determined from this experiment. One possible experiment would be to test the interaction of the proteins *in vitro* using purified proteins. The pull down experiment with these pure proteins would demonstrate a direct interaction because there would not be any other proteins involved in the experiment besides Sec6p and Sec5p/Sec8p.

## Sec8-myc experiments

Similar to the GFP constructs experiments, it was hypothesized that Sec8-myc could pull down Sec6p in an immunoprecipitation experiment. A Sec8-myc plasmid was cut with a restriction enzyme to linearize if the construct. The Sec8-myc protein expressed properly with no degradation via a western blot experiment.

In order produce an efficient immunoprecipitation, an anti-c-myc IP kit was used. The kit was tested with known construct Sec6-myc, producing beautiful results. It was determined that the kit could be used for the Sec8-myc experiments. After numerous IP experiments, Sec6p did in fact pull down with Sec8-myc when probed with anti-Sec6 antibody. This data is further evidence that there is an *in vivo* interaction between Sec8p and Sec6p. Similar to the GFP experiment, it does not prove a direct or indirect protein interaction. However, a direct interaction between purified Sec6p and Sec8p *in vitro* has recently been demonstrated by the Munson Lab at University of Massachusetts Medical School (Sivaram, et al., submitted).

The figures in the western blot or immunoprecipitation experiments were often cloudy. There are several theories as to why this occurred. It is necessary to thoroughly wash all blots to remove any leftover antibody. Allowing the blot to sit longer than the recommended 45 minutes or 1 hour was often beneficial. If the containers that holds the membrane and solutions leak during the blocking stages of the western blot, the exposures are often cloudy. Finally, the ECL solution added to the blots needed to thoroughly dry to minimize patchy dark areas.

Why did the Sec6-myc work well with the anti-c-myc kit? The same protocol was used with the Sec8-myc construct, and the blots were less than optimal. Was the

Sec8-myc construct the proper size? A simple sequencing of the DNA could determine if the given construct is correct. There is a significant size difference between Sec6p and Sec8p which could lead to Sec6p working better in IP experiments. Thirteen c-myc tags were present in Sec6-myc compared to the one to three c-myc tags in Sec8-myc. One will see a stronger signal in the ECL with an increase in the c-myc tags due to the binding of more antibody molecules. More simply, perhaps Sec6-myc expresses better than Sec8-myc in yeast, thus leading to better immunoblots.

Finally, what is the reason for the shift of the protein's molecular weight in the immunoblots in both the GFP and c-myc experiments? The question is baffling, but could be explained by an indirect interaction between the two proteins. Perhaps another exocytic protein is interacting with Sec6p and Sec5/Sec8 causing the molecular weight of the complex to increase. Although, this would not be the case in this experiment as a denaturing gel was used. Are the proteins being phosphorylated? Finally, a possible ubiquitin modification could cause the added molecular weight. Further exploration is needed to explore this mysterious shift.

### **Future Experiments**

As previously stated, it is unclear from the results in the immunoprecipitation experiment if the protein:protein interactions are direct or indirect. Although, Guo et al (1999) demonstrated that the interactions were "direct" (Guo et al., 1999), further exploration is needed. Using purified proteins in *in vitro* characterization experiments should provide evidence for the interactions (Sivaram et al., submitted).

It has been shown by both the GFP and c-myc experiments that there is an *in vivo* interaction between Sec6p with Sec5/Sec8- GFP and Sec8-myc. What domain of Sec6p

specifically interacts? Thus, by testing truncations of Sec6p in the immunoprecipitation experiments, one may locate the domain of Sec6p that interacts with Sec5p and Sec8p. Finally, one may test other exocytic proteins for interactions with either GFP-tagged or Myc-tagged proteins.

In conclusion, the GFP and c-myc experiments have provided more evidence of an interaction between Sec6p with Sec5p and/or Sec8p. Although the relationship between Sec6p with Sec5p and Sec8p was not determined to be direct or indirect, the interactions were shown *in vivo*. This data is another piece of the exocyst puzzle.

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