Using the Auxin-Induced Degradation System to Dissect Factors Within the Exocyst That Localize the Complexes to Sites of Polarized Growth and Secretion

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Abstract

Exocytosis is the delivery of cargo molecules from the Golgi to the plasma membrane . In the budding yeast, *S. cerevisiae*, the proteins regulating the process of exocytosis are found predominantly at the bud tips in G1 phase and mother-bud necks in G2/M phase . The exocyst complex regulates localization and fusion of secretory vesicles at these sites. Exocyst is a multi-subunit tethering complex that is required for post-Golgi vesicle tethering at the plasma membrane. This hetero-octameric protein complex is composed of the following subunits: Sec3, Sec5, Sec6, Sec8, Sec10, Sec15, Exo70, and Exo84. The Munson lab recently showed that each subunit has equal stoichiometry within the complex which is predominantly soluble, and all but Sec3 are essential in maintaining the structure of the complex. Using electron microscopy, the assembly and architecture of exocyst shows that this complex forms an elongated conformation consisting of two modules of four helical rods each.

I am interested in understanding the roles of different subunits in controlling the polarized localization of exocyst. Specifically, I asked what happens to the structure, stability and localization of exocyst when other subunits are degraded. An auxin induced degradation system (AID) will be used with the subunits Sec3, Sec6, and Exo70 to determine if the structure of the complex is affected by this degradation. The AID system allows for rapid depletion of targeted proteins in response to the hormone auxin. I took advantage of the GFP tag on Sec8 in order to determine what subunits are still attached to Sec8 when degradation occurs, by pulling down the complex onto GFP bound nanobody beads. Next, I determined where Sec8-GFP is localized in the cell after Sec6, Sec3, and Exo70 degradation. These results will assist in understanding the nuances of exocyst complex assembly and allow for more accuracy when studying cellular trafficking.

Acknowledgments

Firstly, I would like to thank Dr. Mary Munson and her lab for their tremendous help, encouragement, and support over the last few years. My gratitude and appreciation are not nearly enough to thank the team for helping me accomplish more than I ever expected to. I would also like to give special thanks to my WPI advisor Jagan Srinivasan for his extraordinary guidance and advice throughout my MQP as well as my undergraduate career. In addition, I would like to thank The University of Massachusetts Medical School and Worcester Polytechnic Institute for their dedication to furthering the education of the WPI undergraduate community.

Table of Contents

Introduction

Cellular growth is vital for the maintenance and prosperity of all eukaryotes. In order for cells to reproduce and maintain their size, they needs to add material to their plasma membrane. Exocytosis, in eukaryotes, is the process by which cargo molecules are delivered by vesicles from the Golgi to the plasma membrane. This cellular process aids in the polarized localization of targeted proteins. Polarized localization is regulated by tethering complexes, such as the exocyst. These tethering complexes interact with proteins like SNAREs and members of the Rho and Rab GTPase family, located on the vesicle and plasma membrane to regulate vesicle trafficking in the cell. The exocyst complex binds to several proteins on the vesicle and plasma membrane in order to move from the Golgi to the plasma membrane (Figure 1). Several studies have determined the subunits that bind to these proteins, and make the essential bonds for localization and tethering (Roumanie et al.).

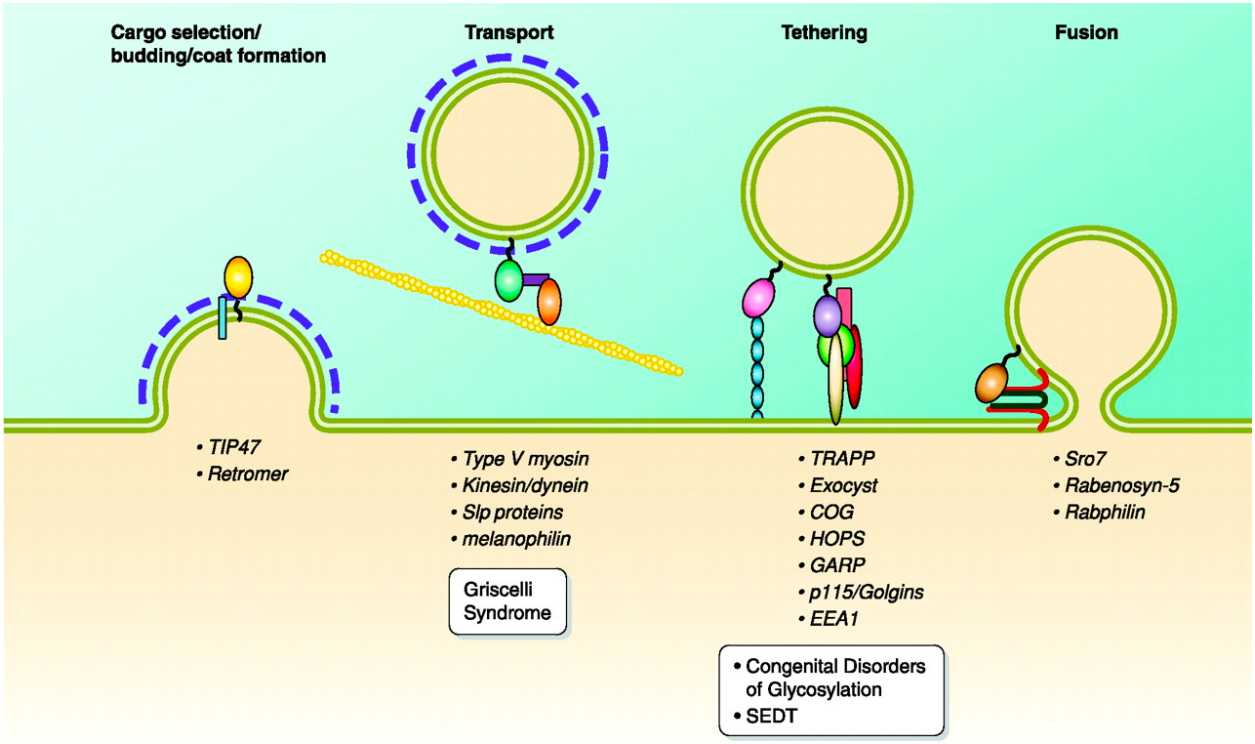


Figure 1: Regulated Exocytosis (Hutagalung & Novick)
Various proteins help regulate cellular trafficking from the ER, through the Golgi, and to the plasma membrane. Here, it is shown that the cargo molecules is transported to the plasma membrane with the help of Tethering Complexes, one of which is the exocyst.

Saccharomyces cerevisiae

Exocytosis is important for analysis of cell polarity and localization. To further understand this process, researchers use an ideal model organism that is easily grown *in vitro*; This model is known as *Saccharomyces cerevisiae*. *S. cerevisiae*, more commonly known as

brewer's or baker's yeast, has a plethora of usages. Yeast reproduce via asymmetric cell division, which functions through cytokinesis and cellular polarization (Hay-Oak Park et al.). *S. cerevisiae* undergo budding, mating, and filamentous growth (Hay-Oak Park et al.). Successful budding requires exocytosis, or the movement of intracellular materials outside of the cell via membrane bound vesicles (Cooper). Exocytosis is regulated by specific proteins in the GTPase family and SNAREs that enhance activation of vesicle budding at the Golgi (Roumanie et al.).

The Exocyst

Exocytosis is the delivery of cargo molecules, such as proteins, by vesicles from the Golgi to the plasma membrane. This process is vital in all cells because the molecules that are too large to passively cross the membrane barrier can be transported through exocytosis. In the budding yeast, *S. cerevisiae*, the proteins regulating the process of exocytosis are located predominantly at the bud tips in G1 phase and mother-bud necks in G2/M phase. The exocyst regulates localization and fusion of secretory vesicles at these sites (Munson). Exocyst is a multi-subunit tethering complex that is required for post-Golgi vesicle tethering at the plasma membrane (Figure 2). This hetero-octameric protein complex in eukaryotic cells is composed of the following subunits: Sec3, Sec5, Sec6, Sec8, Sec10, Sec15, Exo70, and Exo84. The Munson lab recently showed that each subunit has equal stoichiometry within the complex, which is predominantly soluble; all subunits except Sec3 are essential in maintaining the stable structure of the complex (Heider). Using electron microscopy, the assembly and architecture of exocyst shows that this complex forms an elongated conformation consisting of two modules of four helical rods each. The first module contains Sec3, Sec5, Sec6, and Sec8. The second module contains Sec10, Sec15, Exo70, and Exo84 (Figure 2).

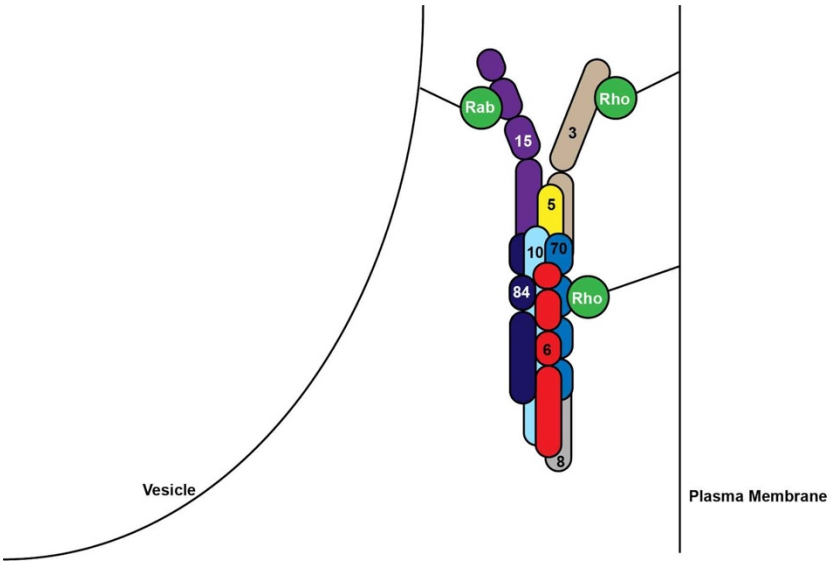


Figure 2: A Schematic Representing Exocyst (Munson & Novick)
All subunits are connected by strong and weak bonds. Each module has at least one subunit that localizes to membranes via binding proteins in the Rho and Rab GTPase family. Sec15 binds to a Rab GTPase on the vesicle membrane, and Exo70 and Sec3 bind to Rho GTPases on the plasma membrane. Rho GTPases regulate cell polarity.

Exocyst Complex Localization

The exocyst complex localizes to sites of polarized growth and secretion in the cell, particularly the plasma membrane. There have been many studies carried out to determine where, when, and how this complex tethers to proteins that specialize in vesicle trafficking, and what subunits in this complex are vital to its fusion to the plasma membrane. The schematic in Figure 2 displays three proteins that interact with the exocyst, however there has been speculation as to how many proteins are actually involved in localization of the exocyst (He et al.). Sec3 and Exo70 localize to the bud tip, and interact with Rho GTP binding proteins at the plasma membrane. Although Sec3 is bound to Rho1 at its N-terminus, it also interacts with Cdc42 at the plasma membrane and this has lead researchers to believe that Sec3 localization is regulated by various membrane bound proteins at different places in the cell cycle (Roumanie et al.). Exo70 interacts with the GTPase Rho3 on the plasma membrane, and does so in a dependent manner (Robinson et al.). Sec6, (shown in red in Figure 2), was found to bind to the SNARE protein Sec9 at the plasma membrane, *in vitro* (Dubuke et al.); however, other studies have concluded that Sec6 interacts with the v-SNARE Snc2 and Sec1, as well as a possible unidentified factor possible located at the plasma membrane (Heider et al.). The exocyst subunit Sec15 binds to the Rab-GTPase Sec4 and Myo2 on the vesicle (Jin et al.), and docks on the target plasma membrane on the site of polarized growth marked by Sec3 (Songer & Munson). In this study, I demonstrate that in *Saccharomyces cerevisiae,* the exocyst subunit Sec8 localizes to the bud tip when Sec3, Sec6, and Exo70 are no longer intact with the complex. This disproves previous findings that Sec6, the direct binding partner to Sec8, requires the stable bond with Sec8 to maintain the complex structure and localize to the plasma membrane. Additionally, it disproves the idea that Sec3 is required for exocyst to localize to the plasma membrane (Finger & Novick).

The Auxin Inducible Degron System

The auxin inducible degron (AID) system allows for fast depletion of targeted proteins in response to the natural plant hormone auxin (Nishimura). The AID system for this project uses the indole-3-acetic acid sequence of auxin (IAA) from *Arabidopsis thaliana*, which is fused with the C-terminus of the subunit that is targeted for degradation. Auxin is produced by plants, which leads to degradation of the transcription repressors via the SCF E3 ubiquitin ligase (Nishimura).

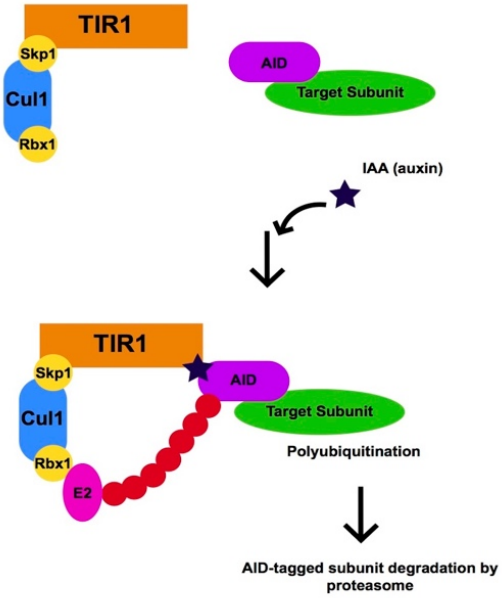


Figure 3: The AID System
The Skp1, Cul1, Rbx1, and TIR1 proteins all form the SCF complex which mediates ubiquitination of proteins targeted for degradation by the proteasome. When Indole-3-Acetic Acid (auxin) is introduced, the AID tag binds to the TIR1 protein. The SCF complex recognizes protein substrates and ultimately target proteins for destruction by the proteasome. E3 ubiquitin ligase then recruits an E2 conjugating enzyme. Rbx1 has a zinc binding domain which allows the E2 enzyme to bind. Ubiquitination occurs, and the proteasomes degrade the AID tagged subunit. Ubiquitination signals targeted proteins for their degradation via the proteasome.

The AID tagged constructs are used alongside a green fluorescent protein tag (GFP) to clearly see where polarized growth is occurring within the budding yeast through fluorescent microscopy.

GFP Bound Nanobody Beads

Anti-GFP nanobodies are coupled with Dynabeads, or magnetic beads, and are used as a method for purifying a protein in solution. In this project, the nanobody beads are used to determine what is still attached to Sec8-GFP after degradation occurs.

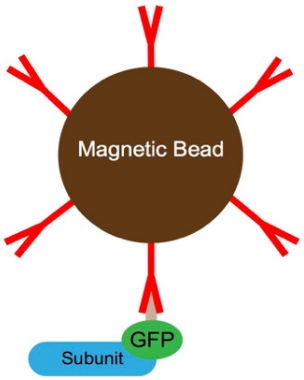


Figure 4: Dynabead Magnetic Beads
There are several steps for achieving a successful protein purification; binding, incubation, washing, and elution. The first step is using an antibody to bind to the magnetic beads. An anti-GFP antibody is used to coat the beads because of the GFP tag integrated in each exocyst yeast construct. Once the beads are anti-GFP bound, the protein sample is added onto the beads. Since Sec8 has a GFP tag the anti-GFP magnetic beads will probe for, and bind this targeted protein after a 1 hour incubation period. The next step is washing the beads with HEPES buffer to discard all unbound protein from the sample. The final step is eluding the protein off of the beads by denaturing it using heat, and an SDS PAGE protein gel that allows for staining and protein identification.

I will explore this particular project step because it is paramount for concluding how the architecture of the complex effects localization to sites of polarized growth and secretion.

Project Goals

I am interested in understanding the roles of different subunits that control the polarized localization of exocyst. Specifically, my hypothesis was to determine how subunit degradation affects the structure, stability and localization of exocyst. Sec3, Sec6, Exo70 and Sec8 were used in this project to determine the outcomes of the AID system when implemented on exocyst. These AID tagged subunits are integrated with Sec8-GFP to accurately conclude what subunits are still attached to Sec8 when degradation occurs. The specific constructs that were created can be found in the Appendix. This project aims to seek out specific factors within the exocyst complex that localize to sites of polarized growth and secretion within the cell, particularly along the plasma membrane. I predict that Sec8-GFP will localize to sites of polarized growth when Sec3, Sec6 and Exo70 are degraded. I will analyze the assembly status of the complex when Sec3, Sec6, and Exo70 are degraded at several time points to determine if the subunits are necessary for maintaining the structure of the complex. I will also determine where Sec8-GFP is localized in the cell after Sec3, Sec6, or Exo70 are degraded using fluorescent microscopy.

A serial dilution growth assay will provide data regarding growth defects of the strains on YPD media as the control, and YPD media with auxin. Degradation of a subunit and no defects during growth are signs of a subunit that is nonessential to the complex, or is not necessary for the complex to stay intact. Unlike the other subunits, Sec3 is considered to be nonessential in the exocyst complex because cells lacking Sec3 do not maintain an optimal growth rate (Wiederkehr et al.). The remaining subunits must be present for exocyst to properly localize.

Next, a series of western blots will be completed to determine if the proteins are being degraded. Primary and secondary antibodies are used to confirm whether or not each subunit is present on the complex. The primary antibody detects if the protein of interest is there. The secondary antibody recognizes specific antigens that are present in the primary antibody, i.e. it detects the presence of the primary antibody signifying that it is bound to the protein. A western blot analysis of the yeast lysates +/- auxin will be completed based on a time course of 10 minute increments. This particular method will allow for observations regarding how long it takes to fully degrade each of the AID tagged subunits in the exocyst complex.

I will take advantage of the GFP tag on Sec8 to determine what subunits are still attached to Sec8-GFP when degradation occurs, by pulling down the complex onto anti-GFP bound nanobody beads. By discerning the combined results of the proposed biochemical assays, the requirements for exocyst complex assembly, localization and secretory vesicle trafficking will be further understood.

Materials and Methods

Building Yeast Constructs

Frozen stocks of yeast strains that contained Sec8-GFP, Sec3, Sec6, and Exo70 were struck out and grown on Yeast Peptone Dextrose -Histidine (YPD -HIS) agarose media plates in a 30°C incubator. The exocyst subunit constructs in this project were built to be used with the Auxin Inducible Degron (AID) system as well as have a way to confirm the success of the AID system. To confirm the success of the AID system and determine where Sec8 localizes in the cell, Sec8 was fused to a Green Fluorescent Protein (GFP) tag and each subsequent strain used was integrated with Sec8-GFP and an AID tag. This allows for confirmation of localization after degradation by fluorescent microscopy. Each tag was integrated using PCR, and purified by using a Qiagen DNA mini spin kit.

Yeast Cell Preparations

Standard yeast preparation methods were used for this project. Cells were grown in Yeast extract Peptone Dextrose (YPD) media comprised of 1% Bacto-Yeast extract (Fisher Scientific), 2% Glucose (Sigma-Aldrich), and 2% Bacto-peptone (Fisher Scientific). Liquid culture media used for time course experiments contained 0.7 mM indole-3-acetic acid (IAA) in addition to the contents listed above. Each strain used has an auxin inducible degron (AID) tag integrated at the C terminus of its genomic locus, by way of linear Polymerase Chain Reaction (PCR) product.

Serial Dilution of AID-tagged subunits in yeast on YPD media +/- auxin

Serial growth dilution assays confirmed the growth of exocyst on YPD media. A 96 well plate was utilized to create a high to low concentration gradient. Cells were grown to an Optical Density (OD) of 1, and diluted ten-fold on plates containing YPD media, and plates containing YPD media and 1mM IAA. Serial dilution growth assay plates were incubated for 3 days in a 30°C incubator and then imaged in an LAS4000.

Auxin Inducible Degradation of exocyst

Yeast lysate powder

Cells were grown in YPD media at 30°C until they reached an OD of .6. IAA was added to the liquid cultures and incubated for 1 hour. These cells were then centrifuged for 15 minutes at 13.2 rpm, and pelleted. The pelleted cells were put into liquid nitrogen through a syringe, to create "noodles" that would then be ground into yeast lysate powder which was stored at -80°C. Noodles were created with the following protocol:
In 2L of autoclaved YPD, add 5mL overnight culture and take the OD. Grow cells to an OD of 0.6. To one flask, add 2.8 mL of IAA (concentration is 1mL ethanol and 88mg IAA powder). Balance each centrifuge bottle before spinning down in the centrifuge for 10 minutes at 3°C. Transfer pellet to 50 mL conical tube. Pour off supernatant. Resuspend pelleted cells in 10 mL of water, and transfer to a 50 mL conical tube. Spin down cells in the centrifuge for 8 minutes, at 3000 RPM, at 4°C. Pour off the supernatant. Resuspend the pellet in 40 mL of sterile water. Spin

down cells for 8 minutes, at 3000 RPM, at 4°C. Pour off the supernatant. Resuspend the pellet in 6 mL of sterile water. Spin down cells for 12 minutes, at 3000 RPM, at 4°C. Remove all liquid, and spin down cells for 15 minutes at 3000 rpm. Obtain liquid nitrogen, and a test tube rack. Appropriately label a 50 mL conical tube, and poke holes in the cap with a needle. Transfer pellet into a sterile syringe. Submerge 50 mL tube in liquid nitrogen, and fill it with liquid nitrogen. Using the syringe, squeeze the pellet into the tube. Cap the tube, and pour out liquid nitrogen. Store in -80°C freezer until they are grinded into powder.

Degradation time courses

Strains were grown on YPD media plates in a 30C incubator for 3 days or until colonies formed. A single colony was mixed with 5mL of liquid YPD media, and grown overnight in a 30°C rotating incubator. The Optical Density (OD) for each culture was measured, and a calculated amount of the overnight culture was added to a 200mL flask of YPD media to continue cell growth until a log phase OD was reached for a specific time. The equation used can be found in the appendices. Each time course produced 8 yeast lysate samples, pertaining to the timepoints necessary for determining when the subunit is degraded. At each 10 minute increment (0 minutes without auxin, 1 minute with auxin, 10 minutes, 20 minutes, etc,), 1 mL of liquid culture was spun down, and cells were snap frozen in liquid nitrogen to the be stored at -20°C. Each pelleted sample was lysed with 100 ul of 0.1M NaOH lysis buffer. Cells were then spun down, and resuspended in 50 ul of 1X SDS dye, and heated for 10 minutes in a 95°C heat block. Once cooled, 10 ul of each sample was loaded into an 8% SDS-PAGE protein gel, and ran for 50 minutes. The gel was transferred onto a PVDF membrane, and proceeded through a standard western blot protocol.

Western Blot analysis

Western blot analysis of yeast lysates was performed with 8% SDS-PAGE protein gels at pH 8.8. 1 mL yeast cell samples were taken at 10 minute increments during a 60 minute time course. Each sample was snap frozen in liquid nitrogen, and stored at -20°C until they were ready for the lysis prep. Cells were resuspended with 100 ul of 0.1 M NaOH lysis buffer and incubated at room temperature for 10 minutes. Samples were spun down for 2 minutes at 13.2 rpm, and then pelleted cells were resuspended in 50 ul of 1X Sodium Dodecyl Sulfate (SDS) loading dye. Samples were put in a 95°C heat block for 10 minutes, and then cooled at room temperature and store at -20°C until ready to load onto an 8% SDS-PAGE protein gel. A Broad Range Color Prestained Protein Standard marker was used to confirm the strains, by their known sizes which can be found in the appendix. The following protocol was used to carry out the Western Blot assays:
Set voltage to 150 V (after samples pass stacking gel, increase to 200 V). Let gel run for 60 minutes. When SDS-PAGE gel is complete, remove gel from glass and place it in transfer buffer. Soak 4 pieces of filter paper and 2 pieces of western blot felt in transfer buffer. Soak PVDF membrane in 10 mL of methanol for 10 minutes on rocker. Remove membrane and let it sit in transfer buffer. Set up "sandwich", with the black square in the back and the clear square in the front. Place 1 piece of felt on the black square. Place 2 pieces of filter paper down. Place the gel on the filter paper and orient the gel so the molecular marker is on the opposite side of the one it ran on. Place the PVDF membrane on top of the gel. Place 2 pieces of filter paper on the membrane. Place 1 piece of felt on the filter paper. Close the sandwich, put it in the gel box with the black square facing the black side of the box. Add an ice pack to the gel box next to black square. Pour remaining transfer buffer into gel box. In the cold room, fill the gel box with transfer buffer. Run gel for 60 minutes at 100 V in the

cold room. When gel is finished, carefully remove the PVDF membrane into container with PBS buffer (to keep it from drying out). Add 10 mL of 5% milk + PBS buffer to container. Cover, and put on rocker for 30 minutes. Pour off milk, and rinse 2X with PBS+Tween buffer. Cut membrane at the appropriate molecular marker. Add 10 mL of the primary antibody, cover and put on rocker for 1 hour. Wash membrane 3X with 10 mL of PBS+Tween buffer. Let membrane rock for 10 minutes for each wash. Add the secondary antibody to correct membrane. Cover, and put on rocker for 1 hour. Wash 3X with PBS+Tween buffer. Let membrane rock for 10 minutes for each wash. After the last wash, add PBS+Tween buffer (to prevent membrane from drying out). Membranes were developed with an ECL Western Blotting Substrate (Thermo Fisher Scientific) and imaged using an LAS4000. Mouse monoclonal antibody Anti-AID was used as the primary antibody. Rabbit Anti-ADH1 was used as the secondary antibody.

Nanobody Pulldowns of Sec8-GFP +/- auxin (exocyst purification)

The following protocol was used to pulldown the exocyst complex onto conjugated nanobody beads: Weigh out 150 mg of powder stored at -80°C, per reaction into eppendorf tubes pre-chilled in liquid nitrogen using a spatula prechilled in liquid nitrogen. Return tubes to liquid nitrogen until ready to resuspend. Buffer used: 50 mM Hepes pH 7.4, 150-300 mM NaCl. Add 600 μl buffer + 15 μl 50X PICS (Roche complete Mini EDTA-Free Tablets, 1 tab in 1 mL water; store at -20 C) and vortex about 30 seconds to get into solution. Spin at 13.2 rpm in cold room in eppendorf centrifuge for 10 minutes.

Prepping Dynabeads

Use 5 μl of slurry per reaction with an additional 1 μl in case of loss. Wash each tube with 500 μl lysis buffer 2x. Wash the beads by adding buffer while the tube is on the magnet, and turning the tube 180 degrees. Wait for the beads to collect on the magnet, and repeat 4 more times. Remove the wash buffer. Resuspend beads in 100 μl lysis buffer per number of reactions (+ 20 μl for the extra beads). Aliquot 100 μl per reaction into screw cap tubes. Before adding lysate, remove all supernatant from the beads. Combine all lysates into a single 15 ml conical tube so all beads will be exposed to same concentration of protein. Add 600 μl lysate to each tube of beads. Nutate 40 minutes in cold room. Wash beads. Remove unbound supernatant. Wash 2x with 500 μl lysis buffer. Resuspend in 250 μl lysis buffer and transfer to a new tube. Remove all supernatant again. Spin down (Galaxy mini) and remove any residual supernatant.

SDS elution (bound material)

Resuspend the beads in 20 μl 1X SDS loading dye (no DTT). Heat samples at 70°C for 10 minutes.

SDS PAGE gel

Load 100% of SDS elution sample onto an 8% SDS PAGE gel. Run at 200V for 50 minutes. Then, use standard Coomassie staining procedures to develop the gel.

Fluorescent Microscopy

The following protocol was used for all fluorescent microscopy assays:

PBS + 15% glycerol was used as the buffer for these preps. PBS + 15% glycerol + 37% formaldehyde was used to fix the cells. Let liquid culture samples grow in the 30°C shaker until they reach log phase growth. Take 1 mL of liquid culture for each tube. Spin down tubes in a centrifuge for 1 minute at 13.2 rpm, and aspirate off supernatant. Resuspend in 1 mL of PBS + 15% glycerol + 37% formaldehyde. Nutate for 10 minutes at room temperature. Spin down cells for 2 minutes in centrifuge at 13.2 rpm. Aspirate supernatant under the hood with biohazard safe conditions. Wash 2 times with 1 mL of PBS + 15% glycerol. Resuspend in 100 ul of PBS + 15% glycerol. Add 6 ul of Vectashield. Use 2 ul of sample per slide for microscopy. Under the microscope, count a minimum of 100 cells per slide, and photograph them using the camera on the microscope. Counting a minimum of 100 cells per slide provides a larger sample size and makes the statistical analysis more consistent.

Statistical Analysis

Data compiled for western blot analysis and microscopy was used for statistical analysis. Each western blot sample was normalized in excel to prevent any data modifications. This calculation scaled the numeric variables to be within the range of 0,1 and comparable to each other. The microscopy data was interpreted by performing cell counts. Each subunit that was analyzed had a minimum of 100 cells counted, and each cell was categorized as being localized or mislocalized. All trials were averaged together to output the percentage of total localized.

Results

Each exocyst subunit construct has a GFP protein marker and an AID tag. The GFP marker allows for fluorescent microscopy detection of Sec8 localization, and the AID tag allows for rapid depletion of the targeted subunit. Once the AID tag was integrated at the C-terminus of the desired subunit, the entire construct including the AID tag and the GFP tag was finished. These exocyst subunit constructs allow the AID system to degrade the targeted Exocyst subunit while GFP is used for tracking the localization of Sec8 in the cell.

Serial Dilution Growth Assays

Altering the natural state of each subunit by integrating multiple tags requires confirmation of growth ability. Cells were grown to an Optical Density (OD) of 1, and diluted ten-fold on agar plates containing Yeast Peptone Dextrose (YPD) +/- auxin to ensure that strains had no defects that were a result of AID tag integration. Growth occurred in a 30°C incubator for 3 days, and then imaged using an LAS4000.

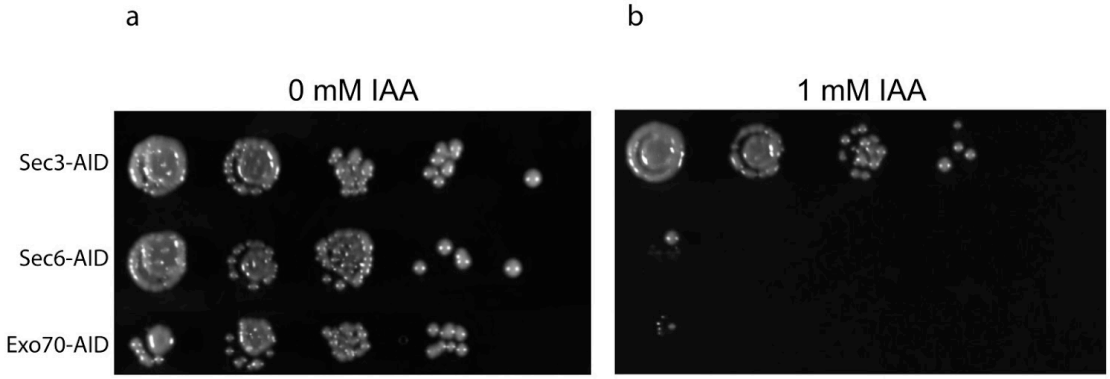


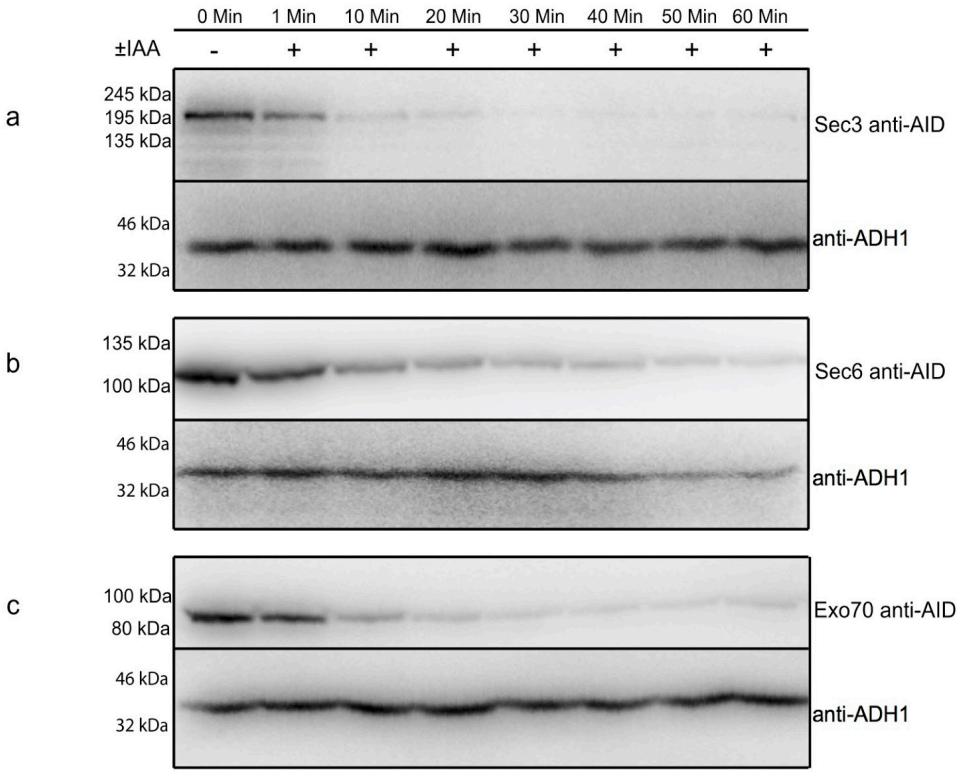
Figure 5: Serial Dilution Growth Assay on Auxin containing YPD media plates
Indicated amounts of indole-3-acetic acid (IAA) was used to degrade the targeted subunits. AID tagged subunit growth on yeast peptone dextrose (YPD) media indicated by 0mM IAA, and AID tagged subunits grown on YPD media with 1 mM of IAA.

Figure 5 confirms that the cells grow with no defects in the absence of the auxin hormone (0 mM IAA) and have growth defects when exposed to auxin (1mM IAA). Previous studies have shown that Sec3 is the only nonessential subunit for the exocyst. This assay confirms these studies, due to Sec3 strains having the ability to maintain its growth rate on plates with and without auxin (Heider).

Degradation of Exocyst Subunits

The AID system was used for this study because it degrades target proteins quickly. The system's functionality and speed was determined by performing a 10 minute-increment time course, and confirmed by using a Western Blot. Since the hormone is known to degrade the target within minutes, the duration of the time course was only 60 minutes. In each case, the target subunit was

degraded within 30 minutes, which confirms that the system is efficient. The amount of degradation is at least 80% in each case, which confirms the AID system's functionality.



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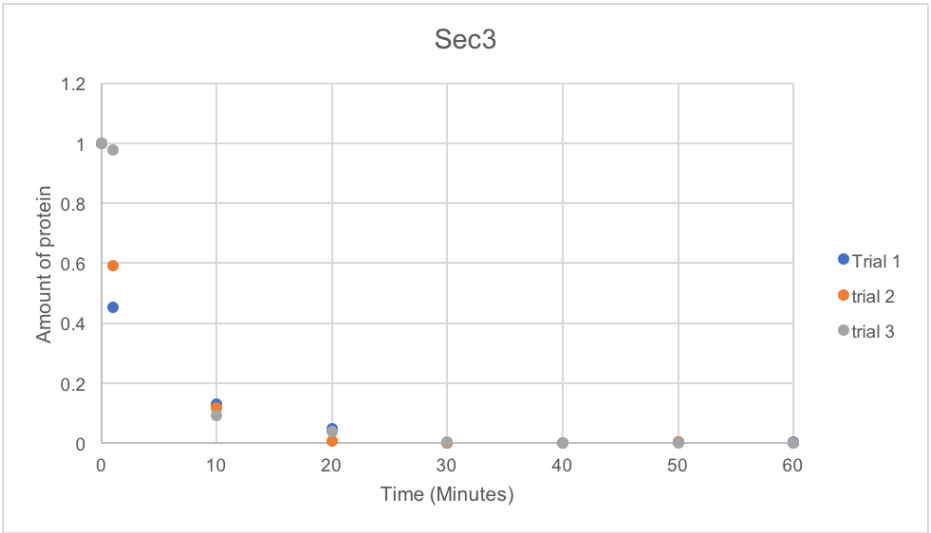

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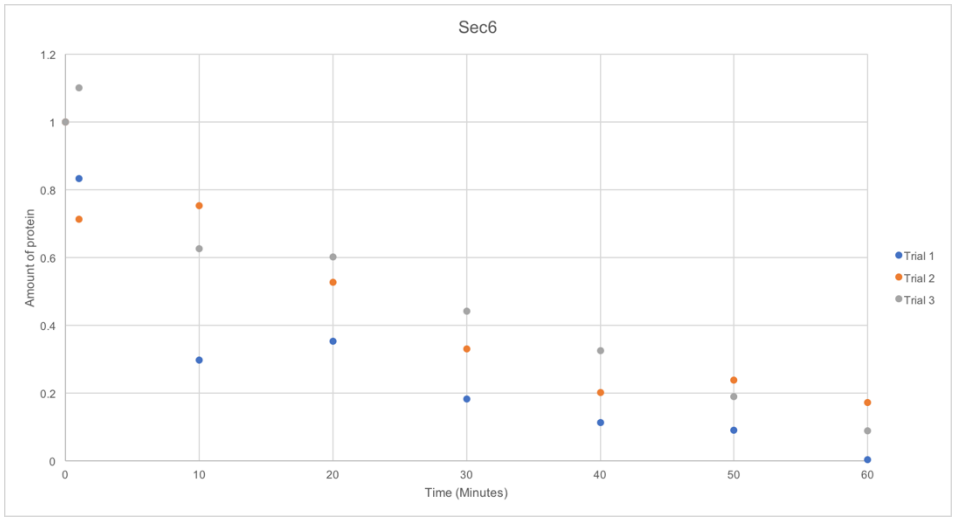

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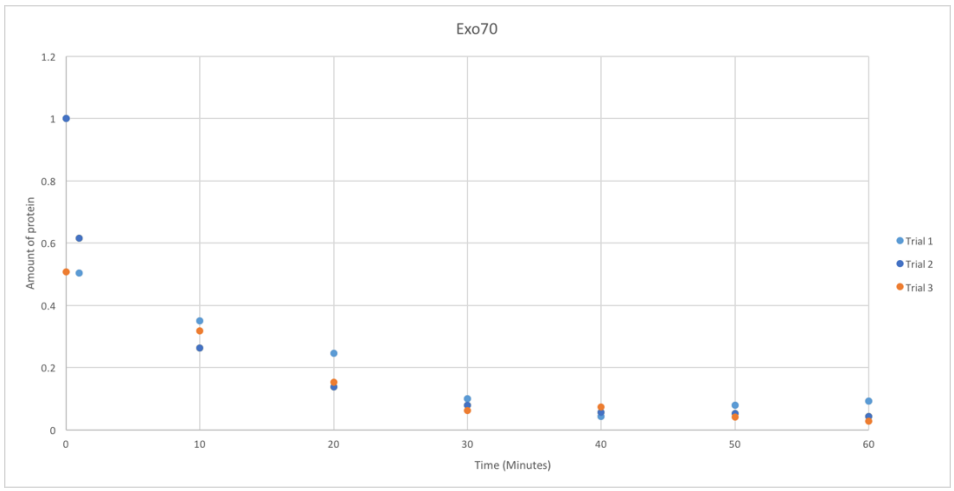

Figure 6: Subunit Degradation
Indole-3-acetic acid was used as the hormone to degrade each targeted subunit. (a) Sec3-AID was probed with an anti-AID antibody, (b) Sec6-AID was probed with an anti-AID antibody, and (c) Exo70-AID was probed with an anti-AID antibody. Each subunit had over 70% degradation from 0 minutes to 60 minutes. The plus symbol indicates that IAA was introduced, and the minus symbol indicates that no IAA was introduced. Anti-ADH1 was used as a loading control in each blot. (d, e, f) Each western blot band was quantified and normalized based on its size and intensity using the GE imaging machine.

The known molecular weight of each subunit, with and without an AID tag, was used to compare the band sizes seen in each blot with the known size and the increased size due to the AID tag. Since each targeted exocyst subunit is able to be degraded, the presence of all other subunits

must be verified. This result will provide more information pertaining to the connections between the subunits in the complex, and the importance of each subunit.

Purification of the Exocyst complex

Once degradation of the target protein occurs, tests must be performed to determine what is still attached in the complex and that only the target protein was degraded. Anti-GFP nanobody beads were used to pulldown the remainder of the exocyst complex. The eight subunits comprising the complex are bound to one another, some bound more tightly than others. These bonds keep the exocyst intact, and losing a subunit causes disruption in its architecture. The confirmation of each target protein being the only subunit degrading is necessary for this study to be valid.

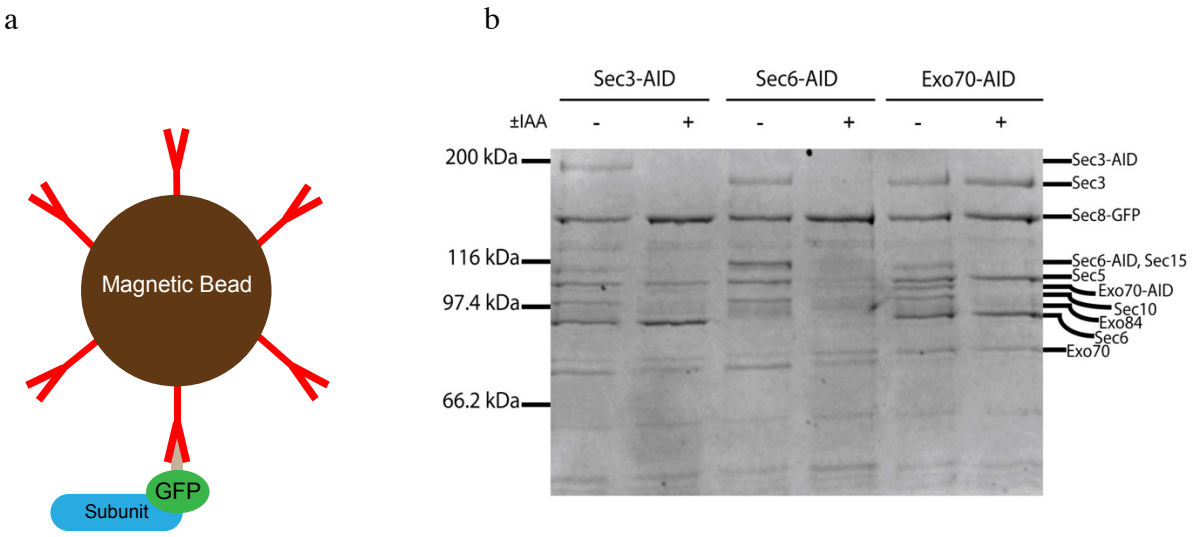

Figure 7: Yeast lysate purification
Purification of yeast lysate using GFP-bound magnetic beads (a) shows what is still attached to Sec8-GFP after degradation occurs. SDS-PAGE gels and Coomassie staining show which subunits are intact with the complex. (b) The AID tagged subunits, as well as the presence of auxin is indicated on the top of the figure. Plus signs denote the presence of auxin and the minus signs denote the absence of auxin. Each subunit is labeled on the right of the figure, as well as the AID tagged subunits. The AID tag increases the original size of the subunit.

Each degraded subunit shows a significant impact on the structural integrity of the complex. Although Sec3 is nonessential, after degradation this subunit causes Sec15 and Sec10 on the opposite module to disappear. Sec15 is essential to cellular trafficking and localization because it binds to the Rho GTPase Sec4 (Figure 2). Degradation of Sec6 causes all other subunit connections to be lost. Without this subunit, the cell should be unable to move from the Golgi to the plasma membrane because the subunits that aid in vesicle trafficking are no longer present. The lack of Exo70 after degradation results in complete loss of the opposite module comprised of Sec15, Sec10, Exo84, and Exo70. Similar to the other subunits, this causes an issue for Sec10 binding to the vesicle during trafficking and localization.

Sec8-GFP Localization by Fluorescent Microscopy

After degradation occurs, one would think that the disappearance of Sec3, Sec6, and Exo70 would hinder any chance of Sec8-GFP localizing to the bud tip or bud neck. This question influenced the next steps in this research, and was answered using microscopy.

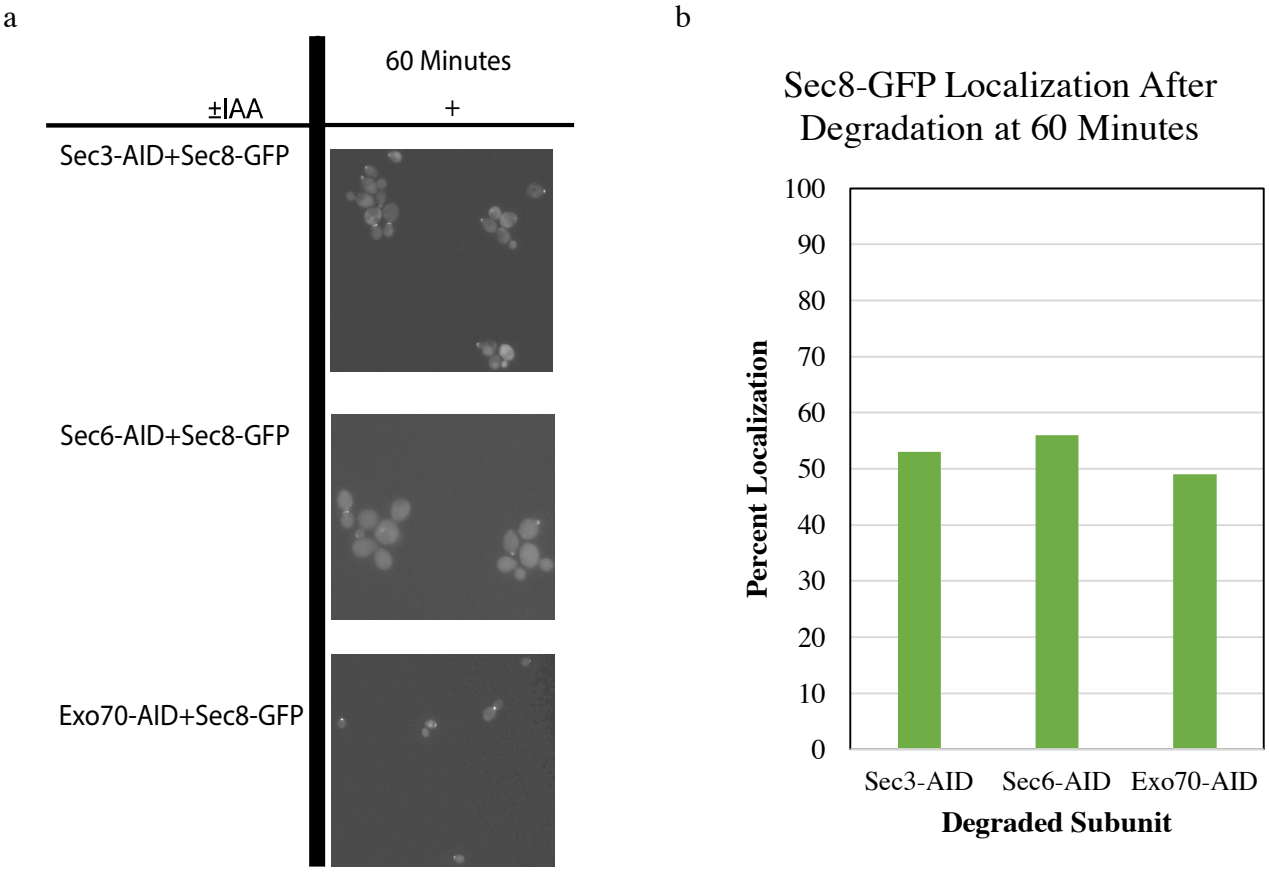

Figure 8: Sec8-GFP localization
(a) Each yeast construct was grown to log phase, and spun down in a centrifuge. The sample was combined with Vectashield to prevent photo bleaching. Images of cells were taken at 40X and 100X magnification. (b) Localization was calculated by counting a minimum of 100 cells for each yeast construct sample. Localization was classified by the GFP tag's location at the bud tip and bud neck of the cell.

Past studies have concluded that the exocyst complex disassembles completely when Sec6 is degraded, and disassembles partially when Sec3 and Exo70 are degraded. However, microscopy revealed that even after the targeted subunit was degraded, Sec8-GFP properly localized in the cell nearly 50% of the time in each strain. Sec6 is known as a strong binding partner for Sec8 within the complex, so this localization is somewhat perplexing. Sec8, without Sec6 (Figure 2), detaches from the entire complex (Heider, Munson). This then suggests that Sec8 can localize without being attached to the complex. This may be due to other Rab GTPases or SNARE interactions that researchers have yet to discover, or just the possibility that this subunit can localize on its own.

Discussion

The research aims of this project were to determine the outcome of specific subunit degradation in the exocyst complex. The subunits degraded have significant importance for the complex binding to the target membrane and vesicle membrane by GTPases and SNARE proteins. The methods used in this study analyzed the speed and amount of degradation (Figure 6), the structure of the complex after degradation (Figure 7), and the location of Sec8-GFP after degradation (Figure 8). The results of these experiments show that the AID system used on the tagged subunits compromises the integrity of the complex structure, and the exocyst complex is required to be fully intact in order to properly localize to the sites of polarized growth. Thus, more research must be done on exocyst localization when the complex is not fully intact. This data correlates to a previous study, regarding the exocyst and its functionality when the whole complex is not fully intact (Heider et al.).

The AID system was used due to its simplicity and accuracy. The AID tag fused to each subunit allowed for its rapid depletion, however there are other methods of deleting a gene in the protein complex. Research studies pertaining to gene deletions have proven that there are simple and effective methods which also degrade a target protein. So what makes the AID system so effective? The purpose of this system is to degrade just a portion of the complex, not the entire DNA sequence. If the genetic code was deleted, the complex wouldn't grow. In Figure 5, strains grown on regular YPD grow without any defects because the media is providing nutrients. However, when the strains are grown in the presence of auxin, they die. The same thing would occur if the genes were deleted, only the cells would die on YPD media as well.

The degradation experiments show that each subunit degraded within 30 minutes of auxin being introduced, and over 80% of the subunit was degraded (Figure 6). This data aligns with the nature of the complex, because the bond between Sec6 and Sec8 is one of the strongest bond in the complex. The complex is known to have two modules bonded together, each comprised of four subunits. One module has Sec6, Sec8, Sec5, and Sec3, and the other module has Sec15, Sec10, Exo84, and Exo70 (Heider et al.). These modules correspond to this studies results in Figure 7, which show the subunits that are still attached to Sec8-GFP after degradation. Although Sec3 is nonessential, the lack of this subunit causes Exo70, Exo84, Sec15 and Sec10 on the opposite module to disappear. Degradation of Sec6 causes all other subunit connections to be lost. The lack of Exo70 after degradation results in complete loss of the opposite module comprised of Sec15, Sec10, Exo84, and Exo70. This particular assay, in tandem with western blot confirmation, is key to having accurate data for each degraded subunit. These assays confirmed each subunit by size using a molecular weight marker, and implemented controls to ensure that only the tagged subunit was being degraded. These findings conclude that the exocyst complex subunits, when targeted for degradation and exposed to auxin, will rapidly deplete and cause the complex to disassemble. This inhibits the exocyst from properly localizing on the plasma membrane to complete exocytosis.

The contradictory results of this study came to light after analyzing the remaining subunits of the complex after degradation and the localization of Sec8-GFP by fluorescent microscopy (Figure 8). The complex localized approximately 50% of the time in each case, which raises many questions pertaining to past research in this field. Although these findings show inconsistencies in localization, Sec8-GFP still localizes to the bud tips and bud necks of the cells. Why does Sec8 localize if its direct binding partners degradation causes the entire complex to fall apart? This result calls for further research into Sec8 to determine if the subunit binds to a

plasma membrane protein on its own. If this were the case, the puzzling results from the fluorescent microscopy assays would correlate with past research in this field.
Although the results for Sec8-GFP localization were fascinating, a new approach to microscopy should be taken into consideration. There were several issues that were troubleshot in regards to photo bleaching. Use of a different microscope, or implementation of other photo bleaching protectants is necessary.

Next steps for research in this field include investigating the roles of the five other subunits in the exocyst as well as the SNAREs and GTPases they interact with on the plasma and vesicle membrane. These subunits will aid in uncovering the mechanisms of the exocyst complex in regards to cellular trafficking and localization. Overall, this study concluded that the exocyst complex must be fully intact for localization and vesicle trafficking in the cell. Without each subunit, the complex is unable to dock to the plasma membrane consistently and cannot localize to the bud tip. Alternative methods for determining what subunits are necessary for localization to sites of polarized growth include testing other subunits that have known GTPase binding partners on the plasma membrane or vesicle membrane. These findings unravel the importance of each subunit for membrane docking and vesicle trafficking.

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Appendix

Table 1: Strains Used

MMY #	General Description	M	Genotype	Made by	Date Frozen
MMY115	Sec8-GFP	a	MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 Sec8-GFP(HIS3)	Invitrogen	
MMY1527	Sec3-AID, Sec8-GFP	a	MATa ura3-1::ADH1-OsTIR1-9Myc(URA3) ade2-1his3-11,15 leu2-3,112 trp1-1can1-100; SEC3::Sec3-AID (KANMX); SEC8::Sec8-GFP (HIS)	RH	8_12_15
MMY1528	Sec6-AID, Sec8-GFP	a	MATa ura3-1::ADH1-OsTIR1-9Myc(URA3) ade2-1his3-11,15 leu2-3,112 trp1-1can1-100; SEC6::Sec6-AID (KANMX); SEC8::Sec8-GFP (HIS)	RH	8_12_15
MMY1529	Exo70-AID, Sec8-GFP	a	MATa ura3-1::ADH1-OsTIR1-9Myc(URA3) ade2-1his3-11,15 leu2-3,112 trp1-1can1-100; EXO70::Exo70-AID (KANMX); SEC8::Sec8-GFP (HIS)	RH	8_12_15

Table 2: Sizes of Each Exocyst Subunit

Exocyst Subunit	Molecular Weight
Sec3	170 kDa
Sec5	112 kDa
Sec6	93 kDa
Sec8	122 kDa
Sec10	100 kDa
Sec15	113 kDa
Exo70	70 Da
Exo84	84 kDa
Sec3-AID	120 kDa
Sec6-AID	200 kDa
Exo70-AID	100 kDa