Stability of ESCRT III Snf7 Polymer Influenced by: Bro1 and Vps2/Vps24

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Stability of ESCRT III Snf7 Polymer Influenced by: Bro1 and Vps2/Vps24

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Protein of Interest

ESCRT III

- Vps20 – Snf7 oligomerization initiation
- Snf7 – monomeric units of the Snf7 oligomer
- Vps2 – part of the Snf7 capping dimer
- Vps24 – part of the Snf7 capping dimer
- Vps4 – ATPase that disassembles the Snf7 complex
- Bro1 – stabilizing element of the Snf7 oligomer
Abstract

The endosomal sorting complexes required for transport (ESCRT) are protein complexes that remodel cellular membranes. The ESCRT III membrane machinery has been implicated in regulating membrane bound receptor proteins through the multi-vesicular body (MVB) pathway, during the end of cytokinesis, and being manipulated by the HIV virus during infection. My thesis project focuses on the in vivo regulation of ESCRT III. Bro1 binding to the Snf7 protein polymer helped stabilize ESCRT III. With this understanding that Bro 1 stabilizes the Snf7 polymer, two questions can be posed: Does it block Vps4 mediated Snf7 disassembly? Or does it block Vps2 and Vps24 from capping the Snf7 polymer? The latter of these two questions is the focus of my thesis. My research has shown the interaction of the capping Vps2:Vps24 dimer to only slightly affect protein sorting in the ESCRT pathway.
Introduction

The compartmentalization of cells with membrane bilayers is crucial to the functionality and survival of any organism. This compartmentalization begins with the plasma membrane and is regulated by the processes of endocytosis and exocytosis. These two systems help regulate the plasma membrane composition so cells can respond to environmental signals. The ability of a cell to traffic proteins through the endocytic pathway is controlled and aided by many membrane remodeling protein systems. One of the most involved organelles with the endocytic pathway is the endosome. The ability to sort and direct proteins for degradation or recycling back to the plasma membrane is aided by the ESCRT machinery.

Endocytic Pathway

The endocytic pathway plays a major role in helping to balance and maintain the composition of the plasma membrane. Plasma membrane equilibrium is controlled by the addition of secretory vesicles after fusion and subtraction of endocytic vesicles after invagination (Vita TA, 1995). Balance of these two events allows for the constant modification and alteration of the plasma membrane. The plasma membranes of organisms are in a constant state of remodeling, therefore the need for a specific and reliable system for membrane protein removal and recycling is needed (Zastrow, 2009). By allowing the plasma membrane to quickly remove or add membrane proteins, a cell can respond dynamically to environmental factors. The

Figure 1: Cartoon representation of the endocytic pathway. The figure includes movement of vesicles from plasma membrane all the way until degradation at the lysosome or vacuole. (Daniel P Nickerson, 2007)
regulation via degradation or temporary suspension in a cytosolic vesicle of membrane proteins allows the cell to choose which option conserves more cellular energy (Zastrow, 2009).

The regulation and control of different membrane proteins begins with the formation of clathrin-coated vesicles from the plasma membrane that travel to and are collected in the early endosome. (Nakano, 2007) Proteins that are targeted and placed into these clathrin coated vesicles are first ubiquitinated by one of the many ubiquitin conjugating enzymes that ubiquitinate the cytosolic side of membrane proteins. Ubiquitin (Ub) is a highly conserved 76 amino acid polypeptide that is linked to specific protein substrates targeted for degradation by a cascade of Ub-conjugation enzymes. Ubiquitination was first characterized to occur on soluble proteins that are polyubiquitinated via the attachment of a chain of four or more Ub subunits, which target these substrates for degradation by the proteasome (Weissman, 2001). Although membrane proteins are mainly regulated through endocytosis, cytosolic proteins can also find their way into endosomes via binding to a membrane-associated receptor. Upon fusion of the clathrin vesicle to an early endosome, the fate of membrane bound proteins is then regulated through the MVB (multivesicular body) biogenesis pathway.

**Multivesicular Body Biogenesis**

The two main forms of protein degradation are through the proteasome or the MVB pathway (Katzmann, 2007) The biogenesis of an endosome begins with an early endosome. Targeting of proteins to the endosome is controlled by the ubiquitination of membrane proteins. Direct evidence that Ub directed MVB sorting was provided in yeast by mutating the lysine residues of MVB cargo such as Cps1 and Phm5, which caused the buildup of these proteins in

![Figure 2: MVB biogenesis cartoon. The maturation of endosomes begins with any early endosome and through the sorting of ubiquitinated proteins into ILVs matures into a MVB, which then fuses with the vacuole or lysosome. (Katzmann, 2007)](image-url)
the vacuole membrane instead of the vacuole lumen. (Katzmann, 2007) After vesicles fuse with the early endosome, the ESCRT machinery is recruited to begin the process of forming intraluminal vesicles (ILVs). Membrane-bound proteins are actively sorted to different places in the cell through tightly choreographed processes, many of which center on the early endosome (James Hurley, 2011). The importance of sorting these proteins into different ILVs that can proceed to degradation or recycling lends to the importance of ESCRT machinery. As the endosome grows larger in size and contains more ILVs it matures into a multivesicular body. These MVBs then fuse with the vacuole in yeast or the lysosome in higher eukaryotic organisms. The ability of this maturation process to occur relies on the efficiency and effective sorting of proteins into the ILVs by the ESCRT complex.

**ESCRT Pathway**

Discovery and elucidation of the ESCRT pathway has been heavily intertwined with the deeper understanding of the multivesicular body. The quest to understand this imperative mechanism in endocytic protein regulation was initiated by the discovery of the first MVBs (Hurley, 2011). The discovery of 60 yeast vacuolar protein sorting (VPS) genes helped to provide direction to understanding endosome and protein sorting. The ESCRT machinery is made up of four complexes: ESCRT 0, I, II, and III. Recruitment of these different ESCRT complexes occurs in a chronological order, and each ESCRT complex requires the previous component to initiate formation of the complex. ESCRT 0 is required for initiation of MVB biogenesis in yeast (Hurley, 2011). The next step in ESCRT recruitment is the formation of ESCRT I and ESCRT II, which create a large protein complex. This complex begins to drive the budding of the limiting membrane which would develop into the ILV.

The main focus of this research project has been to further elucidate ESCRT III in vivo. ESCRT III polymerization is initiated by Vps20 binding that acts as the platform for Snf7 to begin creating the large oligomer complex that drives the scission of the ILV from the endosome membrane. During the polymerization of Snf7, an important stabilizer protein, Bro1, helps to support the large protein complex (Hurley, 2011). The capping of Snf7 by the dimer of Vps2 and Vps24 is the last step before the ILV pinches
away from the limiting membrane and into the lumen of the endosome (Figure 3). The dimer of Vps2 and Vps24 end the polymerization of Snf7 and prime the polymer for recycling by Vps4, an ATPase that hydrolyses ATP to break apart the Snf7 polymer (James Hurley, 2011). After the ILV has budded into the endosome lumen, the ESCRT machinery is recycled away from the surface of the endosome. This occurs so the ESCRT machinery can be recruited again by ubiquitinated proteins that have been shuttled from the plasma membrane to the endosome.

**Figure 3: Cartoon rendering of the ESCRT machinery bound to the endosome lumen. (Teis 2009 Cell)**

**ESCRT III Is Stabilized by Bro1**

Within the dynamic network of ESCRT III, the interaction of Bro1-mediated stabilizing of Snf7 has further been elucidated in how it controls the polymerization or recycling of the Snf7 polymer. Bro1 exerts its stabilization effect toward ESCRT-III downstream of complex assembly. The addition of exogenous Bro1 domain to ESCRT-III was sufficient to protect the complex from Vps4-mediated disassembly (Wemmer M, 2011). The binding of Bro1 to the Snf7 polymer helps provide mechanical stability, and also a barrier to recycling via Vsp4. With the increase of stability in Snf7 caused by Bro1 binding, questions arise about the mechanism of how Bro1 inhibits or limits this recycling process. One option is that the capping dimer of Vps2 and Vps24 are both crucial to initiating disassembly of the Snf7 polymer, so Bro1 binding could be inhibiting these capping proteins or be regulating the recruitment of Vps4.
ESCRT Pathway and Disease

Due to the dynamic nature of the ESCRT pathway and the effect it has on proper cell signaling it has been implicated in many human diseases. The most obvious impact of ESCRT function is through the regulation of receptors such as the EGFR (epidermal growth factor receptor), JAK (Janus Kinase)/ STAT (signal transducer and activator of transcription), and many other important plasma membrane associated receptor proteins (Saksena & Emr, 2009). In cancers such as acute myeloid leukemia, human prostate cancer, mammary carcinomas, squamous carcinomas, and glioblastomas, the ESCRT pathway has been inhibited to upregulate cell growth (Saksena & Emr, 2009). By inhibiting the ability of the ESCRT pathway to remove membrane receptors, cells can continue to grow and divide unchecked, resulting in life threatening diseases. Although increasing signal transduction from the plasma membrane is one avenue for cellular growth, modification in cytokinesis can also drive cancerous growth. The evidence showing the ESCRT III complex function during cytokinesis is due to its interaction with Tsg101 (tumor susceptibility gene) and the recruitment of ESCRT III to the midbody (Saksena & Emr, 2009). The implication of damaged cytokinesis function would lead to multinucleaeic cells. The further elucidation and understanding of how this complex functions in vivo could provide potential drug targets and aid in improving patients quality of life.
Yeast Strains Used

Throughout this project, several yeast strains were used. All of the different knockout strains were created from the parental strain of SEY6210. All of the different gene deletions were made by homologous recombination. Each of the different yeast strains used can be modified and have multiple plasmids transformed to create the needed protein expressions for certain experiments.

<table>
<thead>
<tr>
<th>Yeast Strain</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>SEY6210</td>
<td>MATalpha leu2-3, 112 ura3-52 his3-Δ200 trp1-Δ901 lys2-Δ801 suc2-Δ9</td>
</tr>
<tr>
<td>GOY65</td>
<td>bro1Δ::HIS3</td>
</tr>
<tr>
<td>GOY456</td>
<td>pep4Δ prb1Δ Vps2Δ</td>
</tr>
<tr>
<td>GOY457</td>
<td>pep4Δ prb1Δ Vps2Δ Vps4Δ</td>
</tr>
<tr>
<td>MBY3</td>
<td>Vps4Δ</td>
</tr>
<tr>
<td>MWY24</td>
<td>snf7Δ</td>
</tr>
<tr>
<td>MWY25</td>
<td>Snf7 L231A::Kan</td>
</tr>
<tr>
<td>DMY1</td>
<td>Bro1-GFP</td>
</tr>
<tr>
<td>DMY2</td>
<td>Bro1-GFP Vps4Δ</td>
</tr>
<tr>
<td>GOY184</td>
<td>Vps2Δ Vps24Δ</td>
</tr>
<tr>
<td>GOY282</td>
<td>Vps2-HA pep4Δ</td>
</tr>
<tr>
<td>GOY283</td>
<td>Vps2-HA vps4Δ pep4Δ</td>
</tr>
</tbody>
</table>

Figure 4: Table of yeast strain names and their associated genotypes.
<table>
<thead>
<tr>
<th>Plasmid Name</th>
<th>Insert</th>
<th>Vector</th>
</tr>
</thead>
<tbody>
<tr>
<td>pCR180</td>
<td>Sna3-GFP cloned from CRY117 which has a Longtine Sna3-GFP insert</td>
<td>pRS415</td>
</tr>
<tr>
<td>pGO518</td>
<td>Sac1-Xho1 from pMB139 subcloned into pRS426</td>
<td>pRS426</td>
</tr>
<tr>
<td>pGO534</td>
<td>Spe1-Sal1 fragment from pGO530 subcloned into pRS416</td>
<td>pRS416</td>
</tr>
<tr>
<td>pGO780</td>
<td>Vps2 PCR product from ZW7 and ZW10 gapped into Sac1 site of pGO781</td>
<td>pRS426</td>
</tr>
<tr>
<td>pGO781</td>
<td>Vps23 PCR product from ZW13 and ZW14 gapped-repair into Kpn1 site of pRS 426</td>
<td>pRS426</td>
</tr>
<tr>
<td>pGO835</td>
<td>TK5p and TK6p amplification of MWM3 insered into Sac1 site of pRS423</td>
<td>pRS426</td>
</tr>
<tr>
<td>pMWM3</td>
<td>BRO1 5' UTS::bro1[1-387]</td>
<td>pRS426</td>
</tr>
</tbody>
</table>

Figure 6: Table of plasmids used for these experiments. Included are the names and genotypes and the original pRS series vector used to create them.

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Description</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZW7p</td>
<td>(w)Vps2 5'UTR (500 bp upstream) inserted 5’end of Sac1 site in pRS426</td>
<td>gggatccactagtttagagcgcacccgctggGGAGCTGATGAATCAAACAG</td>
</tr>
<tr>
<td>ZW10p</td>
<td>(C) Vps2 3'UTR (500bp downstream) (inserts 3’end of Sac1 in pRS426 and regenerates the Sac1 site)</td>
<td>gcaattaccacctactaatgggaacaaaagctggagCTTCCATTAGAAGTCTGG</td>
</tr>
<tr>
<td>ZW13p</td>
<td>(W) Vps24 5’UTR (500 bp upstream)overhang anneals 5’end of Kpn1 site in</td>
<td>agtgagcgcgcgtaatacgactcactatagggcgaattgCATTCTTAAAGCGTTCTGTG</td>
</tr>
<tr>
<td>ZW14p</td>
<td>(C) Vps24 3'UTR (500 bp downstream) overhang anneals 3’end of Kpn1 site in pRS426</td>
<td>aattcgatatcagcttatgacccctgcacccctgaaCTGATACGATTACCGTGGG</td>
</tr>
<tr>
<td>TK5p</td>
<td>BOD 5’ UTR anneals 500bp upstream tail vps2</td>
<td>aacccctaggtctctgtctcgcactgccttcagagaaAAAGGCACAGTTGG</td>
</tr>
<tr>
<td>TK6p</td>
<td>BOD 3’ UTR anneals 500 BP downstream tail in pgo780</td>
<td>ggcgccaattaccctacaaaggggaacaaaagctggagCATCTATGTTTTCCTCCTTT</td>
</tr>
<tr>
<td>DN320p</td>
<td>ML 1° fwd, KO VPS2</td>
<td>N/A</td>
</tr>
<tr>
<td>GP262p</td>
<td>(C) VPS23 3’UTR</td>
<td>N/A</td>
</tr>
</tbody>
</table>

Figure 5: Table of primers used when creating plasmids or gene knockout yeast strains. The figure includes a description of their use and the primer sequence.
**Methods**

**Yeast Culture**

The culturing methods used to grow yeast for experiments consisted of a two-step process. Depending on the biochemical deficiency of the yeast strain, either minimal media (lacking specific amino-acids) or rich media was used to grow cells overnight. Then diluted an optical density of (OD$_{600}$) .2 for yeast strains in rich media, and 0.3 O.D.s for cultures being grown in minimal media. Yeast cultures were then harvested between 0.5 – 0.8 O.D.s/ml and used for different experimental purposes.

**Yeast Transformation Protocol**

A standard yeast transformation protocol was performed when transforming a linear plasmid and PCR product, circular plasmid, or to create a knockout yeast strain with a PCR product. Cells were grown to mid-logarithmic stage of OD$_{600}$= 0.5 – 0.8 and then harvested. These cells were then incubated at 30˚C in a solution of 0.1 M Lithium Acetate/TE. After the incubation period, DNA of interest is added with carrier DNA and incubated an hour at 30˚C. Immediately following the second incubation, the cells are heat shocked at 42˚C for 20 minutes. These transformed cells are spread on minimal media plates lacking the appropriate nutrients to select for the plasmid. For genetic deletions, transformations were plated on YPD agar plates containing 200μg/mL kanamycin.
**Escherichia coli Transformation Protocol**

To transform bacteria with plasmids, the chemically competent XL1 Blue strain of *e.coli* cells are thawed on ice and added to pre-chilled closed circular DNA. Depending on the concentration of the DNA to be transformed, usually 100ng is added to the thawed bacteria. These cells are incubated on ice, and then heat shocked for 2 minutes. The bacterial cultures then recover in a liquid Luria broth (LB), at 37˚C. These transformed cells are then plated on LB agar plates containing 50μg/mL ampicillin.

**Plasmid Creation**

The plasmids created for the purpose of studying ESCRT III were all pRS series vectors (Chee MK, 2012). This style of vector requires yeast auxotrophic for adenine, leucine, tryptophan, uracil, or histidine with the option of expressing these plasmids using a centromeric or 2μ high copy number (Chee MK, 2012). The pRS series of vectors also have an *Escherichia coli* driven promoter so plasmid can be purified from bacteria. The plasmids created consisted of a 2μ Vps2 and Vps24, a 2μ Vps24 alone, a centromeric Vps2 – HA tagged vector, and a 2μ BOD (Bro1 Domain) expression vector.

The gene of interest was amplified from yeast genomic DNA. The PCR product was then transformed into wildtype *S.cerevisiae* along with a pRS series vector that was linearized with a specific endonuclease. Using the endogenous machinery of the *S.cerevisiae*, the gene of interest homologously recombines into the pRS vector and is selected using the yeast marker within the pRS vector. These would then be purified from yeast using zymolase and transformed into bacteria. This then allows for the selection of one amplified plasmid per bacterial colony, which can be checked via PCR and gene knockout rescue experiments using the newly created plasmid.
Yeast Deletion Creation

The yeast strains created for these experiments were made by homologous recombination. This method of quickly knocking out genes in *Saccharomyces cerevisiae* takes advantage of using PCR to make an integration cassette (Longtine MS, 1998). PCR primers are designed to homologously recombine with the gene of interest by replacing the gene with a selectable marker (Longtine MS, 1998). This system allows for specific and precise recombination and quick confirmation of knockouts.

Yeast Lysate Preparation

Yeast was inoculated from an overnight culture to a density of OD600 0.2/ml and grown to log phase OD600: 0.5 – 0.8/ml. Two different yeast lysis protocols were used one, a mechanical lysis and the other using osmotic pressure. For mechanical lysis, the cultures were frozen in liquid nitrogen, and then crushed under liquid nitrogen with a mortar and pestle, fracturing the cell walls of the yeast with mechanical grinding. The yeast cell powder was then lysed using a buffer containing TritonX – 100 and protease inhibitor cocktail, centrifuged to remove the insoluble portions of the cell, and the concentration of this lysate was measured using a BCA protein concentration assay. Osmotic lysis begins with removing the cell wall from log-phase yeast cultures with zymolase, resuspending the cells in low-osmotic buffer containing TritonX-100. Centrifuge these cultures to remove residual material.
Immuno-Precipitation of Yeast Lysates

The purpose of using immunoprecipitations is to pull down proteins of interest using antibodies specific to the protein you wish to study. For these immunoprecipitations, protein G sepharose beads were used. After washing the beads in lysis buffer, primary antibody specific to our protein of interest was added. 5μgs of anti-HA monoclonal antibody was added to the washed beads and rotated at 4˚C. Unbound αHA antibody is washed away and protein lysate is added. For each immunoprecipitation, 5 ODs or 500μgs were used. After letting this solution rotate at 4˚C, the beads were gently centrifuged, then washed using buffers containing TritonX-100 and NaCl to remove non-specific binding to the protein G beads. The beads were then dried and resuspended in Laemmli buffer, and all 5 ODs or 500μgs were loaded into a SDS-page gel.

Western Blot Analysis of Protein Lysates

Western blots run in this thesis are 8 or 10% polyacrylamide gels. These gels help keep the boiled, and sample buffer prepared samples denatured and negatively charged while running with the electric current. Depending on the concentration of the samples, 10-15μl of sample were loaded each time and run in a SDS-page running buffer of a tris-glycine solution. These were then transferred to nitrocellulose paper overnight at 4˚C. The nitrocellulose blots were blocked with a 10% milk solution, then exposed to primary antibodies specific to the protein of interest. Both monoclonal and polyclonal antibodies were used, depending on the protein of interest. These blots were exposed for 1.5 hours at 21˚C or overnight at 4˚C. A secondary antibody with conjugated horseradish peroxidase (HRP) was applied to bind the primary antibody and allow for detection by chemiluminescence.
**Results**

**Snf7 Co-Immunoprecipitation with HA Tagged Vps-2**

When attempting to understand how Bro1 helps stabilize the Snf7 polymer, the best technique to elucidate relative amounts of Snf7 polymer would be using an immunoprecipitation. By using an HA tagged Vps2 I pulled down Vps2 and then probed for a co-immunoprecipitation of Snf7 when Bro1 is expressed normally or expressed at in increased level, in hopes of seeing the levels of Snf7 protein change depending on the expression of Bro1 in the different yeast strains.

For this experiment new, yeast knockout strains needed to be created. An already created yeast strain possessed knockouts of two important vacuole peptidases, pep4Δprb1Δ, and another had both these peptidases knocked out along with Vps4Δ, which is the ATPase that recycles the Snf7 complex. Using homologous recombination GOY456, pep4Δ prb1Δ vps2Δ, and GOY457, pep4Δ prb1Δ Vps2Δ Vps4Δ, were created. With these new knockout strains, Vps2-HA could be expressed on a plasmid so it could be pulled down using G-sephorase beads. A cartoon representation of this knockout process is described in figure 8. After the kanamycin gene was homologously recombined, a PCR check was done to knock out Vps2 and then test to ensure a complete knockout was created.

![Figure 8: Longtine knockout system in yeast. This system was used to knockout Vps2 and then test to ensure a complete knockout was created.](image)

**Figure 8.**

![Figure 9: Agarose gel showing the knockout of Vps in several colonies. The kanamycin resistance gene is 1559 bps long while Vps2 is only 798bp so the difference could be observed.](image)

**Figure 9.**
performed using primers specific to the kanamycin resistance PCR product. ZW7 and ZW10 would amplify the gene locus, providing a longer fragment if the Vps2 gene had been homogously removed with the Kan resistance gene. This is shown in figure 9, the larger bands in this image are the colonies possessing the kanamycin resistance gene while, the shorter fragments are the Vps2 gene. After performing these reactions, the PCR gel showed that 3 of the colonies tested were positive for the kanamycin resistance gene. These could then be checked via western blot to ensure that the knockout was complete.

With these knockouts made, I could begin to answer the question of how this capping protein causes a slight change in ESCRT III function. As discussed previously, Vps2 and Vps24 cap the Snf7 polymer, and this capping dimer recruits the ATPase Vps4 to begin recycling the complex (James Hurley, 2011). In an attempt to understand how Bro1, an effector protein that was shown to stabilize the Snf7 complex (Wemmer M, 2011), might be inhibiting the recruitment function of the capping dimer a co-immunoprecipitation of Vps2-HA was devised. By immunoprecipitating Vps2 while overexpressing the Bro1 domain, I hoped to see altered levels of Snf7 polymer being pulled down. These constructs were transformed into, pep4Δ prb1Δ Vps2Δ and pep4Δ prb1Δ Vps2Δ Vps4Δ, which are two yeast strains created specifically for this experiment. In figure 10 you can see the proof of concept for this experiment where Snf7 CO-IP is being performed when I pull down Vps2-HA. The presence of Snf7 in immunoprecipitation lanes with bands at 37Kd shows that a co-immunoprecipitation occurred when Vps2-HA was pulled down. Figure 10 shows the difference in

**Figure 10**: Western blot of Snf7 and HA showing the levels of expression of Snf7 when Bro1 is overexpressed.
expression of Snf7 when the Bro1 Domain is overexpressed. When overexpressed, there is more stabilization of the Snf7 polymer and Snf7 is able to be pulled down. When the BOD domain is overexpressed we could see similar levels of Snf7 in both of the yeast strains, but a larger amount may be present in the strain lacking Vps4 due to disassembly of Snf7 being inhibited. Moving forward efforts to pull down Vps2-HA in both overexpression and endogenous expression backgrounds to compare the levels of Snf7, but issues with these experiments have made the data un-conclusive. When comparing these two expression levels of Bro1, the endogenous and the overexpression, I will have a very clear picture of the effect of Bro1 on Snf7 stability.

**pGO780 and pGO781 Plasmid Creation**

The ESCRT pathway has been well studied for the past 10 years, but there are still many aspects of this fascinating pathway that have yet to be explored. As stated previously, the goal of this thesis was to understand the interactions of the Vps2 and Vps24 protein dimer and how it interacts with the Bro1 and the Snf7 polymer. It has been previously shown that Vps4 localizes to Snf7 both independently and via assistance from the capping dimer (Hurley, 2011). Previous work showed the increase of Snf7 polymer formation in the presence of the BOD, but there are still more questions to be answered.

In an attempt to further delve into this aspect of the ESCRT pathway 2μ high expression vectors needed to be created that contained Vps24 only, and Vps2 and Vps24 together. Creation of these constructs began with designing oligonucleotide primers to amplify these genes out of genomic yeast DNA. For the creation of pgo780 both of these amplified elements were inserted into the empty pRS vector, pRS426 which is a Uracil Section- 2μ plasmid. The Vps2 gene was inserted into the SacI site and the primer design allowed for reconstruction of the SacI site in the event it was needed for future experiments. As you can see from figure 1, the intron in Vps2 was included in the creation of this plasmid.
Also inserted into this same vector was the Vps24 gene that was amplified with primers ZW13 and ZW14. After cutting the now inserted Vps2 plasmid with Kpn1, the amplified Vps24 gene was cloned into this cut site. Again, this cut site was regenerated in the event it would be needed for future experiments.

These two plasmids were checked for proper gene insertion by using the same primer sets to amplify the now inserted gene out of the plasmid. The primer pairs of ZW7p and ZW10p were used for Vps2. While the primer pair of ZW13p and ZW14p was used to amplify Vps24. The resulting amplification of these two genes out of plasmid is shown below in figure 2. This figure contains an 8% agarose and 3% ethidium bromide gel that has been loaded with two PCR reactions using the described primer sets from above. The first 2 bands after the base pair ladder are the Vps24 gene amplification primers, followed by the next two bands which are the Vps2 amplification primers.
Recovery of Vps24Δ Mutants Using pGO781

To test the effectiveness and functionality of pGO781, a yeast strain containing a Vps2ΔVps24Δ double mutation (GOY184) were transformed with this newly created 2μ Vps24 overexpression vector. GOY184 and a wild type strain SEY6210 were transformed with pGO781 (Vps24 overexpression) and pgo518 (Vps2 overexpression). The resulting yeast strains were subjected to western blot analysis using a αVps2 and αVps24 primary antibody. As a positive control, a previously designed vector for Vps2 overexpression was used. From this experiment you can see the Vps2 vector rescues the expression of Vps2 in the GOY184 strain and is also overexpressed in wild type. This can be concluded due to the dark bands present at 37kD, which is the molecular weight of both Vps2 and Vps24. Also, the bands present in the αVps24 portion of this gel show that the created Vps24 plasmid rescues Vps24 and produces a larger quantity in the wild type lysate. This experiment confirms the creation of this plasmid and its ability to overexpress Vps2.

Figure 13: Western blot showing the recovery of Vps24 expression with pgo781 in Vps24 knockout yeast strains.
Rescue of GOY184 with pGO780

When confirming the creation of pGO780 (the 2μ Vps2 and Vps24 expression construct), the different isolated constructs were transformed into GOY184 (Vps2Δ Vps24Δ). As a positive control both SEY6210 lysates and GOY184 transformed with a pGO518 (2μ Vps2) were included in this western blot analysis. In figure 4, you can see the 3 controls in the first 3 lanes and then the 6 different constructs isolated from a bacterial purification of pGO780. The presence of a large overexpression band in the GOY184 positive control (and this band absent in the negative control lane at 37 Kd) shows that our controls are working. The 3 other bands in lanes 7, 8 and 9 at 37kd are all yeast expressing pgo780. We can, therefore, conclude that the Vps2 expression portion of pGO780 is incorporated correctly. The 7th band colony in the western blot analysis was purified from the original bacterial colony and saved as a glycerol stock for later use. The difference in the bands between pgo518 expression and pgo780 expression could be due to the size of the vectors and the rate at which they are expressed and copied.
**Effect of Sna3 sorting with overexpression of Vps24**

With the newly created overexpression plasmids of Vps24, a question was posed on the impact on Snf7 polymerization when the Vps24 capping element was overexpressed. To test the effect of this capping protein we used Sna3-GFP. This membrane protein is trafficked through the ESCRT pathway of yeast to be degraded in the vacuole (Zastrow, 2009). In the event of an early capping of the ESCRT III complex, mis-sorting at the endosomal membrane would occur, and this protein would not be targeted to the vacuole for degradation. Therefore, we can study the impact of membrane protein sorting when using this GFP-tagged protein. Yeast strains MWY25 and MWY24 were transformed with Sna3-GFP and the Vps2 and Vps24 overexpression plasmid. You can see in the MWY25 and GOY65 lanes a stronger expression of the GFP protein correlating to more mis-sorted Sna3-GFP protein. This shows that, in an event were capping proteins are overexpressed, we see a slight change in ESCRT sorting patterns due to the increase of GFP expression since Sna3-GFP is not being degraded and can be measured via western blot.

**In Vivo Imaging of Snf7 Complex with GST/GFP Immunoprecipitation**

The Snf7 polymer is a very flexible and transient structure. Therefore the visualization of this complex with x-ray crystallography cannot be performed. Previous studies have shown the polymerization and disassembly of this complex using isolated protein and electron microscopy to view the Snf7 polymer in an in vitro setting (Shaogeng Tang, 2015). Ideally an in vivo image of nascent proteins would be the most ideal for understanding how the ESCRT III complex functions. The goal of this
experiment was to visualize the endogenous state of the ESCRT III complex using negative stain electron microscopy.

The yeast strains used for this experiment were DMY1, DMY2, and MBY3. The two DMY strains both have Bro1 with an integrated GFP tag, which we can use GFP specific nanobody for immunoprecipitation. The nanobody can still be isolated using glutathione sepharose beads and elution from the bead8s can be done using an excess glutathione solution to replace the protein bound nanobody. Figure 15 shows the proof of concept experiment that was performed. The top bands are the GFP protein expressed in the protein isolates. The presence of Snf7 on the glutathione bead fractions in this figure lead me to believe that a small amount of Bro1-GFP is needed to pull down the Snf7 complex. With this valuable information, I can begin to optimize this reaction so that we can pull down large quantities of the Snf7 complex. These will then be subjected to negative stain electron microscopy for visualizing the Snf7 polymer in its native state.
Discussion & Future Directions

The ESCRT pathway is a crucial element to the health and longevity of any cell or organism. Manipulation and remodeling of the plasma membrane is important so cells can adapt and modify their extracellular receptors to respond to new stimuli and growth factors. By understanding how the ESCRT machinery assembles and disassembles, we can begin to apply this knowledge to elucidating novel therapies and solutions to diseases that are caused by mutations in this pathway. Although overexpression of the capping dimer does not specifically alter the ability of ESCRT III, you can see from the data that it modifies the levels of trafficked proteins slightly. These small buildups of protein concentrations in the endosome could eventually lead to further inhibition of the endocytic pathway. Future experiments to further understand this interaction would be using the double expression plasmid of Vps2 and Vps24 to see how overexpression of both of these capping proteins together affects protein sorting. Additionally, the use of a high-speed centrifugation and sucrose gradients to see how the levels of cytosolic Snf7 compare to the heavier Snf7 polymer could give us important information on how this cap functions. Binding Snf7 polymers prematurely can lock Snf7 in a polymer state and inhibit sorting or cap the polymer and target it for recycling to cytosolic monomers. This would provide an excellent insight into the interaction between the capping dimer and ESCRT III function.

The interplay between the capping dimer of Vps2 and Vps24 and their possible antagonist Bro1 is vital to understanding the dynamic nature of ESCRT III. If you could control the polymerization and functionality of Snf7, ESCRT III targeted protein degradation could occur more efficiently. From the data, you can see that, when pulling down Vps2-HA, Snf7 is co-immunoprecipitated. This interaction shows how the capping and stabilizing the Snf7 polymer is important to the function of ESCRT III. Throughout the yearlong attempts to pull down Vps2-HA, several different approaches were used. The first approach used an integrated HA tag that was not expressing properly in the different yeast strains. The preliminary findings were drawn from a Vps2 knockout strain with a transformed Vps2-HA plasmid. Moving forward, attempts to understand this interaction would be to integrate a new Vps2-HA into the yeast genome and see how
the Snf7 polymer is co-immunoprecipitated. Another avenue would be using a different tag, such as a Flag tag, to try and pull down the Vps2 protein and look for a co-immunoprecipitation of Snf7. By further studying the interaction of Bro1 with Sfn7 we can continue elucidate how the Sfn7 polymer is maintained and manipulated to finalize the ILV formation process.

Understanding of how the different elements of the ESCRT III complex interact lacks one crucial piece of evidence about how Sfn7 complex orients itself in a 3 dimensional space. The pulldown of Bro1-GFP has been shown to be possible, but the next step needs to be optimizing the experiment to produce the largest amount of the Sfn7 polymer. The purpose of using negative stain EM would be to make the Sfn7 polymer stand out amongst the background when visualizing the polymer. The applications and implications of the ESCRT pathway are just beginning to be elucidated. Interactions between Vps2, Vps24, and Bro1 might create different Sfn7 conformations. As we continue to understand how this dynamic system works and what we can do to manipulate it, these manipulations could lead to better treatments of human diseases associated with ESCRT mutations and also new technologies that can improve scientific research in ways we cannot foresee.
Works Cited


