ANALYSIS OF THE TRANSPORT OF *SACCHAROMYCES CEREVISIAE* CHITIN SYNTHASE 3 BY THE EXOMER SECRETORY VESICLE CARGO ADAPTOR

A Dissertation

Presented to the Faculty of the Graduate School

of Cornell University

In Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

by

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August 2014

ANALYSIS OF THE TRANSPORT OF *SACCHAROMYCES CEREVISIAE* CHITIN SYNTHASE 3 BY THE EXOMER SECRETORY VESICLE CARGO ADAPTOR

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Cornell University 2014

A major feature of a eukaryotic cell is its ability to compartmentalize its functions by sequestering components into distinct membrane-bound organelles. Since membrane-embedded proteins cannot diffuse through the cell to travel between organelles, they must be sorted into transport vesicles, typically by coat complexes and their adaptors. The transmembrane protein Chitin Synthase 3 (Chs3) in *Saccharomyces cerevisiae* is an excellent model system to study some of these complex sorting and transport processes. Chs3 cycles between specific locations on the plasma membrane (PM) where it synthesizes chitin for the yeast cell wall, and retention in *trans*-Golgi network (TGN) compartments where it is inactive.

Exomer, a novel protein complex found in fungi, acts as an adaptor complex for the transport of Chs3 and several other proteins from the TGN to the PM. The exomer complex is composed of the core subunit protein Chs5 and paralagous adaptor proteins known as ChAPs (Chs5-Arf1-binding Proteins), and is recruited to the membrane by the small GTPase Arf1. I have determined the minimal functional fragment of Chs5, which I have shown interacts with Arf1, likely contributing to exomer recruitment. The ChAPs are responsible for binding cargo, and Chs6 is required for transport of Chs3. Therefore, I examined Chs6 protein levels throughout the cell cycle and incorporation into complexes. When Chs6 levels were held constant by replacing its promoter with another, there was an effect on Chs3 transport only in one yeast

background, indicating this regulation is only required under certain conditions.

I also show that different segments of the Chs3 N-terminus mediate distinct trafficking steps. I present a crystal structure of residues 10-27 bound to the exomer complex, which are residues known to mediate retention and also seem to play a role in internalization. Residues 28-52 are involved in transport to the plasma membrane and recycling out of the endosomes to prevent degradation. Together, these findings contribute to our understanding of how proteins are transported by exomer, and how cycling of a transmembrane protein can be regulated.

BIOGRAPHICAL SKETCH

Amanda was born in Rochester, New York to parents Sandra and Mark. She grew up in the suburban town of Hilton. Amanda had an early interest in nature and how things work. She rediscovered this love for science in high school biology class, and while exploring college options before she graduated in 2002, she learned there were opportunities to combine this with her love of computer programming and mathematics.

At Rochester Institute of Technology, Amanda majored in bioinformatics. Her first research experience was in the lab of Irene Evans, using microarrays to study gene expression in yeast. Amanda entered the combined B.S./M.S. degree program, and did her Masters research in the lab Suzanne O'Handley. The focus of the laboratory was biochemistry, specifically bacterial enzymology. Amanda soon discovered she preferred being at the lab bench purifying proteins and assaying enzymatic activity, over sitting in front of a computer analyzing sequences. Her mentor Dr. O'Handley was understanding enough to nurture this desire.

After graduating with highest honors and completing her Masters research, Amanda decided she would continue her education by earning a Ph.D., this time in the field that had truly captured her heart: biochemistry. She settled on Cornell University, where she developed a strong interest in cell biology, specifically membrane trafficking. Amanda joined the lab of Chris Fromme, where she has studied the exomer complex, and trafficking of Chs3. Ithaca has brought Amanda new friends, new hobbies, and a new husband. However, she is eager to find a postdoctoral position and continue the adventure, wherever it may be.

To Jake – and a dissertation was formed!

ACKNOWLEDGMENTS

First I would like to thank my advisor, Chris Fromme, for his support and mentorship. Chris has perfectly balanced providing guidance and allowing me to solve problems independently. I am a better scientist, better writer, and better thinker because of his help. I also thank my other committee members: Scott Emr, who has highlighted the importance of imagination and visualization in science, and Ruth Collins, who has always cared about my development as a scientist and as a person. Many other scientists in the Weill Institute for Cell and Molecular Biology have been excellent sources of advice, collaboration, and friendship.

I feel honored to be one of the first few members of the Fromme lab, and to help establish an environment for all of us to learn and develop as scientists. I would like to thank all of the members of the lab: Jon Paczkowski who has been my colleague in studying exomer, Brian Richardson who has always provided excellent advice, and the moral support from Caitlin McDonold and Maggie Gustafson. Thank you to all current and previous undergraduates, especially Jodie Chang and Bertheleau Ngakam for giving me the chance to share my skills with the next generation of scientists.

Science cannot occur without the very practical components of facilities and funding. Therefore, I thank the staff of the Cornell High Energy Synchrotron Source (CHESS) specifically the Macromolecular Diffraction (MacCHESS) facility, for help with structure determination. I also thank the Emr and Bretscher labs for use of their microscopes, and the Smolka lab for the use of their mass spectrometry equipment. Funding for the work described was provided by a NIH training grant (T32GM07273) and startup funds from Cornell University to Chris Fromme.

Finally, I thank my friends and family. I credit my parents, Mark and Sandy, and my late grandmother, Janet, for raising me with a love of learning. Thanks to my siblings, extended family, and in-laws. I also thank my former in-laws in the Strassner family who continue to support my efforts, especially my “honorary mother-in-law” Karen. Finally, my deepest gratitude to my husband, Jake, the most loving and helpful partner I could imagine.

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CHAPTER 1

Introduction

1.1. Membrane trafficking in eukaryotes

All life on earth is made up of cells, but cells are far from static building blocks. Cells are dynamic, active, and complex. They constantly create, destroy, modify, and move their components as necessary. Eukaryotic cells organize themselves by sequestering materials and functions into membrane-bound compartments called organelles. Many proteins and lipids are synthesized in one organelle, reside in another, and eventually need to be degraded in yet another. This necessitates transport between organelles (Figure 1.1). For lipids and proteins that are associated with membranes, this is achieved primarily by small membrane-bound carriers called vesicles, which will be discussed further, and sometimes by extended membrane protrusions known as tubules. One could imagine the constant flow of material would eventually lead to homogeneity among the organelles. However, cells maintain organelle identity through careful sorting and regulation. Several common routes of transport within the cell have been well characterized, although within all of these routes, there are many questions left to be answered. Since these transport processes are often conserved among all eukaryotes, many insights can be gained by studying them in single-celled organisms that are easily manipulated. The yeast *Saccharomyces cerevisiae* has been used as a model organism for many years, and tools have been developed to allow for easy genetic manipulation.

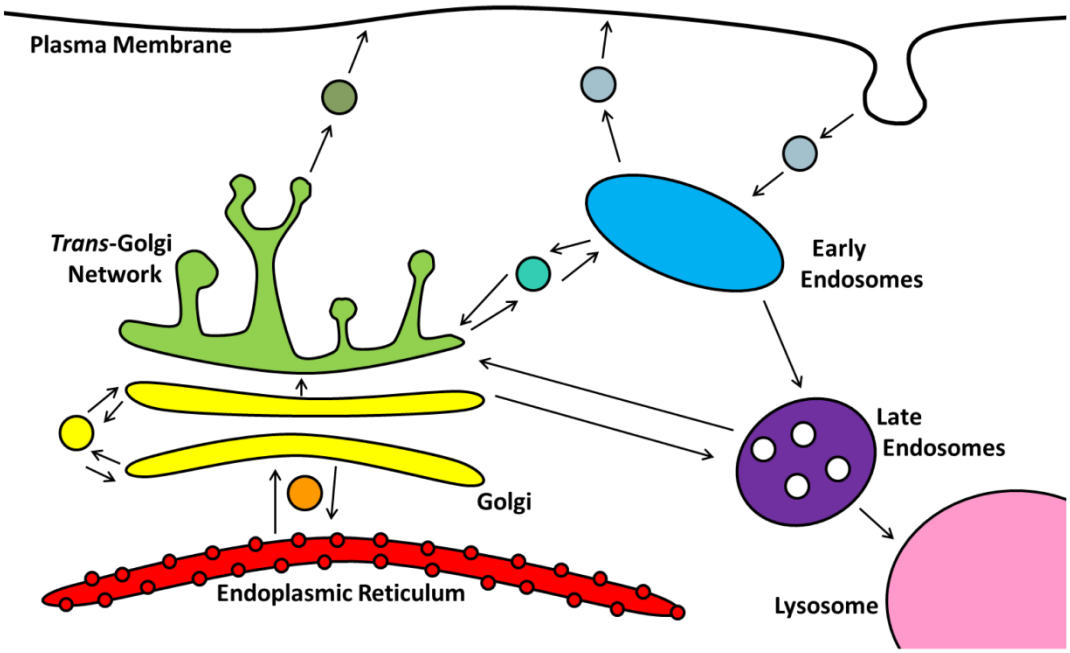

Figure 1.1: Membrane trafficking. A simplified schematic of membrane trafficking in eukaryotic cells.

1.2. The secretory pathway

The series of organelles through which proteins pass on their way to reach the cell surface is known as the secretory pathway. This is not to say only proteins that will be released into extracellular space follow this pathway – proteins destined for many compartments within the cell follow this route, in whole or part. During or after synthesis, proteins destined for transport to the cell surface are translocated into the lumen of the ER or its membrane. They then are incorporated into vesicles at ER exit sites (ERES) and transported to the dynamic cluster of tubular and vesicular compartments known as the ER-Golgi intermediate compartment (ERGIC) before continuing on to the Golgi (Lee et al., 2004; Appenzeller-Herzog & Hauri, 2006). The Golgi in mammalian cells appears as stacks of flat compartments known as cisternae, while in *S.*

cerevisiae the "stack" formation is less apparent and there is no ERGIC compartment. The termini of the Golgi are named from the point of view of the ER: new secretory cargo enter at the *cis*-Golgi and eventually reach the *trans*-Golgi. Whether proteins move through the stacks or the stacks mature from *cis* to *trans* has been a subject of debate (Glick, 2000; Lavieu et al., 2013; Rizzo et al., 2013), but either way, proteins traversing the secretory pathway eventually reach a sorting station at the end of the Golgi known as the *trans*-Golgi network (TGN).

A subset of the proteins that reach the TGN are sorted into secretory vesicles, and carried to the plasma membrane. Secretory vesicles deliver integral membrane proteins to the PM, and release soluble materials within the vesicle to the outside of the cell. Other proteins that reach the TGN are sorted into vesicles bound for compartments within the endosomal system. Yet others are directed back into the Golgi. Even within the population of secretory vesicles destined for the PM, there have been two varieties identified in yeast, each of which carries a different subset of secretory cargo (Harsay & Bretscher, 1995). In mammalian cells there is additional complexity since many cell types are polarized. Different vesicles are formed to transport proteins to the apical or basolateral domains of cells, and the detailed mechanisms can vary between cell types (Weisz & Rodriguez-Boulan, 2009). In yeast, polarization of secretion is much simpler: cells reorient their cytoskeleton to direct secretion toward the tip of new buds, and toward the mother-bud neck just before cytokinesis (Pruyne & Bretscher, 2000).

1.3. The endocytic pathway

The retrieval of proteins and lipids from the PM, as well as soluble extracellular material, is described as the endocytic pathway. Endocytosis serves a few major functions in the cell. One of these functions is downregulation of proteins that are active at the PM. Endocytically

downregulated membrane proteins in yeast include transporters like the amino acid permease Gap1 and receptors like the mating pheromone receptor Ste2 (Hein & André, 1997; Dunn & Hicke, 2001). Another function of endocytosis is to maintain polarization of proteins within the PM. Proteins delivered to a specific region, such as the bud tip, will diffuse throughout the PM. If this diffusion were allowed to continue unimpeded, the protein would eventually be distributed along the entire surface of the cell. When cells must maintain a more precise localization, the protein must be actively endocytosed from regions where it is not required. For example, when endocytosis is disrupted in actively growing yeast cells, they fail to properly maintain the markers that guide bud site selection (Tuo et al., 2013).

Newly endocytosed material enters compartments known as early endosomes or sorting endosomes, and from these compartments there are many pathways a protein can take. If it is destined for degradation, the protein travels to the late endosome. The membrane of the late endosome buds into the interior of the endosome to form a multivesicular body (Hurley, 2008). This compartment fuses with the lysosome, which is called the vacuole in yeast, where its contents can be degraded (Futter et al., 1996). However, proteins that make it to late endosomes can still be retrieved back to the Golgi. Some proteins in yeast that are transported from the late endosome to the TGN require retromer. The retromer complex is conserved between mammalian and yeast cells, and recycles proteins from early endosomes in yeast back to the TGN. Retromer is composed of two subcomplexes: the sorting nexin dimer containing proteins Vps5 and Vps17, and the cargo-selective subcomplex containing Vps26, Vps29, and Vps35 (Seaman, 2012). Most endocytosed proteins that are not meant to be degraded never reach the late endosome. In mammalian cells, they can recycle quickly from the early endosome, or more slowly through a compartment known as the endocytic recycling compartment or recycling endosome (Hao &

Maxfield, 2000). The recycling endosome is a sorting station not unlike the TGN in its ability to direct proteins to several destinations (Maxfield & McGraw, 2004). There is no evidence of a recycling endosome in yeast, however, and the sorting that normally occurs there seems to be carried out at the early endosome itself. The sorting nexin proteins Snx4, Snx41 and Snx42 are required for the transport of some proteins from the early endosome in yeast to the TGN (Hettema et al., 2003). Though several pathways out of the endosomal system have been discussed, there are many more proteins involved in directing traffic through, and out of, the endosomal system. In addition, many of these pathways compensate for each other when disrupted, allowing cargo protein to reach its destination even if it requires taking a nontraditional route.

1.4. Vesicle formation and coat protein complexes

Some of the membrane trafficking in cells is carried out by membrane tubules (Lippincott-Schwartz et al., 2000), but the majority is conducted by vesicles. Vesicles are small, spherical membrane enclosed compartments. Vesicles can simultaneously carry soluble proteins, metabolites, or other materials in their aqueous interior, and membrane-associated proteins and lipids in their membrane. Vesicles are directed to their target compartments by SNARE proteins. SNAREs on the vesicle are matched with SNAREs on the target membrane that promote fusion of the two membranes (Chen & Scheller, 2001). Since I am interested in how membrane protein transport is signaled and initiated, I will focus on the formation of vesicles and not their targeting and fusion.

Formation of a transport vesicle requires bending of the membrane into a vesicle and gathering of the cargo to be transported. The budding of a vesicle cannot happen spontaneously,

as it requires significant deformation of the lipid bilayer into a highly-curved structure, which is energetically unfavorable. Coat complexes can provide the energy by forming curved oligomers that interact with the membrane (Farsad & Camilli, 2003). This is often accomplished by the outermost layer of a coat. The inner layers, or sometimes interchangeable adaptor protein complexes, select the cargo to be packaged into the vesicle by a direct interaction. Recruitment of the coat itself is sometimes accomplished by small GTPases. Small GTPases act as molecular switches that are "on" when bound to GTP, allowing effectors to interact with them, and "off" when bound to GDP (Cherfils & Zeghouf, 2011).

The coat protein complex II (COPII) creates vesicles at the ER that are transported to the ERGIC or the Golgi. Following activation by the guanine-nucleotide-exchange factor (GEF) Sec12, the small GTPase Sar1 associates with the membrane and recruits the inner coat layer, the Sec23-Sec24 subcomplex (Barlowe & Schekman, 1993). Sec23 is a GTPase activating protein (GAP) for Sar1, enhancing its GTP hydrolyzing activity (Yoshihisa et al., 1993). Sec24 interacts with the cargo proteins to sort them into the nascent vesicle (Miller et al., 2002). This inner coat recruits the outer coat, made up of Sec13 and Sec31. The Sec13/31 coat oligomerizes into a cage, bringing multiple Sar1-Sec23/24-cargo complexes together and contributing to membrane deformation (Fath et al., 2007).

The COPI complex, also known as coatomer, follows a similar pattern of vesicle formation as COPII. This coat mediates transport from the *cis* Golgi to the ER, to retrieve resident ER proteins, give misfolded proteins a second chance, and move proteins to earlier Golgi cisternae. COPI is recruited by the small GTPase Arf1 which, like Sar1 in COPII, binds the membrane only in its active form. Assembly also requires the GAP for Arf1, ARFGAP1, whose homologue is Gcs1 in yeast (Yang et al., 2002). COPI is made of five proteins assembled

into two subcomplexes, and those subcomplexes have been named B-COP and F-COP (Fiedler et al., 1996). F-COP shares similarity with adaptor complexes for other coats. However, unlike COPII, these subcomplexes do not assemble sequentially, but rather simultaneously (Lee et al., 2004).

A third well-studied coat is clathrin. Clathrin is composed of a heavy chain and light chain that form a triskelion shape, and these triskelions assemble into higher order cage-like structures (Wilbur et al., 2005). Unlike COPII and COPI, however, clathrin coats can be recruited by a number of adaptors, and traffic proteins from several locations in the cell. There are three adaptor protein (AP) complexes conserved in all eukaryotes: AP-1, AP-2, and AP-3. Mammals (and some other types of organisms) also have a fourth, AP-4, but it is absent from yeast (Boehm & Bonifacino, 2001). These adaptor complexes each are made up of two large subunits, one medium subunit, and one small subunit. AP-1, which will be discussed further for its role in Chs3 trafficking, is recruited to membranes by the small GTPase Arf1 which causes a structural rearrangement in AP-1 that allows it to bind cargo at the TGN and endosomes (Ren et al., 2013). AP-2 is the adaptor responsible for clathrin-mediated endocytosis, and is recruited by the membrane itself. AP-2 binds the phospholipid $PI(4,5)P_2$, promoting an open conformation that can bind cargo at the PM (Collins et al., 2002). AP-3 transports cargo from the TGN and possibly endosomes directly to the lysosome, and while it can recruit clathrin, some of its functions may not require clathrin at all (Lewin & Mellman, 1998).

In addition to the AP complexes, there are other proteins capable of acting as clathrin adaptors. The GGA proteins, Gga1 and Gga2 in yeast, are monomeric clathrin adaptors that mediate trafficking from the TGN to the endosomes (Boehm & Bonifacino, 2001). While this seems to overlap with the function of AP-1, the GGAs may transport their cargo to later

endosomal compartments (Black & Pelham, 2000). The proteins Ent3 and Ent5 were initially discovered as accessory proteins that bind to AP-1 and the GGAs and are required for normal CCV formation at the TGN and endosomes, but they are also capable of interacting with clathrin themselves (Duncan et al., 2003). All of these examples highlight the common features of coat complexes: adaptor complexes, often recruited by GTPases or the membrane itself, bind to cargo and recruit cage-like coats to deform the membrane and cluster cargo proteins.

1.5. Cargo sorting signals

Adaptor proteins and complexes identify their cargo by conserved sorting signals. The clathrin adaptors bind several conserved motifs in their cargo proteins. These can be separated into signal sequences containing tyrosine, and those containing a pair of leucine residues (Bonifacino & Traub, 2003). The motif YXXφ was initially identified in the cation-independent mannose-6-phosphate receptor (MPR) (Canfield et al., 1991). The medium-sized subunits of all of the the AP complexes can bind the YXXφ sequences, although the residues surrounding the motif can lead to preferential binding to specific AP complexes (Ohno et al., 1995; Ohno et al., 1998; Aguilar et al., 2001). Another tyrosine-containing motif, NPXY, was discovered for its role in internalization of the LDL receptor and binds specifically to AP-2 (Chen et al., 1990; Boll et al., 2002). One type of dileucine motif, [D/E]XXXLL, was identified in a chain of the T-cell receptor as a sequence that drives internalization (Letourneur & Klausner, 1992). Depending on the surrounding residues, it can be recognized by AP-1 and AP-2, or by AP-3 (Höning et al., 1998; Hofmann et al., 1999). The other dileucine motif is recognized by the GGA proteins, and contains the residues DXXLL (Bonifacino, 2004).

Many of the transmembrane proteins that reside in the ER contain KKXX motifs (Lee et

al., 2004). This sequence is bound by COPI to mediate retrieval of these proteins to the ER from the Golgi (Cosson & Letourneur, 1994). COPII sorting signals are far less simple than the others. There are multiple sorting signals that have been shown to COPII to allow exit of cargo from the ER (Barlowe, 2003). These include, but are not limited to, a di-acidic [D/E]X[D/E] motif as found in VSV-G, and a di-aromatic FF, FY, or YY like the FF found in ERGIC53 (Nishimura & Balch, 1997; Kappeler et al., 1997). Having many different sorting signals that can mediate the same or similar transport routes could allow for differential regulation. It is not surprising that sorting signals can be so complicated, as membrane trafficking is so complicated.

1.6. Cycling as a regulatory mechanism

Some membrane proteins employ all of the pathways described previously – the secretory pathway, endocytosis, and recycling – to cycle between multiple locations in the cell. One example is the GLUT4 glucose transporter protein. This protein is found in adipocytes and skeletal muscle, and when located at the PM, allows glucose to enter the cell (Birnbaum, 1989; James et al., 1989). Mice that are heterozygous for a mutation disrupting the *GLUT4* gene develop phenotypes similar to humans with type 2 diabetes mellitus (Stenbit et al., 1997). Therefore, it is of interest both as a model system to study transport of transmembrane proteins, and as a medically relevant protein whose regulation could become a therapeutic target.

Under normal conditions, very little GLUT4 is located at the cell surface, but upon insulin signaling, it is rapidly transported to the PM (Figure 1.2) (Bryant et al., 2002). This transport is the result of signaling cascades activating the molecules involved in transport, such as Myosin 5a which moves compartments along actin toward the PM (Yoshizaki et al., 2007). Most of the internally stored GLUT4 is in unique membrane compartments called GLUT4-

storage vesicles (GSVs), but in cells not stimulated with insulin, approximately 40% GLUT4 is also seen throughout the endosomal compartments (Martin et al., 1996). This suggests GLUT4 traverses the endocytic pathway, rather than the GSVs being derived directly from the PM.

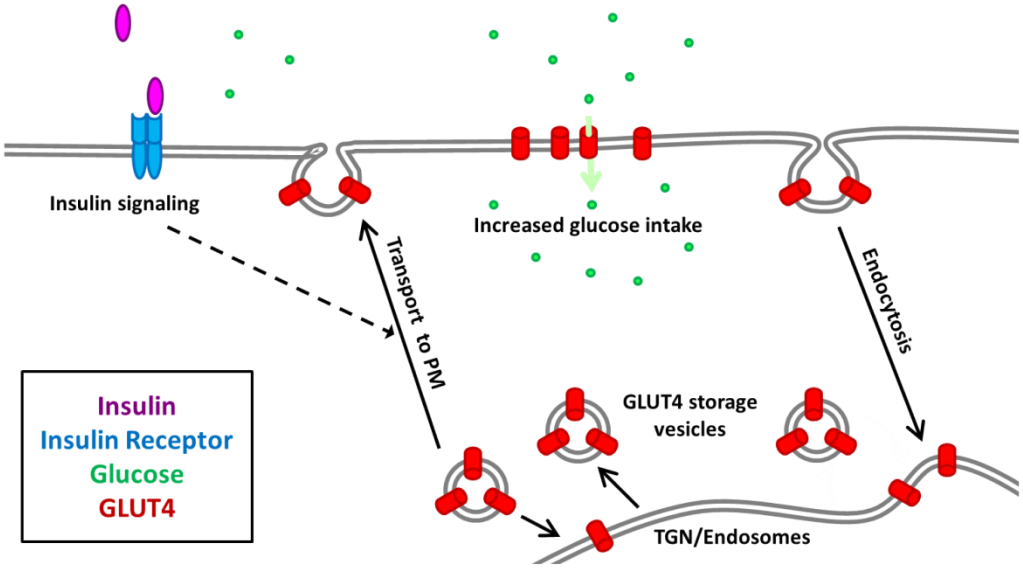

Figure 1.2: GLUT4 cycling. Trafficking of the GLUT4 glucose transporter between the plasma membrane and GLUT4 storage vesicles.

Several targeting motifs have been identified in GLUT4, located at both the N- and C-termini of the protein, and directing several different trafficking steps. At the N-terminus, there is a well-studied FQQI motif. This motif is required for efficient endocytosis of GLUT4 (Garippa et al., 1994), and also for transport from endosomes into GSVs (Melvin et al., 1999). At the C-terminus, there are two required motifs: a pair of leucines, and a longer TELEYLGP sequence. Mutation of the dileucine motif was shown to affect transport out of the TGN (Melvin et al., 1999), but also internalization from the PM (Garippa et al., 1996). Residing just downstream

from the dileucine motif, the TELEYLGP motif was required for transport out of endosomes (Shewan et al., 2000). This system of multiple sequences each mediating multiple steps in GLUT4 trafficking make it clear how complicated it can be for cells to execute the precise cycling of some proteins.

1.7. Chs3 trafficking and the exomer complex

Another protein with such a pattern of cycling between the cell surface and internal compartments is Chitin Synthase 3 (Chs3) in yeast (Figure 1.3A). This protein is an enzyme that synthesizes chitin, a polymer of N-acetylglucosamine found in the yeast cell wall. Chitin interacts with the other molecules in the cell wall to create an insoluble barrier against the environment (Hartland et al., 1994). Chs3 is a polytopic membrane protein, and both its N- and C-termini face the cytoplasm (Figure 1.3B) (Sacristan et al., 2013).

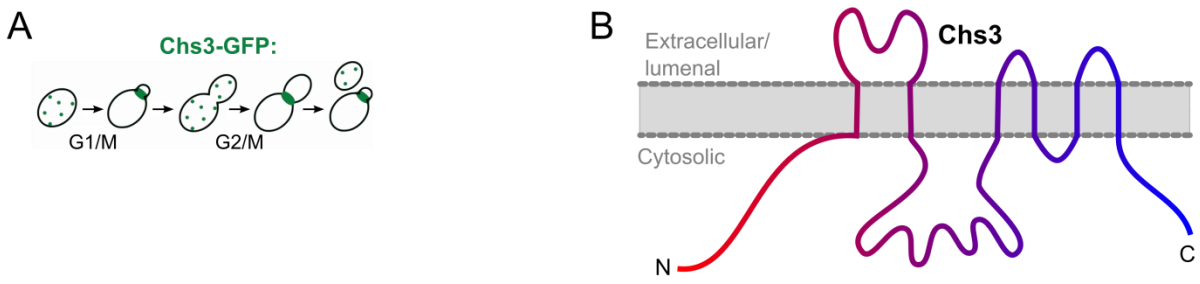

Figure 1.3: Chs3 characteristics. (**A**) Chs3 localization throughout the cell cycle. (**B**) Chs3 topology, as determined in (Sacristan et al., 2013).

Chs3-GFP localizes to the bud site, and remains at the bud neck as a small bud emerges and begins to grow, presumably to help build the cell wall around the bud. It is then endocytosed and retained internally, in multiple small punctae. These compartments were originally referred to as “chitosomes” (Leal-morales et al., 1988) but were later found to correspond to the TGN, as

they colocalize with the TGN marker Kex2 (Santos & Snyder, 1997) . Chs3 remains internal while the bud grows. Just before cytokinesis, Chs3 is again transported to the mother-bud neck, where it might contribute to formation of the primary septum. Immediately after cell division, Chs3 is again endocytosed, and remains at the TGN until the next budding event occurs. The Chs3 localization is not due to rounds of synthesis and degradation – in fact, the protein has a very long lifetime in the cell (Chuang & Schekman, 1996), and therefore undergoes multiple rounds of transport through the secretory and endocytic pathways. Chs3-GFP is not seen in the vacuole under normal conditions.

Chs3 is one of three chitin synthases in yeast. It is the only synthase transported to the new bud at the time of formation. The Chs2 synthase is transported to the bud neck prior to cytokinesis, similarly to Chs3, where it is required to deposit a disk of chitin into the primary septum to allow the cells to separate. However, Chs2 is newly synthesized each cell cycle and stored in the ER until its release is triggered by the mitotic exit network (Zhang et al., 2006). After cytokinesis, Chs2 is endocytosed and travels through the endosomal system to the vacuole for degradation (Chuang & Schekman, 1996). Chs1 is also transported around the time of cytokinesis, where it functions in the daughter cell. Chs1 contributes chitin to rebuild the cell wall at the birth scar, which is weakened after division from the mother cell (Cabib et al., 1992; Powell, 2003). Chs1, like Chs3, is long-lived and cycles between the PM and the TGN (Ziman et al., 1996).

There are several proteins in yeast that appear to be devoted solely to the transport and activation of Chs3. The only known function of Chs7 is that it is required for Chs3 to exit the endoplasmic reticulum (Trilla, 1999). It may be a chaperone responsible for proper folding, but the exact mechanism is unknown. Chs4, also known as Skt5, activates Chs3 enzymatic activity

(Trilla et al., 1997) and influences its localization to the bud neck by interacting with Bni4, a protein that interacts with septins (DeMarini, 1997). A homologue of Chs4, Shc1, also activates Chs3 but is only required during sporulation or in high pH conditions (Sanz et al., 2002; Hong et al., 1999). Despite *CHS3* being a nonessential gene under laboratory conditions, it appears very important to the yeast to regulate the transport of Chs3 carefully.

When Chs3 is not required at the PM, it is retained in the TGN. However, the TGN is a transient structure that seemingly fragments into transport compartments heading toward their destinations. Therefore, retention within the TGN requires active transport of protein out of the TGN to the endosomal system, and back to the TGN by recycling pathways (Lemmon & Traub, 2000). The clathrin adaptor AP-1 complex is required for retention of Chs3. Introducing an AP-1 mutation into a strain lacking Chs6 can restore Chs3 levels at the PM (Valdivia et al., 2002). AP-1 is largely localized to the TGN, where it recruits clathrin to form CCVs that travel from the TGN to early endosomes. In yeast, there is evidence for AP-1 mediated transport both anterograde to the endosomes, and retrograde back to the TGN (Foote & Nothwehr, 2006; Liu & Surendhran, 2008). Therefore, it is possible AP-1 is involved in both directions of transport of Chs3. Both the Gga1 and Gga2 clathrin adaptor proteins, and the epsin-related proteins Ent3 and Ent5, are also required for Chs3 retention (Copic et al., 2007). These proteins are involved in transport from the TGN to late endosomes. It is unknown how Chs3 is retrieved from the endosomes back to the TGN, but it presumably requires one or both of the retromer and Snx4/41/42 recycling complexes discussed previously.

Chs5 and Chs6 were originally identified for their role in transport of Chs3 to the PM, as a deletion of either the *CHS5* or *CHS6* gene trapped Chs3 at the TGN (Santos & Snyder, 1997; Ziman et al., 1998). It was then determined that Chs5 and Chs6, together with Chs6 homologues

Bch1, Bud7, and Bch2, are subunits of a protein complex (Sanchatjate & Schekman, 2006). This complex was named exomer, and was initially referred to as a coat complex (Wang et al., 2006). As described previously, an important feature of coat protein complexes is the ability to deform membranes into vesicles, and exomer lacks this ability. Therefore, it either acts as an adaptor complex, or promotes vesicle formation in a different manner. Chs6, Bud7, Bch1 and Bch2 are paralagous proteins that have been termed Chs5-Arf1-binding proteins (ChAPs) (Trautwein et al., 2006). Chs5 is the core protein of this complex, which dimerizes through its N-terminal domain (residues 1-50). The long alpha-helix just C-terminal of the dimerization domain (residues 50-77) interacts with the ChAP proteins (Paczkowski et al., 2012) creating a heterotetramer with two copies of Chs5 and two ChAP proteins. Each ChAP protein can pull down all of the other ChAP proteins from cell lysates (Trautwein et al., 2006), indicating an individual exomer complex can contain two different ChAP proteins, resulting in a variety of possible conformations (Figure 1.4A). Similar to other coat adaptor proteins discussed above, exomer is recruited to the TGN membrane by the small GTPase, Arf1, and interacts directly with cargo proteins (Figure 1.4B).

There are two other characterized cargo proteins whose transport to the PM is mediated by exomer. One of these is a mating protein, Fus1, which localizes to the shmoo tip upon treatment with mating pheromone (Santos & Snyder, 2003). The ChAPs that mediate transport of

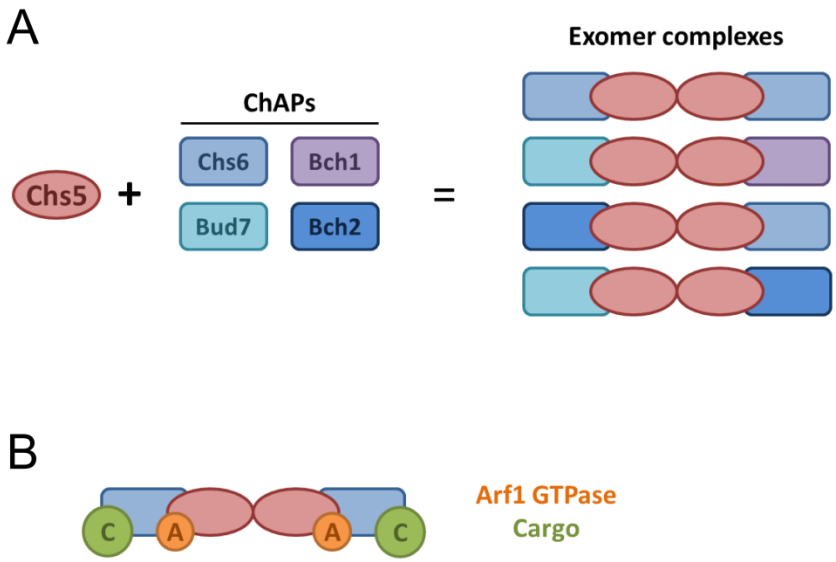

Figure 1.4: The exomer complex. (**A**) Each exomer complex contains two Chs5 subunits, and two of the same or two different ChAP proteins. (**B**) Exomer complex is recruited by Arf1 and binds directly to cargo proteins.

Fus1 are Bud7 and Bch1 (Barfield, 2009). Exomer recognizes an IXTPK sequence in the cytosolic domain of Fus1. The retention of Fus1 requires AP-1, and disruption of this retention will rescue transport to the PM in a strain with exomer mutated, just as with Chs3.

The most recent exomer cargo identified is a prion-like domain-containing protein of unknown function, Pin2. The exomer-mediated transport of Pin2 requires the ChAPs Bch1 or Bch2. As with the other two exomer cargo proteins, AP-1 is required for retention of Pin2. However, there is an additional level of retention of Pin2: in response to environmental stress, the prion-like domains of Pin2 aggregate in the TGN and prevent export of the protein to the PM.

Chs3 makes an interesting model system for studying the cycling of a protein from the cell surface to internal compartments. There are still many mysteries left to solve about its

trafficking, and about exomer in general. If exomer is acting as an adaptor protein, is there a corresponding coat, or another mechanism for generating vesicles? Does regulation of transport to the PM occur at the level of the cargo proteins like Chs3, or at the level of exomer itself? How does exomer transport different cargo at different times? How exactly does Chs3 interact with its corresponding ChAP, Chs6? How is Chs3 recycled from endosomes? Here I present work I have done on many aspects of exomer regulation and function, and Chs3 transport. In Chapter 2, I focus on the exomer core protein Chs5, determining the minimal functional fragment and identifying several other proteins with which this fragment interacts. Chapter 3 is focused on the ChAP Chs6, characterizing the variation in its expression levels and incorporation into complexes throughout the cell cycle. These studies were undertaken in hopes of determining how transport of Chs3 responds to the cell cycle. Chapter 4 examines the interaction between Chs3 and exomer, and identifies regions in the Chs3 N-terminus that mediate its different trafficking steps.

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CHAPTER 2

The molecular architecture and interactions of Chs5[1]

2.1. Abstract

Transport of Chs3 from the TGN to the PM requires the exomer complex. Exomer is composed of the core protein Chs5, and the four interchangeable ChAPs. Different exomer cargo proteins require different subsets of the ChAPs for transport, but all of the exomer cargo require Chs5. I determined that residues 1-274 comprise the minimal functional fragment of Chs5, and determined that this fragment binds to the small GTPase Arf1 to contribute to recruitment of exomer to the TGN. The fragment also interacts with retromer, a complex that I've shown contributes to recycling of Chs3. Unlike Chs3, the two other chitin synthases in *S. cerevisiae* do not require exomer for transport. These results contribute to our understanding of the functions of Chs5 within the exomer complex.

[1] Portions of this work are reproduced from: Jon E. Paczkowski, Brian C. Richardson, Amanda M. Strassner, and J. Christopher Fromme. 2012. "The Exomer Cargo Adaptor Structure Reveals a Novel GTPase-Binding Domain." *The EMBO Journal* 31 (21): 4191–4203. Several experiments performed by J.E.P. and B.C.R. are included for clarification and indicated in the text.

2.2. Introduction

The core of the exomer complex is the protein Chs5. As its name implies, Chs5 was discovered for its role in transport of Chs3 (and therefore chitin synthesis), but it is necessary for transport of all of the exomer cargo. Correspondingly, a deletion of the *CHS5* gene confers all of the phenotypes seen from deletion of each of the ChAPs (Trautwein et al., 2006). Prior to the availability of the crystal structure of exomer, I conducted many experiments to study the functional architecture of Chs5.

Chs5 contains several domains identifiable by homology (Figure 2.1). Near the N-terminus there is a fibronectin type 3 (FN3) domain immediately followed by a breast cancer suppressor protein 1 (BRCA1) C-terminal (BRCT) domain. Found in many types of organisms, FN3 domains are often in pairs or longer arrays of repeats, and most are involved in binding proteins on the cell surface (Campbell & Spitzfaden, 1994). The structure of the FN3 domain is a β sandwich made up of 7 β sheets (Leahy et al., 1992). BRCT domains are also often found in pairs, often in DNA damage repair related proteins (Glover et al., 2004). BRCT domains mediate protein-protein interactions with targets including phosphoproteins (Yu et al., 2003) and other

Figure 2.1: Domain architecture of Chs5. Chs5 domains as predicted by early biochemical studies (for 'O' and 'C') and by sequence similarity. 'O': oligomerization; 'C': ChAP-binding; 'FN3': Fibronectin type 3 domain; 'BRCT': BRCA1 C-Terminal domain; 'PEST': degradation signals rich in proline, glutamate, serine, and threonine; 'K': lysine-rich tail.

BRCT domains (Masson et al., 1998). Thus far, Chs5 is the only non-nuclear protein known to contain a BRCT domain. Taken together, these descriptions make it unclear why either a FN3 or BRCT domain would be present in Chs5. Particularly puzzling is that the two domains are closely apposed, with only a short linker sequence between them. Do these domains promote the same interactions as their counterparts in well-studied proteins, or do they create a yet undescribed structure with its own unique function?

The C-terminal portion of Chs5 is very different. It contains no known structural motifs. It is composed mostly of PEST sequences, and a short conserved lysine-rich segment at the C-terminus. PEST sequences are named for the amino acids enriched within them – proline, glutamate, serine and threonine – and they promote ubiquitin-mediated degradation of the proteins that contain them (Rechsteiner & Rogers, 1996). This portion of Chs5 has little predicted secondary structure, implying it may be a flexible, unstructured region. Unstructured proteins are difficult, often impossible, to crystallize. We predicted it would be beneficial for our efforts to obtain a crystal structure of exomer to truncate the Chs5 protein to its minimal functional portion, reducing the amount of unstructured protein.

The most well-understood exomer cargo, and the focus of most of the research described here, is Chs3. Chs3 is one of the three chitin synthase enzymes found in *S. cerevisiae*. Chs1 trafficking is similar to that of Chs3 in that it is a long-lived protein that cycles between the PM and the TGN (Ziman et al., 1996). However, it localizes to the PM at a different point in the cell cycle, synthesizing chitin after cell separation to replace that digested by chitinases (Cabib et al., 1992). Chs2 is transported to the bud neck of large buds just before cytokinesis concurrently with Chs3, but not transported to small buds like Chs3 is. Chs2 is degraded after each cell cycle, in

contrast to Chs3 (Chuang & Schekman, 1996). It was not known whether either Chs1 or Chs2 require exomer for trafficking, or interact with exomer components.

In this work, I determine the minimal functional fragment of Chs5, which allowed for more appropriate constructs to be used in determining the crystal structure of exomer. I also examined some of the interactions between Chs5 and its regulators. The FBE domain was found to interact with the small GTPase Arf1 in a nucleotide-independent manner, and to interact with the retromer complex. In addition, Exomer was determined not to be required for transport of the other chitin synthases, Chs1 and Chs2.

2.3. Methods

Plasmids, strains, and antibodies

See Tables 2.1 and 2.2 for a list of plasmids and yeast strains used. The CHS5 gene used as a source for all constructs was derived from the SEY6210 yeast strain. The SEY6210 Chs5 ORF sequence differs slightly, in both sequence and length, from that of the S288C yeast strain used for the published genomic sequence. Antibodies were used against Myc (Sigma), Arf1 (gift from Randy Schekman), G6PDH (Sigma), Chs3 (Schekman lab).

Table 2.1. Yeast strains used in Chapter 4.

Name	Genotype	Source
SEY6210	MATα his3-Δ200 leu2-3,112 lys2-801 trp1-Δ901 ura3-52 suc2-Δ9	(Robinson et al., 1988)
SEY6211	MATa his3-Δ200 leu2-3,112 lys2-801 trp1-Δ901 ura3-52 suc2-Δ9	(Robinson et al., 1988)
CFY264	SEY6211 chs5Δ::KanMX Chs3-GFP::His3	(Paczkowski et al., 2012)
CFY885	SEY6211 chs5Δ::KanMX Sec7-Mars::Trp1	(Paczkowski et al., 2012)
	SEY6210 Vps5Δ::His3	Emr lab
	SEY6210 Vps26Δ::His3	Emr lab
	SEY6210 Vps29Δ::His3	Emr lab
	SEY6210 Vps35Δ::His3	Emr lab
CFY247	SEY6211 chs5Δ::KanMX	(Paczkowski et al., 2012)
CFY613	SEY6211 chs5Δ::KanMX Chs2-GFP::Trp1	This study
CFY614	SEY6211 Chs2-GFP::Trp1	This study
CFY668	SEY6211 Chs5-3HA::KanMX Chs2-GFP::Trp1	This study
CFY664	SEY6211 chs5Δ::KanMX Chs1-GFP::Trp1	This study
CFY665	SEY6211 Chs1-GFP::Trp1	This study

Microscopy

Cells were grown in synthetic dropout media and imaged in log phase (OD600 ~ 0.5). Live cells were imaged at room temperature on a DeltaVision RT wide-field deconvolution microscope (Applied Precision) using a PlanApo 100x objective (1.35 NA; Olympus), FITC, rhodamine, and DIC filters, and a digital camera (Cool Snap HQ; Photometrics). Images were deconvolved using softWoRx 3.5.0 software (Applied Precision). Images were further processed in Adobe Photoshop, adjusting only min/max light levels for clarity, and using equivalent processing for all images within an experiment. Exposure times for the FITC, rhodamine, and DIC channel were 1 second, 0.5 second, and 0.1 second, respectively, unless otherwise noted in the figure panel.

Table 2.2: Plasmids used in Chapter 2.

Plasmid	Description	Backbone	Source
pRS416	Centromeric URA3 plasmid		(Sikorski & Hieter, 1989)
pCF1027	Chs5 (full length)	pRS416	(Paczkowski et al., 2012)
pAS26	Chs5(51-658)	pRS416	This study
pAS27	Chs5(74-658)	pRS416	This study
pAS13	Chs5(1-299)	pRS416	This study
pAS14	Chs5(1-274)	pRS416	This study
pAS15	Chs5(1-249)	pRS416	This study
pAS16	Chs5(1-224)	pRS416	This study
pAS25	Chs5(1-268)	pRS416	This study
pAS24	Chs5(1-262)	pRS416	This study
pAS23	Chs5(1-258)	pRS416	This study
pAS22	Chs5(1-253)	pRS416	This study
pAS46	13xMyc-Chs5 (full length)	pRS416	This study
pAS61	13xMyc-Chs5(1-258)	pRS416	This study
pAS59	13xMyc-Chs5(1-262)	pRS416	This study
pAS60	13xMyc-Chs5(1-299)	pRS416	This study
pAS57	13xMyc-Chs5(1-324)	pRS416	This study
pAS64	13xMyc-Chs5(51-658)	pRS416	This study
pAS65	13xMyc-Chs5(74-658)	pRS416	This study
pETDuet1	T7-promoter driven expression plasmid		Novagen
pJP13	Chs5(1-299)/Chs6-6xHis	pETDuet1	
pGEX-2T	GST-fusion expression plasmid		GE Healthcare
pAS96	GST-Chs5(76-299)	pGEX-2T	This study
pCF1219	GST-Chs5(1-56)	pGEX-2T	(Paczkowski et al., 2012)
pET28a	T7-promoter driven expression plasmid		Novagen
pCF1053	6xHis-TEV-DN17-Arf1 (18-181)	pET28a	(Paczkowski et al., 2012)
pJP14	Chs5(51-299)/Chs6-6xHis	pETDuet1	(Paczkowski et al., 2012)
pCF1004	Chs6-6xHis	pETDuet1	(Paczkowski et al., 2012)
pAS95	Myc-Chs5(W244A)	pRS416	This study
pAS108	GFP-Chs5(W244A)	pRS416	This study
pMBS28	PATH tag vector	pET21a	Smolka lab
pAS98	PATH-Chs5(76-299,W244A)	pET21a	This study
pAS99	PATH-Chs5(76-299)	pET21a	This study
CFY1186	Myc-Chs5(1-79)	pRS416	This study
CFY1180	GFP-Chs5(1-79)	pRS416	(Paczkowski et al., 2012)

Preparation of synthetic liposomes

Unilamellar liposomes were generated from a mixture of lipids, using either Folch fraction I (Sigma), or a synthetic mixture approximating the endogenous TGN lipid composition determined in a published lipidomics study (Klemm et al., 2009; Richardson et al., 2012), with 1% DiR near-infrared dye (Avanti Polar Lipids) to aid in visualization and quantification of lipids. Following vacuum drying, lipid films were hydrated in 20 mM HEPES pH 7.4, 150 mM KOAc, followed by extrusion through 400 nm filters to generate liposomes. Due to some batch-to-batch variation, each figure panel represents data collected using a single batch of liposomes.

Liposome pelleting assay

A binding reaction consisting of 2 µg of protein with or without 300 µM liposomes in 40 µl total volume of HKM buffer (20 mM HEPES pH 7.4, 150 mM KOAc, and 1 mM MgCl2) was incubated at room temperature for 15 minutes. The samples were spun at 15,000 g for 10 minutes at 4oC. The supernatant was removed, a sample was taken for gel analysis, and the pellet was resuspended in 40 µl 1X SDS sample buffer/HKM. The samples were heated at 55oC for 15 minutes with frequent vortexing. The samples were run on SDS-PAGE and stained with IRDye (Li-COR) before scanning on an Odyssey imager (Li-COR). Band intensities were determined using Odyssey software to determine the relative amount of protein in the pellet (P) or supernatant (S) fractions. The background-subtracted percentage of protein pelleted was calculated using the formula: $[P_{liposomes} / (P_{liposomes} + S_{liposomes})] - [P_{no\ liposomes} / (P_{no\ liposomes} + S_{no\ liposomes})]$.

Co-Immunoprecipitation

Yeast culture was grown to log phase and 25 OD600 of yeast cells were pelleted. Cells were washed with 1 ml cold TE, transferred to a 2 ml tube, and pelleted. After removing supernatant, pellet was flash frozen in liquid nitrogen (and in some cases, stored at -80°C). 1 ml lysis buffer (50 mM Tris pH 7.5, 0.2% Tween-20, 150 mM NaCl, 5 mM EDTA, 1X Protease Inhibitor (Roche), 1 mM PMSF) was added to pellet with 500 µl glass beads and sample was vortexed 3 times for 10 minutes each time, with at least 1 minute on ice between. Sample was centrifuged 13,000 rpm at 4°C for 5 minutes to clear debris. 10 µl of α-HA resin was added to lysate and incubated overnight. Beads were pelleted at 4,000 rpm for 1 minute and washed 3 times with 1 ml lysis buffer. 10 µl of 2x urea sample buffer was added and samples heated at 55°C for at least 15 minutes before analysis by SDS-PAGE and Western Blot.

GST-pulldown Arf1 interaction assay

GST-fusion proteins were expressed from plasmids derived from the pGEX-2T vector (GE Healthcare) in Rosetta2 (DE3) E. coli cells (Novagen). Cells were harvested by centrifugation, resuspended in 50 ml 1X PBS with 1 mM DTT per 1 L original culture, and lysed by sonication. Glutathione resin was equilibrated and added at 100 µl per 10 ml lysate, then rotated at 4°C for at least 1 hour. Resin was washed 3 times with 1 ml PBS+DTT, then resuspended in 950 µl PBS+DTT. 50 µl of ΔN17-Arf1 at 33µM, having either GDP or GTP exchanged in using EDTA, was added to the resin and incubated at least 1 hour rotating at 4°C. Resin was washed 3 times with 1 ml PBS+DTT, then analyzed by SDS-PAGE.

SILAC mass spec

PATH-fusion proteins were expressed in Rosetta2 (DE3) *E. coli* cells (Novagen). Cells from 100ml of culture were harvested by centrifugation and resuspended in lysis buffer (50 mM Tris pH 7.5, 0.2% Tween-20, 150 mM NaCl, 5 mM EDTA, 1X protease inhibitor from Roche, 1X PMSF). Cells were lysed by sonication. Cleared lysate was incubated with 100 µl equilibrated IgG resin (GE Healthcare) for 1 hour rotating at 4°C. Resin was washed 3 times with 5 volumes of lysis buffer.

Two cultures of yeast cells were grown, either with normal ("light") or heavy isotope labeled amino acids. Cells were harvested from 200 ml of cells at 0.4 OD_{600} by centrifugation, washed in cold TE, transferred to a 2 ml tube, and pelleted. After adding 1.2 ml lysis buffer and 600 µl glass beads, samples were vortexed in 3 intervals of 10 minutes with rest on ice between each. Lysate was cleared by centrifugation at maximum speed for 5 minutes at 4°C.

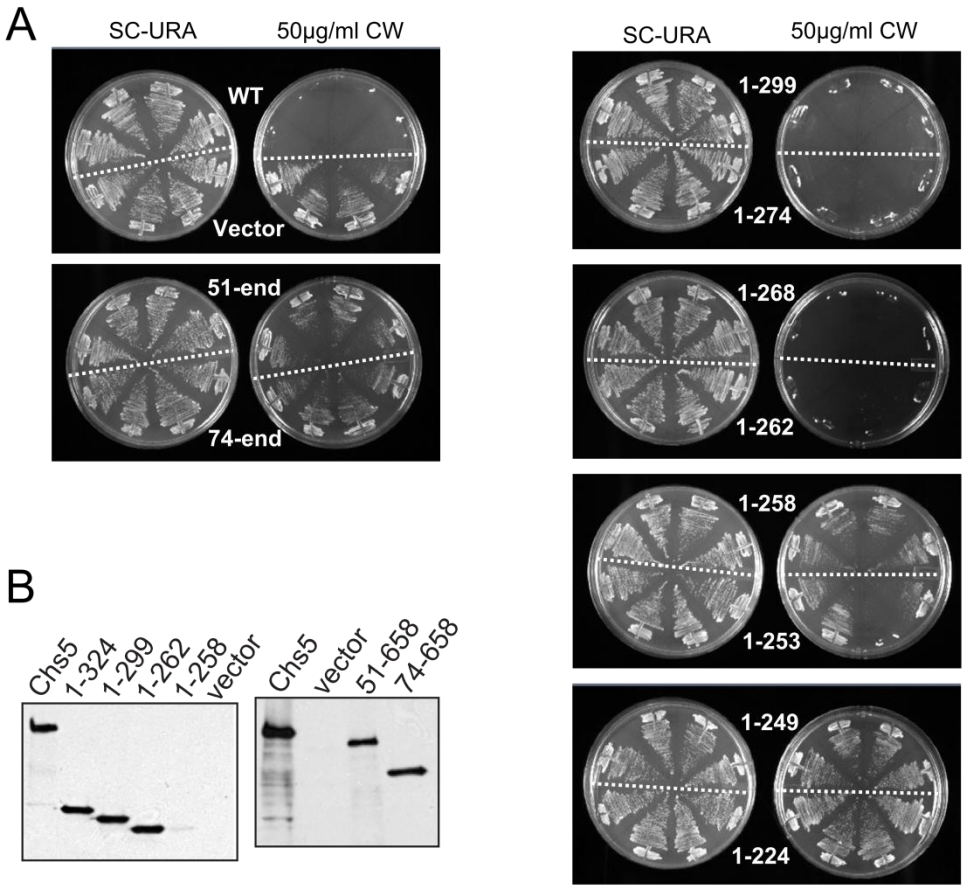
One sample of yeast lysate was added to each sample of IgG-PATH-fusion resin, and incubated with rotation for 1 hour at 4°C. Resin was washed 3 times with 10 volumes of lysis buffer and resuspended in 200 µl lysis buffer. Proteins were eluted from resin by TEV cleavage at room temperature. Light and heavy samples were combined and SDS added to 1% concentration, DTT to 5 mM, and water to a final volume of 400 µl. Samples were boiled 5 minutes, alkylated with iodoacetamide, then precipitated. After pelleting and washing with precipitant solution, proteins were resuspended in 8M urea and 10 mM Na_2PO_4. Protein was incubated with 200 µl of cobalt resin (Sigma) to remove tagged fusion protein. Proteins were digested with 4 µg Trypsin Gold (Promega) overnight. Peptides were desalted using a Sep-Pak C18 column (Waters), separated by hydrophilic interaction liquid chromatography (HILIC), and analyzed by mass spectrometry, as described (Albuquerque et al., 2008).

2.4. Results

Determination of the minimal functional fragment of Chs5

To better understand the architecture of Chs5, and to inform the development of constructs for crystallization, I attempted to identify the shortest functional fragment of Chs5. A series of C-terminal truncations of Chs5 were created, and assayed for function by CW resistance. All fragments 262 residues and longer restored CW sensitivity, indicating proper Chs3 transport to the PM, while all fragments 258 residues and shorter were resistant to CW (Figure 2.2A). These fragments were all stably expressed at an equal level to full-length Chs5 (Figure 2.2B). The boundary between residues 258 and 262 corresponds to an abrupt decrease in sequence conservation among other Chs5 homologues (Figure 2.2C).

The same Chs5 truncations were also assayed for function by Chs3-GFP localization. Chs5(1-274) was the shortest fragment capable of fully rescuing function in this assay (Figure 2.3). Constructs ending at residue 268 or 262, which were both functional based on CW sensitivity, had an intermediate phenotype with some small buds lacking Chs3-GFP. Together, these two assays indicate that residues 1-274 are capable of forming a functional exomer complex that can mediate the transport of Chs3.



C

```
                  :* . ***:: ***: *** *  *****.:*:
N_fisheri         …LWAKAVEMNIPVVVPEWVDACEAEGTIVGVRGYYLN----ADPKARQLGPIHAS…
A_fumigatus       …LWEKAVEMNIPVVVPEWVDACEAEGTIVGVRGYYLN----ADPKARQLGPIHAS…
P_stipitis        …EFVKANDMNIPIIRPEWLKACERERRIVGVRDFYV------------------
C_albicans        …EYVKANDMNIPIIRPEWLKACERERRIVGVRDFYVKDCVLPDIFAKNYWQKTTS…
K_lactis          …ELAHARDNNIPIVRPEWIRACELEKRIVGVRDFYLD-SDPSNFLNYKFTAAAPS…
S_cerevisiae      …ELIRAKHNNIPIVRPEWVRACEVEKRIVGVRGFYLD-ADQSILKNYTFPPVNEE…
                                  249 253  258 262     268    274
```

Figure 2.2: Determining the minimal fragment of Chs5 necessary for Chs3 transport. (**A**) Cells expressing the indicated Chs5 fragments from a plasmid were plated on calcofluor white (CW) and imaged after 2 days of growth at 30°C. Resistance to CW indicates a lack of Chs3 at the PM. (**B**) Expression levels of N-terminal Myc-tagged Chs5 fragments, as determined by anti-Myc antibody (Sigma) immunoblotting of TCA-precipitated whole-cell lysates resolved by SDS-PAGE. (**C**) Sequence alignment of Chs5 homologues from several fungi. Symbols indicate identity (*), high similarity (:), and moderate similarity (.).

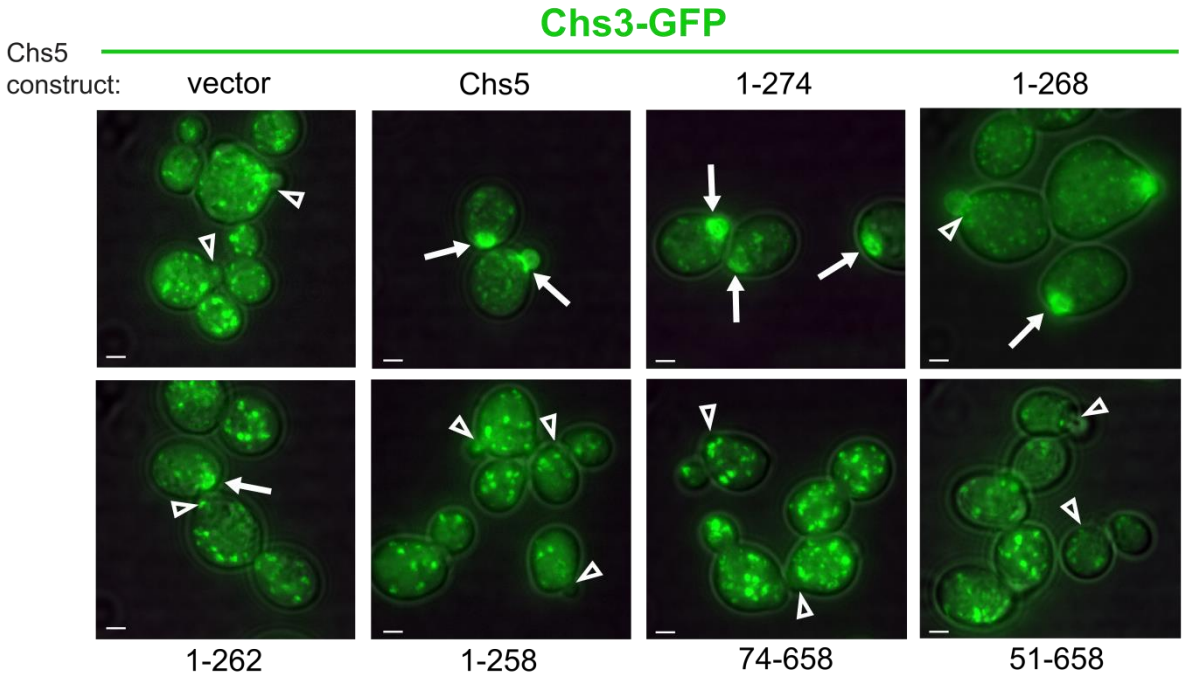
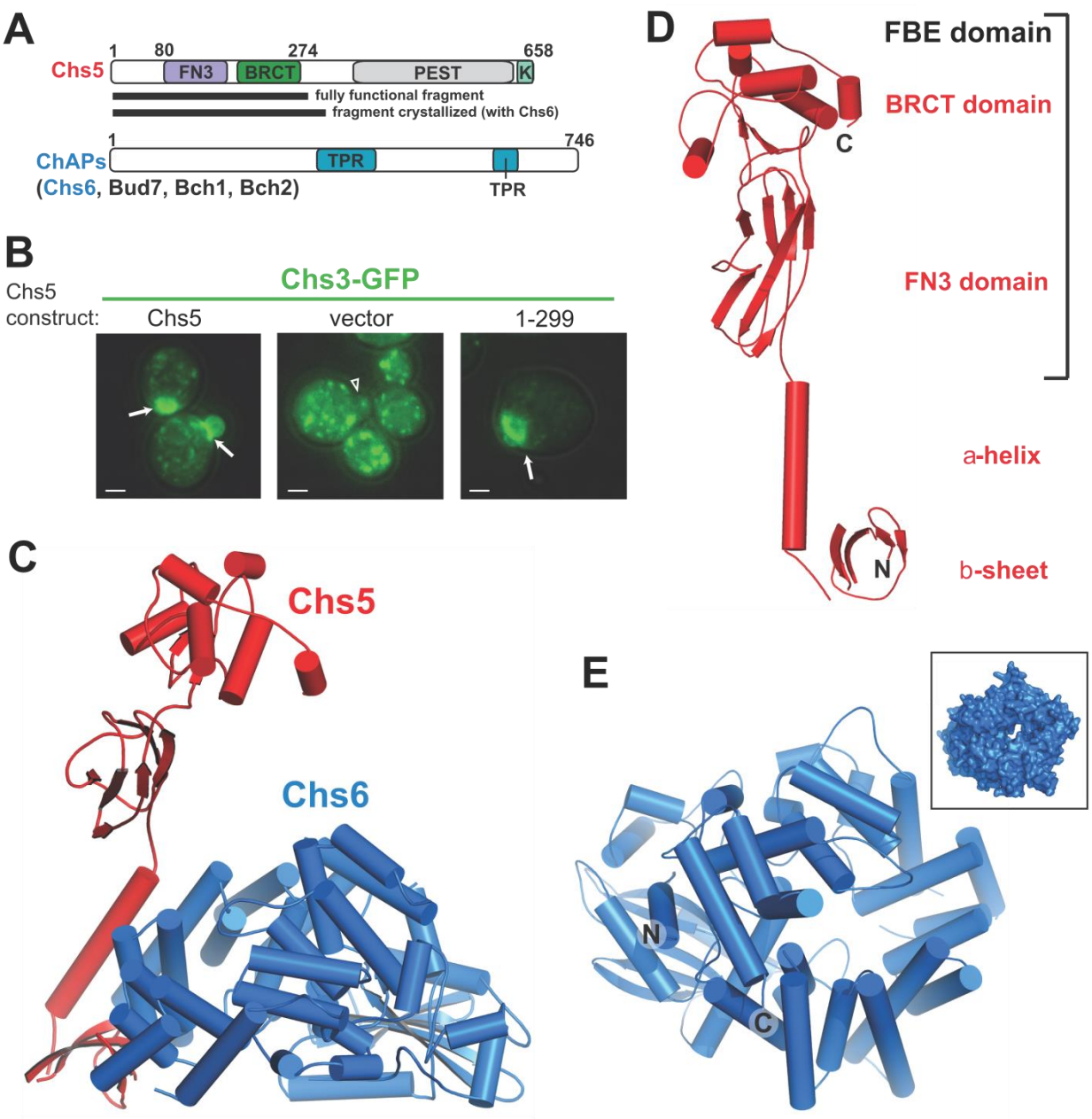


Figure 2.3: Localization of Chs3 in Chs5 truncation mutants.
Chs3-GFP localization in *chs5Δ* yeast cells expressing various fragments of Myc-tagged Chs5. CFY264 cells (Chs3-GFP::HIS3; *chs5Δ*::KANMX) expressing Chs5 constructs from centromeric URA3 plasmids were visualized on a DeltaVision wide-field microscope. The images shown combine a single focal plane from the GFP channel and the DIC channel. Arrows indicate proper Chs3-GFP localization at incipient buds and at the mother-bud neck of very small buds. Arrowheads indicate lack of proper Chs3-GFP localization at these sites.

Experiments performed by B.C. Richardson had indicated the N-terminal 51 residues of Chs5 were required for homodimerization, and residues 52-74 were required for interaction with the ChAPs. I assayed these constructs for Chs3-GFP localization as well, and consistent with their important roles in exomer complex formation, they completely trapped Chs3-GFP at the TGN (Figure 2.3A) despite being well expressed (Figure 2.2B). These N-terminal truncations also failed to rescue CW sensitivity (Figure 2.2A). While these assays cannot tell us the exact role of the N-terminus of Chs5, they confirm its requirement for transport of Chs3.

The fully functional fragment of Chs5, comprising residues 1-274, ends just after the FN3 and BRCT domains (Figure 2.4A). Attempts to crystallize the exomer complex were conducted using Chs5 fragments at least 174 residues in length. The fragment used to obtain the crystal structure was 299 residues in length. This fragment was functional in both the CW resistance assay (Figure 2.2A) and Chs3-GFP localization assay (2.4B). This allowed B.C. Richardson and J.E. Paczkowski to determine the crystal structure of a Chs5(1-299)/Chs6 complex refined to 2.75 Å (Figure 2.4C). Chs5 contains 4 small structural motifs: an antiparallel β sheet, an extended α-helix that mediates interaction with Chs6, the FN3 domain, and the BRCT domain (Figure 2.4D). The FN3 and BRCT domains interact with each other, but are projected away from the complex. This portion has been named the FN3-BRCT of exomer (FBE) domain. Chs6, which is discussed in detail in Chapter 3, is mostly α-helical and forms a ring shape, with a solvent-accessible channel running down the center (Figure 2.4E).

Figure 2.4: Molecular architecture of the Chs5/6 exomer heterodimer.
(A) Schematic of exomer subunit constructs used, with domains and motifs identified by searching the SMART and TPRpred databases. Exomer complexes form through association of Chs5 with one or more of the ChAPs (Chs6, Bud7, Bch1, and Bch2). **(B)** Chs3-GFP localization of yeast cells expressing residues 1-299 of Chs5 phenocopies that of cells expressing full-length Chs5 (residues 1-658). Plasmids expressing Chs5 constructs or empty vector were introduced into *chs5Δ* Chs3-GFP cells (yeast strain CFY264). Images show a single focal plane, with GFP and DIC channels merged. Arrows indicate proper localization of Chs3-GFP to incipient bud sites and the mother-bud neck of very small buds. Arrowhead indicates lack of proper localization in cells lacking functional Chs5. Scale bar, 2μm. **(C)** The structure of the Chs5(1-299)/Chs6 heterodimer asymmetric unit, as determined by J.E.P. and B.C.R., shown as a ribbon diagram. Chs5 is red and Chs6 is blue. **(D)** Ribbon diagram of the Chs5(1-299) structure, indicating the four different structural motifs. **(E)** Ribbon diagram of the Chs6 structure, from a top-down perspective relative to (C). Inset shows the same perspective as a surface diagram to visualize the solvent channel in Chs6.


A
Chs5
1
80
274
658
FN3
BRCT
PEST
K
fully functional fragment
fragment crystallized (with Chs6)
ChAPs
(Chs6, Bud7, Bch1, Bch2)
1
746
TPR
TPR
B
Chs5
construct:
Chs3-GFP
Chs5
vector
1-299
C
Chs5
Chs6
D
FBE domain
BRCT domain
FN3 domain
a-helix
b-sheet
C
N
E
N
C

The FBE domain mediates exomer recruitment

A FN3 domain followed by a BRCT domain is a phenomenon unique to Chs5. However, the structure formed resembles the "appendage" domains in other coat protein adaptors (Figure 2.5). The appendage domains of clathrin adaptors and COPI have an N-terminal immunoglobulin-like β-sandwich subdomain, and some also have a C-terminal α/β platform subdomain. In the case of Chs5, the FN3 domain resembles the β-sandwich subdomain, and the BRCT domain resembles the platform subdomain.

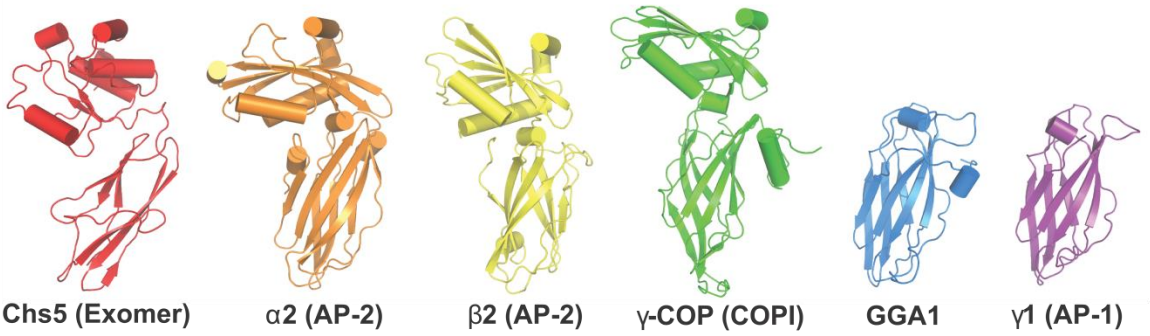


Figure 2.5: The Chs5 FBE domain resembles appendage domains of other cargo adaptors. Structural resemblance of the Chs5 FBE domain (this work) and the appendage domains of the α2 subunit of AP-2 (PDB: 1B9K)(Owen et al., 1999), the β2 subunit of AP-2 (PDB: 1E42)(Owen et al., 2000), the γ-COP subunit of COPI (PDB: 1PZD)(Hoffman et al., 2003), GGA1 (PDB: 1OM9)(Collins et al., 2003), and the γ1 subunit of AP-1 (PDB: 1GYU)(Kent et al., 2002).

To determine whether the FBE domain contributes to the interaction of exomer with membranes, which could affect its recruitment to the TGN in cells, I performed a liposome pelleting assay. This work was done as part of a collaborative project to determine what portions of Chs5 mediate recruitment, so I simultaneously tested the N-terminus of Chs5 for membrane

interaction. GST-FBE and GST-N-terminus did not interact with liposomes significantly above the GST protein alone (Figure 2.6A,B). Correspondingly, J.E. Paczkowski found that an exomer complex lacking the FBE domain, Chs5(1-80)/Chs6, interacted with membranes just as well as the functional complex, Chs5(1-299)/Chs6 (Figure 2.6C,D). This indicates the FBE domain does not directly participate in interaction of exomer with the membrane.

Arf1 is the small GTPase that recruits exomer to the TGN in cells. Therefore, I examined the interaction of the FBE domain with soluble ΔN17-Arf1, pre-loaded with either GTP or GDP. The GST-tagged FBE domain interacted strongly with Arf1 in both its GTP- and GDP-bound states (Figure 2.7A). However, in a liposome pelleting assay performed by J.E. Paczkowski, the GST-FBE was not pelleted by Arf1-loaded liposomes (Figure 2.7B,C). Therefore, while the FBE domain does interact with Arf1 when present in very high concentrations *in vivo*, it is not sufficient to recruit exomer to the membrane. The ChAP Chs6 can interact directly with the membrane but this interaction is not strengthened by the presence of Arf1 (Figure 2.7B,C and additional information in (Paczkowski et al., 2012)). Taken together, this suggests a cooperative mechanism for exomer recruitment, with Chs6 binding the membrane and the Chs5 FBE binding to Arf1.

Analysis of the BRCT domain of Chs5

The presence of a BRCT domain in Chs5 is curious. Before the exomer structure was solved, it was unclear whether this predicted domain would even adopt a typical BRCT fold. Comparison with other BRCT domains, such as the C-terminal of the two BRCT domains found in BRCA1, shows a very similar structure (Figure 2.8A,B). Most BRCT domain pairs bind phosphoproteins. The residues in BRCA1 that bind the phosphorylated serine in the interaction

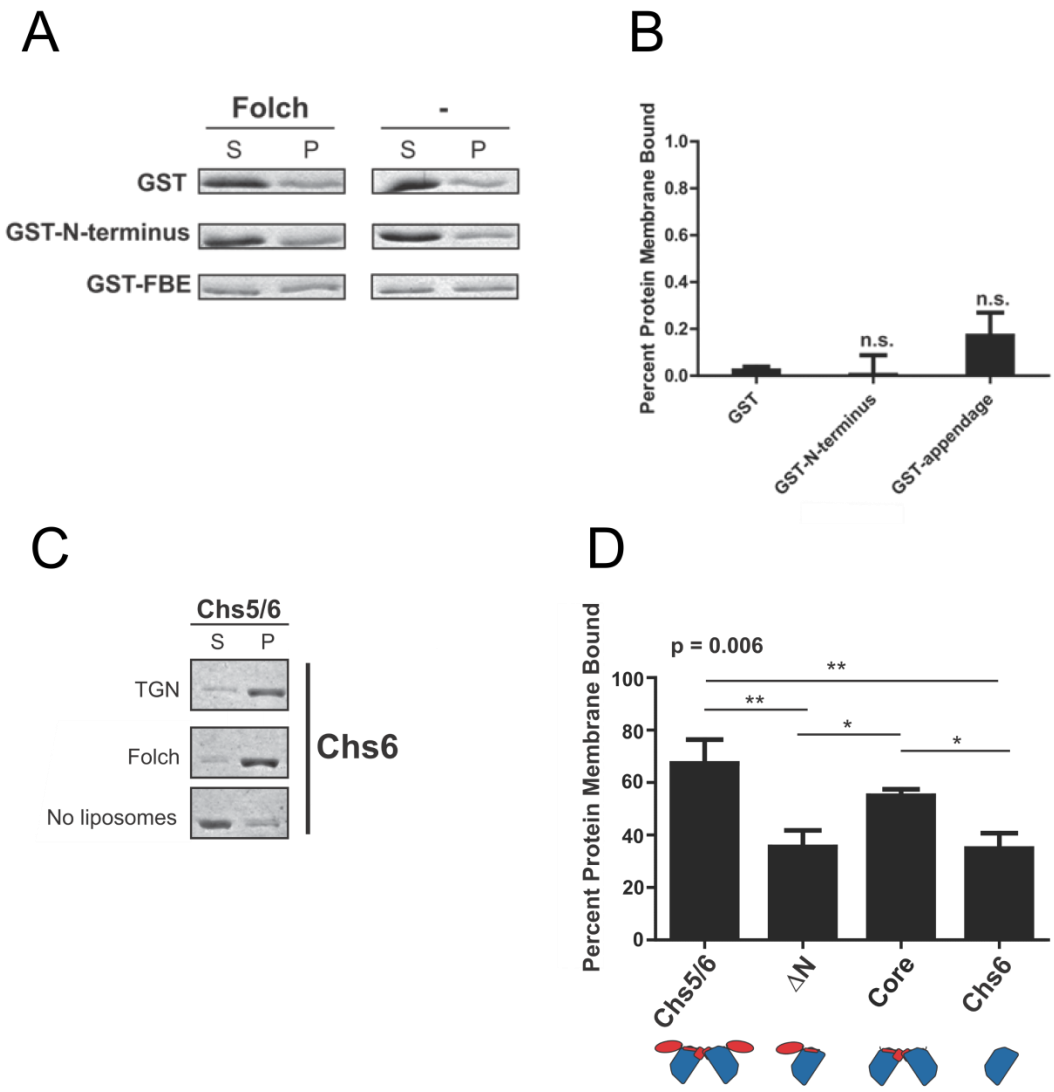


Figure 2.6: The FBE domain does not bind membranes directly. (**A**) Example liposome pelleting assay comparing membrane binding of GST, GST-N-terminus (Chs5 residues 1-55), and GST-FBE (Chs5 residues 76-299) to Folch fraction I liposomes. (**B**) Quantification of (A). Error bars represent s.e.m., n=3, with significance determined by one-way ANOVA with post-processing to correct for multiple comparisons. (**C**) Liposome pelleting assay measuring Arf1-independent membrane binding. 'TGN', TGN-like liposomes, 'Folch', Folch fraction I liposomes. 'S', supernatant fractions, 'P', pellet fractions. (**D**) Quantification of Arf1-independent membrane binding. Error bars represent 95% confidence intervals, n=3, with significance determined by one-way ANOVA with post-processing to correct for multiple comparisons. The overall P-value for this statistical model is indicated. Comparisons not labelled with asterisks were not statistically different.

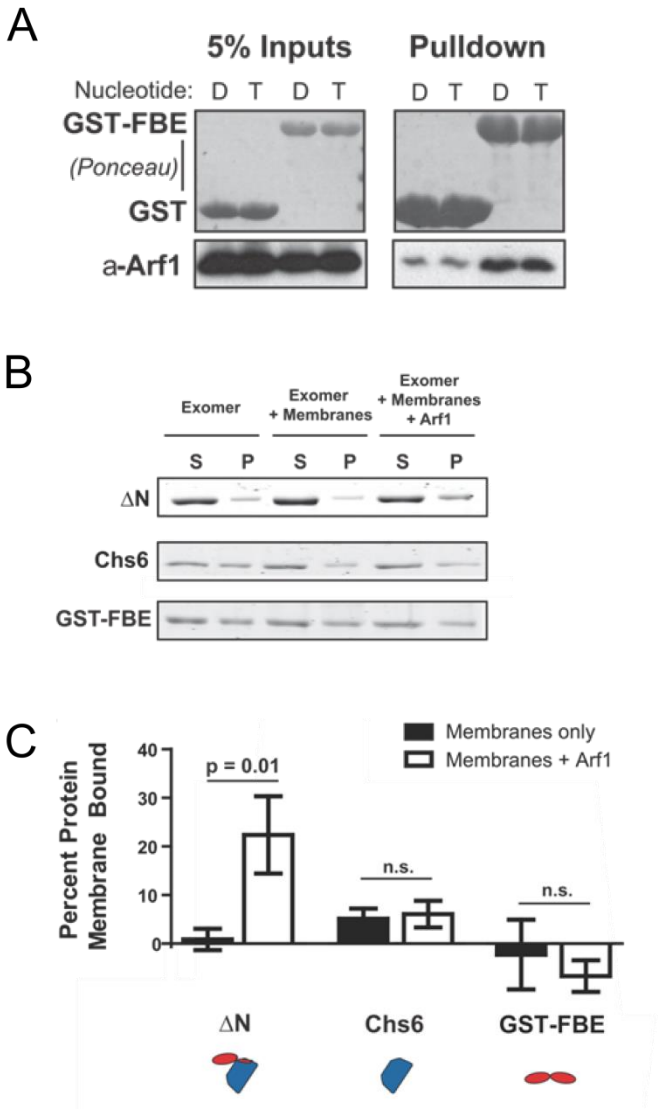


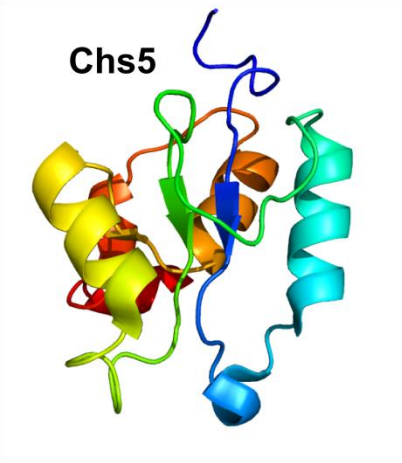
Figure 2.7: The FBE domain binds Arf1 weakly. (**A**) A GST-pulldown was performed to compare binding of purified ΔN17-Arf1 (preloaded with GDP or GTP) to either the GST-FBE domain construct or GST alone. Arf1 was detected with anti-Arf1 antibody and the GST proteins were detected by Ponceau staining. (**B**) Example liposome pelleting assay comparing exomer constructs in the presence or absence of membrane-bound Arf1-GTP. Pelleting was performed using a different batch of Folch liposomes. Occasionally, liposome batches bound proteins more weakly; we took advantage of one such batch to measure Arf1-dependent binding. Before adding exomer constructs to the "+Arf1" experimental condition, Arf1 was loaded with GTP in the presence of liposomes. (**C**) Quantification of (B). Error bars represent s.e.m.; signficance was determined using a two-tailed t-test, n=3.

partner have been identified by structural methods, and are well conserved in other BRCT pairs that bind phosphoserine-containing proteins (Glover et al., 2004). The residues that form the phosphoserine binding pocket in BRCA1 are located in only one of the two BRCT domains, suggesting a lone BRCT domain like the one in Chs5 might also be capable of binding phosphorylated residues. However, the BRCT domain from Chs5 does not contain the residues found in this binding pocket in BRCA1 (Figure 2.8C). When compared to BRCT domains that bind various other proteins, including phosphoproteins and other BRCT domains, there are many similar and identical residues present in Chs5 (Figure 2.8D). These could be residues important for structure, function, or both.

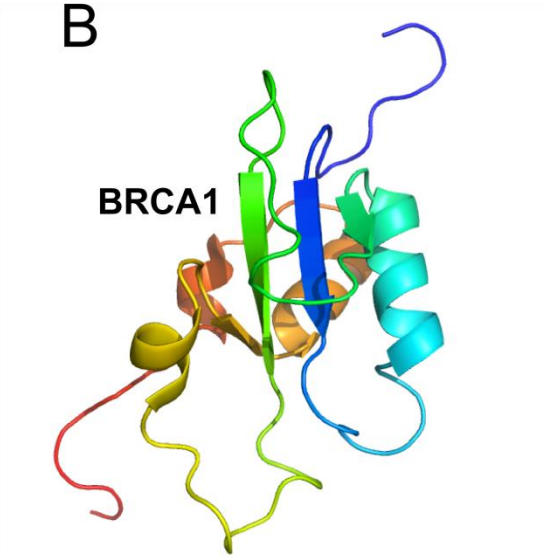
Based on the residues in the Chs5 BRCT domain that are conserved within other BRCT domains (Figure 2.8D), I designed several mutations. The only mutation that affected the function of Chs5 was W244A, which completely abolished transport of Chs3-GFP from the TGN to the PM (Figure 2.9A). This mutant also affected Chs5 localization, as there was significantly more localization of Chs5(W244A)-GFP to the cytoplasm than Chs5-GFP, which is seen only at TGN punctae (Figure 2.9B,C). This mutation does not affect the level of protein in the cell (Figure 2.9D), as you might expect if the protein were drastically misfolded and therefore prone to degradation. Making the W244A mutation in GST-tagged FBE domain does not affect its interaction with Arf1, which is still robust and nucleotide-independent (Figure 2.9E). It is possible this mutation disrupts an interaction with other proteins required for exomer function.

Figure 2.8: Comparison of Chs5 BRCT domain to those in other proteins. (**A**) BRCT domain of Chs5 (residues 166-270) colored red to blue, N to C (PDB ID: 4GNS)(Paczkowski et al., 2012). (**B**) C-terminal BRCT domain from BRCA1 colored as in (A) (PDB ID: 1OQA)(Gaiser et al., 2004). (**C**) Alignment of Chs5 with N-terminal BRCT domain from BRCA1 with important residues indicated. (**D**) Alignment of BRCT domain from Chs5 with those from a few well-studied human proteins. Grayscale shading indicates similar residues in aligned sequences, green shading indicates residues conserved in Chs5 homologues.

A



B



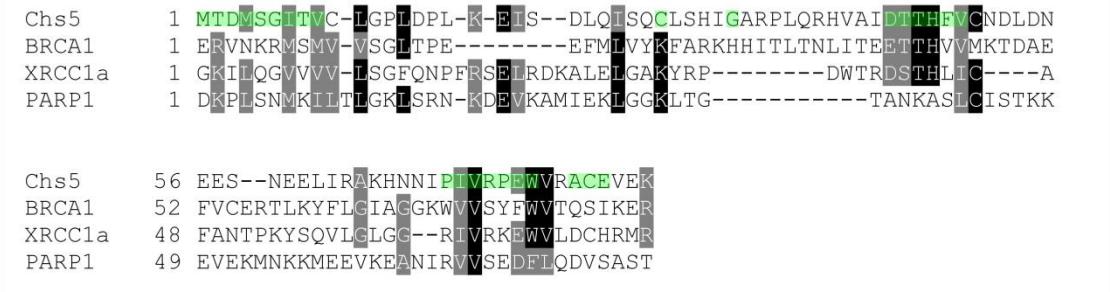
C

```
Chs5       MTDMSG-ITVCLGPLDPLKEISDLQISQ--
BRCA1_1    -ERVNKRMSMVVSGLTPEEFMLVYKFARKH
              :.  ::: :. * * : :   ::::

Chs5       --CLSHIGARPLQRHVAIDTTHFVCNDLDN
BRCA1_1    HITLTNLITEETTHVVMKTDAEFVC-----
              *::: :.   : *    :.***

Chs5       EESNEELIRAKHNNIPIVRPEWVRACEVEK
BRCA1_1    ERTLKYFLGIAGGKW-VVSYFWVTQSIKER
           *.: : ::    .:  :*   **  .  *:
```

Phospho-serine binding pocket

Conserved among Chs5 homologues

D



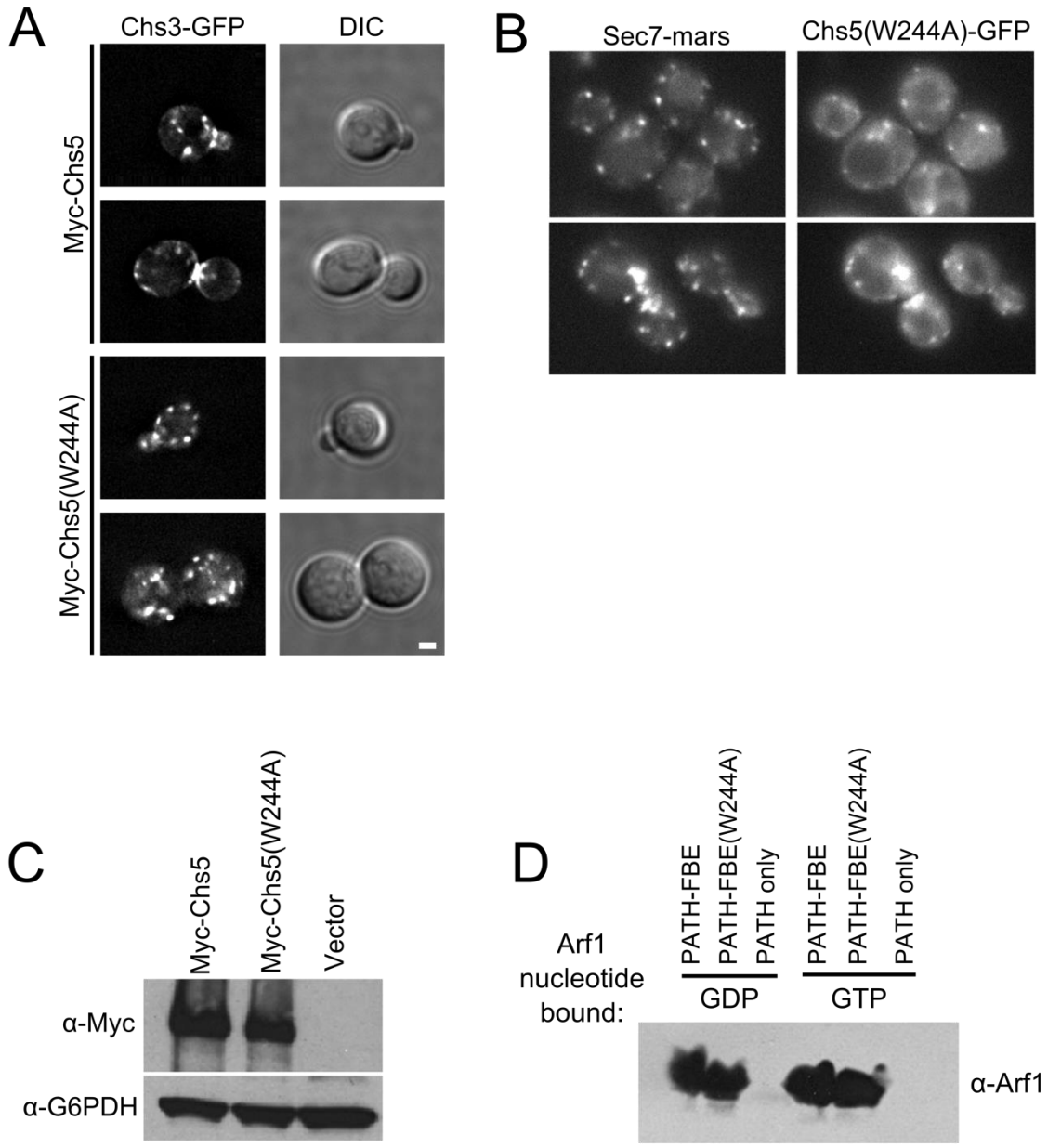


Figure 2.9: The W244A mutation in the Chs5 FBE domain disrupts Chs3 transport. (**A**) Chs3-GFP localization in *chs5Δ* cells expressing Myc-tagged Chs5 or Chs5(W244A) from a plasmid. Scale bar = 1μm. (**B**) Chs5(W244A)-GFP localization. Sec7-mars images are shown to confirm the TGN is intact. (**C**) Chs5-GFP localization. (**D**) Expression level of Myc-Chs5 and Myc-Chs5(W244A) in cell lysates, assayed by Western blot. α-G6PDH blot is included as a loading control. (**E**) GST-pulldown to compare binding to ΔN17-Arf1 of PATH-tagged FBE domain with or without W244A mutation. Pull-down was assayed by α-Arf1 blot.

Retromer contributes to recycling of Chs3

To discover other proteins that might be interacting with the FBE domain, we used stable isotope labeling by amino acids in cell culture (SILAC) mass spectrometry. This technique allows the comparison of the levels of proteins in two populations. For instance, one can grow cells under two different conditions of interest, labelling one condition by growing the cells with heavy isotope amino acids, then compare the levels of all isolated proteins. For this experiment, I used purified PATH-tagged Chs5 FBE domain (PATH-FBE) and PATH-FBE(W244A) to pull down proteins from yeast lysates, in order to find interactions that are disrupted by the W244A mutation. There were no proteins strongly enriched in the wild type PATH-FBE interacting population versus the PATH-FBE(W244A) interacting population that were likely to have any role in membrane trafficking or other related processes. However, both the wild type and mutant FBE domains pulled down several components of the retromer complex. Since Chs3 needs to be recycled back from the endosomes to the golgi, it is possible this requires the retromer complex, and this led to the interaction seen in the SILAC mass spectrometry. I observed the localization of Chs3-GFP in cells lacking retromer components. Components of the cargo selective complex Vps35, Vps26 and Vps29 all led to increased GFP in the vacuole, while one of the subunits of the Snx-BAR dimer, Vps5, did not affect Chs3-GFP localization (Figure 2.10A). It is possible retromer interacts directly with the Chs5 FBE domain, mediating the recycling of Chs3 that is bound to the exomer complex. Consistent with this hypothesis, though by no means conclusive, Chs3-GFP localization to the vacuole is increased in cells expressing Chs5 lacking the FBE domain (Figure 2.10B). Chs5 itself is partially mislocalized to the limiting membrane of the vacuole when the FBE domain is missing (Figure 2.10C). These data support the speculation that

an interaction between exomer and retromer could be involved in maintaining proper trafficking of Chs3.

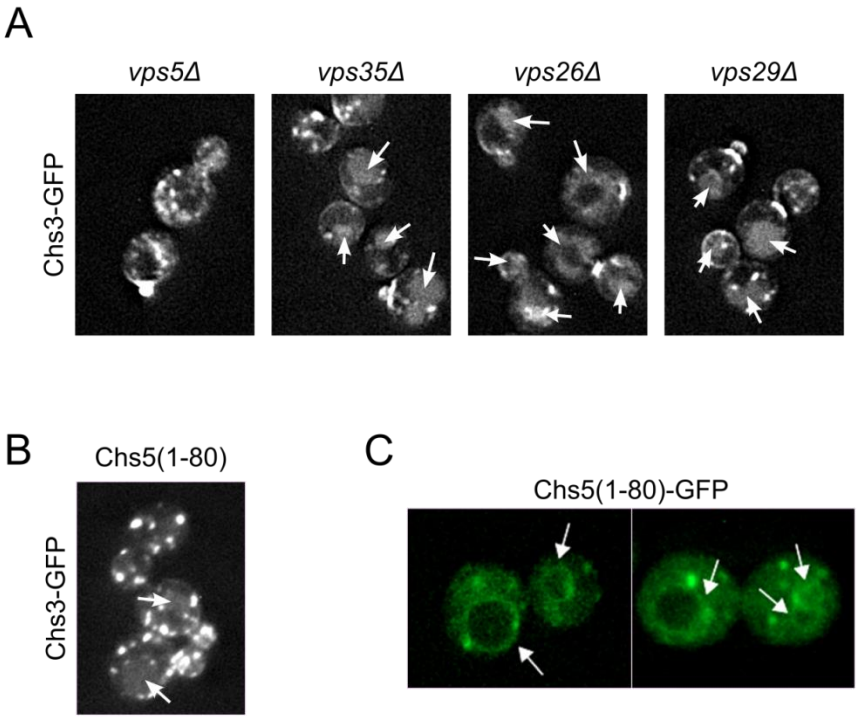


Figure 2.10: The retromer complex contributes to recycling of Chs3. (**A**) Chs3-GFP localization in cells with the indicated retromer genes deleted. Arrows indicate vacuolar localization. (**B**) Chs3-GFP localization in *chs5Δ* cells expressing Chs5(1-80) on a plasmid. (**C**) Localiation of Chs5(1-80)-GFP in *chs5Δ* cells. Arrows indicate vacuolar membrane localization.

Trafficking of other chitin synthases does not require exomer

SILAC mass spectrometry was used in our lab by J.E. Paczkowski to screen for new exomer cargo proteins. In this screen, he detected an interaction between exomer and the chitin synthase Chs2 (unpublished data). Chs2 is synthesized each cell cycle and trapped at the ER until the mitotic exit network triggers its release, then is transported to the bud neck before

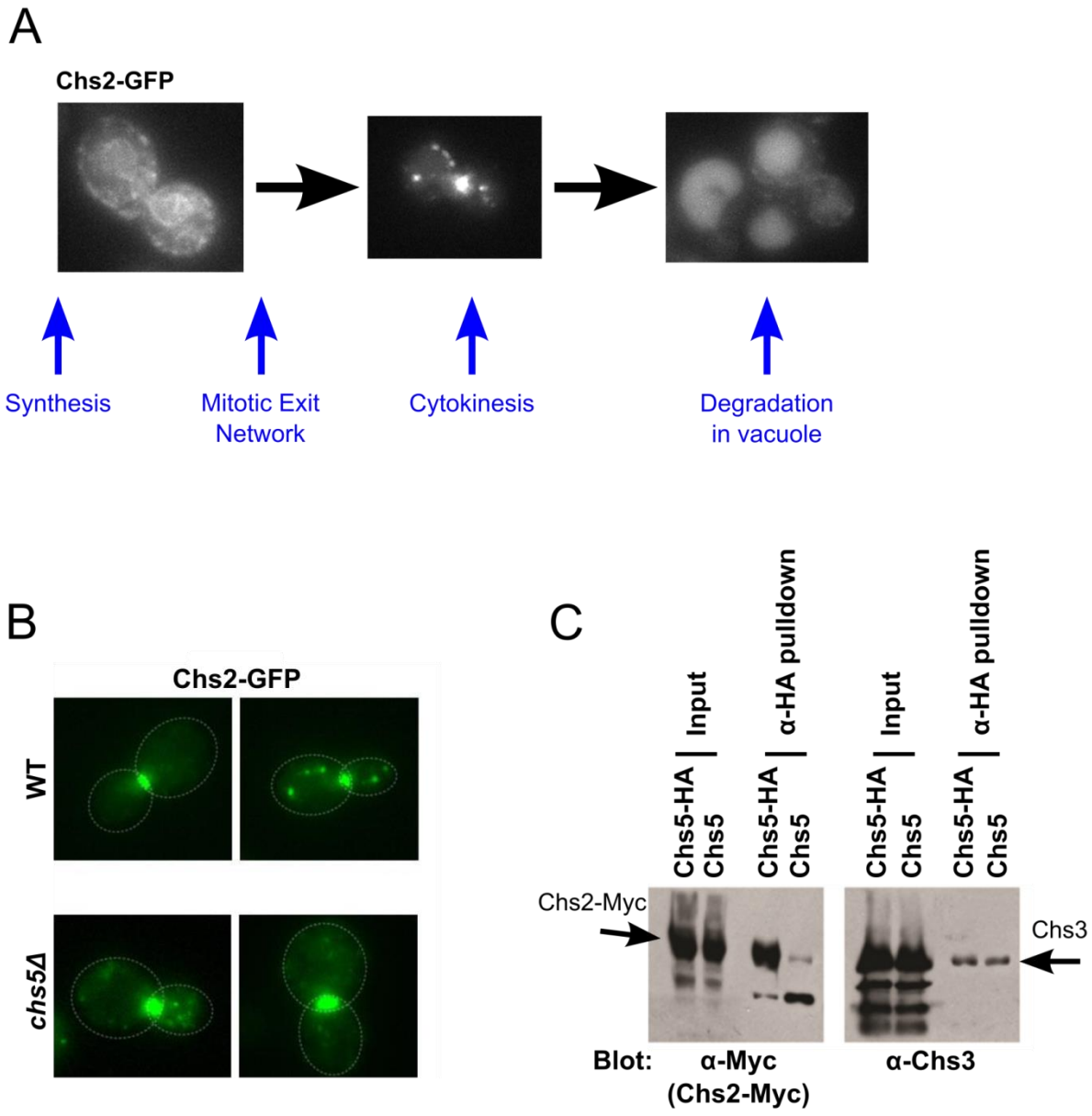


Figure 2.11: Chs2 trafficking does not require exomer. (**A**) The itinerary of Chs2 localization throughout the cell cycle. (**B**) Chs2-GFP localization to the primary septum in normal and *chs5Δ* cells. Cell boundaries indicated by dotted lines. (**C**) Co-immunoprecipitation using an α-HA antibody to pull down Chs5-HA was analyzed by SDS-PAGE and blotting with α-Myc to recognize Myc-tagged Chs2 (left) or α-Chs3 (right).

cytokinesis, and after endocytosis is internalized and degraded (Figure 2.11A) (Chuang & Schekman, 1996; Zhang et al., 2006). It was not known whether exomer was required for the step of Chs2 transport to the PM. Therefore, I examined Chs2-GFP localization in *chs5Δ* cells. The lack of exomer did not impact the localization of Chs2-GFP to the primary septum (Figure 2.11B). The high level of Chs2-GFP localization leads to such bright GFP signal that it is possible exomer is mediating transport of some small percentage of the Chs2, but this assay is not sensitive enough to measure it. To confirm the interaction between Chs2 and exomer, I precipitated Chs5-HA with α-HA antibody, and blotted for Chs2-Myc. I saw an interaction between Chs5-HA and Chs2-Myc which was stronger than the interaction between Chs5-HA and the known exomer cargo Chs3 (Figure 2.11C). This confirms that Chs2 does indeed interact with exomer, though the purpose of this interaction remains unknown.

There are three chitin synthase enzymes in S. cerevisiae. The interaction of exomer with two of these proteins implies that transport mediated by exomer may be a common feature of all of the chitin synthases. However, localization of Chs1-GFP to the bud neck was not perturbed by a deletion of the CHS5 gene (Figure 2.12).

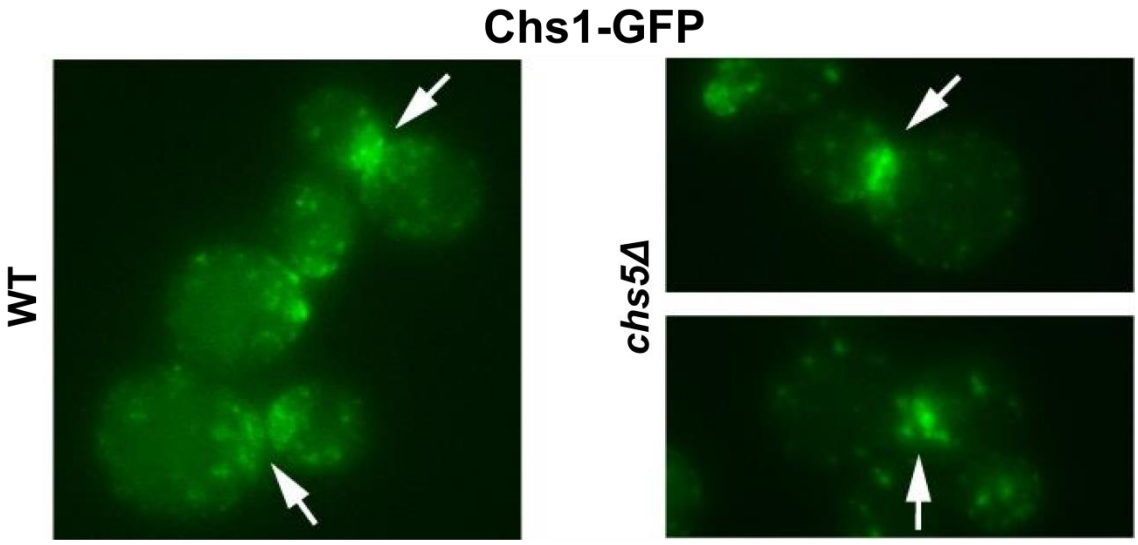


Figure 2.12: Chs1 trafficking does not require exomer. Chs1-GFP localization at the site of bud division (arrows) in cells immediately preceding or following cell division.

2.5. Conclusions and discussion

To inform crystallography trials and to learn more about the functional architecture of Chs5, I determined the minimal functional fragment. This fragment, residues 1-274, represents only 41% of the total length of the Chs5 protein. These studies confirmed that the segment used to obtain the crystal structure of exomer, residues 1-299, is functional, and therefore the structure accurately depicts a functional exomer unit. The predicted FN3 and BRCT domains of Chs5 form one structural module that we termed the FBE domain. This domain does not interact with membranes directly, but does interact with the membrane-associated small GTPase Arf1, which can contribute to exomer recruitment to the TGN. Surprisingly, this interaction is nucleotide-independent. Most effectors bind to Arf1 in its "switch" region, which undergoes a structural rearrangement upon nucleotide binding. However, the FBE domain of Chs5 interacts with a

region outside of the switch region (Paczkowski and Fromme, in press), providing an explanation for the nucleotide independent binding. We predict exomer recruitment occurs by cooperativity, requiring interaction of the ChAP with the membrane and interaction of the FBE domain of Chs5 with Arf1.

The BRCT subdomain within the Chs5 FBE domain does not contain the residues that would be expected of a BRCT domain that binds phosphoserine residues within proteins, as many other BRCT domains do. One point mutation (W244A) was found that disrupted Chs3 trafficking, but it did not appear to disrupt interaction of the FBE domain with any proteins involved in membrane trafficking. The FBE domain did appear to interact with several retromer components, and the cargo-selective subcomplex of retromer was found to be required for diverting Chs3 away from the vacuole. Another interaction was discovered between exomer and the chitin synthase Chs2, but exomer is not required for transport of either Chs2 or Chs1 to the PM.

Our lab and another each independently found that the C-terminus of Chs5, predicted to be largely unstructured, is not required for function (Martín-García et al., 2011). Why has this portion of the protein been preserved if it is not needed? This segment contains PEST motifs, which mediate degradation of proteins (Rechsteiner & Rogers, 1996), and could promote degradation of Chs5 to maintain the proper levels in the cell. While not required under our normal laboratory conditions, the C-terminus may be required in the harsher environments faced by yeast in nature. If the cell wall were stressed – by osmotic pressure, temperature, pH, or other conditions – careful regulation of Chs5 may be required to properly transport Chs3 to the cell surface and maintain appropriate levels of chitin production.

The mere 41% of Chs5 that is required for function is mostly composed of the FBE domain. While the FBE domain bears resemblance to appendage domains, its role appears to be quite different, as no adaptor appendage domains bind to the small GTPases that recruit them. Appendage domains typically interact with regulatory accessory proteins, but the only possible interaction identified has been that of the FBE domain with retromer. It is interesting that exomer employs a FN3 domain and a BRCT domain to create this shape, when each of those domains are usually found in other contexts.

The interaction of the FBE domain with retromer introduces an interesting, if purely speculative, model: exomer could act as an adaptor to bridge the interaction between retromer and Chs3. There is no such system known so far, as the retromer subcomplex Vps26/29/35 usually interacts directly with cargo, through many different recognition motifs (Seaman, 2012). Whether the interaction between exomer and retromer is significant *in vivo*, retromer does seem to play a role in recycling of exomer. However, only the cargo selective complex appears to play a role. There is some precedent for only one of the two retromer subcomplexes being required for transport of certain proteins, but in the other reported cases, the Snx1/2 subcomplex (homologous to Vps5 and Vps17 in yeast) was required (Nisar et al., 2010; Prosser et al., 2010).

Another interesting interaction with exomer was that of Chs2, the chitin synthase that helps to build the primary septum during cytokinesis. I demonstrated that the most obvious hypothesis, that exomer may be required for sorting and transport of Chs2 to the PM, was incorrect. Why would exomer interact with Chs2? The three chitin synthases in yeast display high sequence similarity in the regions responsible for enzymatic activity, so it is possible exomer interacts with a sequence in those regions. However, the sequences of Chs1 and Chs2 are

not similar to those of Chs3 in the regions that are known to bind exomer: the Chs3 C-terminus (Rockenbauch et al., 2012) and N-terminus (Chapter 4 and (Starr et al., 2012)).

2.6. Acknowledgements

I would like to thank the Smolka lab, particularly Yi Liu for protocols and Patrice Ohouo for assistance with SILAC mass spectrometry. I also thank the Emr laboratory for use of their microscope, as well as to Chris Stefan and Bret Judson for technical assistance with microscopy.

For the crystallography project very briefly described: we thank the laboratory of Steve Ealick for the gift of heavy atom compounds. We are grateful for the use of beamline A1 at CHESS, and beamline X25 at the NSLS. We received expert technical advice from Marian Szebenyi at CHESS A1 and Annie Heroux at NSLS X25. CHESS was supported by the National Science Foundation and the National Institutes of Health/National Institute of General Medical Sciences under NSF award DMR-0225180, using the Macromolecular Diffraction at CHESS (MacCHESS) facility, which was supported by award RR-01646 from the National Institutes of Health, through its National Center for Research Resources. NSLS was supported principally by the Offices of Biological and Environmental Research and of Basic Energy Sciences of the US Department of Energy, and by the National Center for Research Resources of the National Institutes of Health Grant number P41RR012408.

On the manuscript from which methods and figures were reproduced here: We thank Robyn Barfield, Tony Bretscher, Scott Emr, and Holger Sondermann for critical reading of the manuscript. We thank Randy Schekman for encouragement and advice, and Harvey McMahon for a helpful discussion regarding the FBE domain. We were supported by startup funds to JCF from Cornell University, and by NIH Training Grant (T32GM07273) awards to the

author and J.E. Paczkowski. I thank J.E. Paczkowski, B.C. Richardson and J.C. Fromme for preparation of the manuscript.

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CHAPTER 3

Regulation of exomer-mediated transport at the level of the ChAPs

3.1. Abstract

The exomer complex is composed of the core protein Chs5, and four interchangeable adaptor proteins known as Chs5-Arf1-binding proteins (ChAPs). Chs6 is the ChAP required for transport of Chs3 to the PM, and thus, represents a possible level of regulation of Chs3 transport. The level of Chs6 protein varies in response to the cell cycle, and may have an impact on Chs3 transport under certain conditions. Using sizing of complexes by gel filtration, I showed that the ChAP proteins are differentially incorporated into exomer complexes. Several mutations in Chs6 disrupting the transport of Chs3 are analyzed.

3.2. Introduction

The exomer core protein Chs5 and the ChAP Chs6 are so named because they were identified as being required for the synthesis of chitin by Chs3 (Cid et al., 1995; Santos et al., 1997). After *in vivo* results showed that these proteins were required for transporting Chs3 to the PM (Santos & Snyder, 1997; Ziman et al., 1998), biochemical studies showed that they form a complex, along with the other ChAP proteins Bud7, Bch1 and Bch2 (Sanchatjate & Schekman, 2006).

Before our lab solved the structure of the exomer complex (Paczkowski et al., 2012), our knowledge about the ChAP proteins came from sequence analysis, biochemical studies, and *in vivo* findings. The only domains identifiable by homology in the ChAP proteins are several tetratricopeptide repeat (TPR) motifs (Figure 3.1A). A TPR motif is a 34 amino acid sequence that folds into two antiparallel α-helices, and is often involved in protein-protein interactions (Goebl & Yanagida, 1991; Blatch & Lässle, 1999). Even outside of the TPR motifs, the secondary structure of the ChAPs was predicted to be mostly alpha-helical, which the crystallographic structure now clearly confirms. The C-terminus of each ChAP is necessary for its function and a deletion of this segment perturbs the interaction with Chs5 (Trautwein et al., 2006) though the ChAP C-terminus is not well conserved and its exact role is unclear.

All of this information provides some insight into the ChAP proteins in general – but what makes them different from each other? The exomer cargos each require a different subset of ChAP proteins for their transport. Chs3 is not transported if either CHS6, or BUD7 and BCH1, are deleted (Trautwein et al., 2006). Fus1 is not transported if BUD7 and BCH1 are deleted (Barfield et al., 2009). Pin2 can be transported at low levels by any of the ChAPs, but Bch1 or Bch2 allow normal levels of transport (Ritz et al., 2014). Chs6 and Bch2 are closely

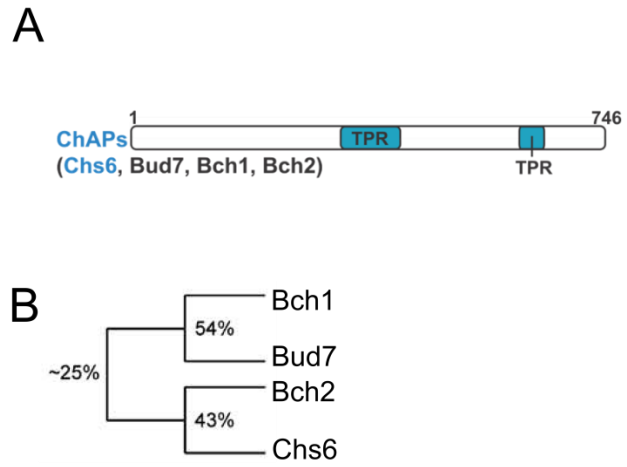


Figure 3.1: Characteristics of the ChAP proteins. (**A**) Domains identified by sequence homology in the ChAP proteins. (**B**) Sequence identity of the four ChAP proteins.

related, as are Bud7 and Bch1, as each of these pairs arose from the whole genome duplication. Some other species of fungi only have two ChAP proteins – one similar to Chs6/Bch2, and one similar to Bud7/Bch1. The ChAP proteins are about 25% identical to each other overall (Trautwein et al., 2006) (Figure 3.1B), so the clues to what makes each ChAP unique are likely in the other 75% of residues. The exomer cargo proteins are transported during slightly different parts of the cell cycle, or in the case of Fus1, only in response to the presence of mating pheromone. These differences could be due to signals in the cargo itself, the ChAPs, or other accessory proteins.

The goal of this work was to explore possible features of Chs6 or mechanisms of its regulation that could contribute to the trafficking pattern of Chs3. I determined that the level of Chs6 protein in the cell varies throughout the cell cycle, although this effect is not necessary in every yeast strain background. The incorporation of the ChAPs into exomer complexes was examined by sizing, finding that different ChAPs show different patterns. Before our lab had obtained the structure of Chs6, many mutations were made based on sequence analysis, and

reported here are the two mutations that affected Chs3 transport to the PM. Together, these results suggest several mechanisms by which Chs6 could be regulated and, in turn, regulate the trafficking of Chs3.

3.3. Materials and methods

Plasmids, strains and antibodies

See Tables 3.1 and 3.2 for a list of plasmids and yeast strains used. Antibodies used were obtained from the following sources: α-GFP from Torrey Pines Biolabs, α-Myc from Sigma, α-Chs5 from Randy Schekman, α-G6PDH from Sigma.

Table 3.1: Yeast strains used in Chapter 3.

Plasmid	Description	Source
SEY6210	MATα his3-Δ200 leu2-3,112 lys2-801 trp1-Δ901 ura3-52 suc2-Δ9	(Robinson et al., 1988)
SEY6211	MATa his3-Δ200 leu2-3,112 lys2-801 trp1-Δ901 ura3-52 suc2-Δ9	(Robinson et al., 1988)
CFY256	SEY6211 chs6Δ::KANMX	This study
CFY280	SEY 6211 bud7Δ::TRP1 bch1Δ::KanMX	This study
CFY287	SEY6211 chs6Δ::KanMX Chs3-GFP::his	This study
BY4741a	MATa ura3-Δ0 his3-Δ1 leu2-Δ0 met15-Δ0 GAL+	Research Genetics
CFY239	BY4741a chs6Δ::KanMX	This study
YPH499	MATa ade2-101 his3-Δ200 leu2-Δ1 lys2-801 trp1-Δ63 ura3-52	(Sikorski & Hieter, 1989)
TSY49	YPH499 chs6Δ::HIS3	(Starr et al., 2012)
RSY3540	SEY6210 Chs6-GFP::HIS3	Schekman lab
CFY340	SEY6211 Chs6-Myc::TRP1	This study
CFY182	SEY6210 Bud7-GFP::KanMX	This study
CFY262	SEY6211 Bch1-GFP::KanMX	This study

Table 3.2: Plasmids used in Chapter 3.

Plasmid	Description	Backbone	Source
pRS416	Centromeric URA3 plasmid		(Sikorski & Hieter, 1989)
pCF1028	Chs6	pRS416	This study
pCF1029	Bud7	pRS416	This study
pCF1030	Bch1	pRS416	This study
pAS28	Chs6 - NheI restriction site before start	pRS416	This study
pAS29	Bud7 - NheI restriction site before start	pRS416	This study
pAS30	Bch1 - NheI restriction site before start	pRS416	This study
pAS40	Bud7 promoter - Chs6	pRS416	This study
pAS42	Bch1 promoter - Chs6	pRS416	This study
pAS62	Chs6-Myc	pRS416	This study
pAS63	Bud7 promoter - Chs6-Myc	pRS416	This study
pJC4	Chs6 F590A Y594A D596K	pRS416	This study
pJC7	Chs6 F590A	pRS416	This study
pJC8	Chs6 Y594A	pRS416	This study
pJC9	Chs6 D596K	pRS416	This study
pAS90	Chs6-Myc R146E R148E D150K D152K	pRS416	This study
pAS91	Chs6-Myc R146A R148A D150A D152A	pRS416	This study

Cell synchronization

Nocodazole

Yeast were grown in either YPD or synthetic complete (SC) dropout media with 1% DMSO added, at 30°C, until early log phase (~0.3 OD600). 1.5 mg/ml Nocodazole (Sigma) in DMSO was added to a final concentration of 15 μg/ml and cultures were incubated for 1.5 hours until at least 95% of cells had buds nearly the size of the mother cell. To release from synchronization, cells were centrifuged at 3,000 rpm for 2 minutes, washed in at least 1 volume media warmed to 30°C, and resuspended in warmed media to 0.3 OD600.

α-Factor

Yeast were grown in either YPD or synthetic complete (SC) dropout media at 30°C until early log phase (~0.3 OD600). α-Factor (Sigma) was added to a final concentration of 5 μg/ml

and cultures were incubated for 1.5 to 2 hours until less than 5% of cells had buds and most had adopted a "shmoo" conformation. To release from synchronization, cells were centrifuged at 3,000 rpm, washed in at least 1 volume media warmed to 30°C, and resuspended in warmed media to an OD600 of 0.3.

Microscopy

Cells were grown to log phase (OD_{600} ~ 0.5) in synthetic dropout media, and imaged on a DeltaVision RT wide-field deconvolution microscope (Applied Precision). Images were deconvolved in SoftWoRx 3.5.0 software (Applied Precision) and min/max light levels adjusted for clarity in ImageJ (Abramoff et al., 2004) with levels kept consistent within each experiment.

Sizing by gel filtration

150 OD600 of yeast cells were pelleted from culture at log phase (~0.5 OD600). Cells were washed with 10 ml cold TE, separated into six tubes, and pelleted. After removing supernatant, pellets were frozen in liquid nitrogen. 1 ml PBS with 2X Protease Inhibitor (Roche) was added to each tube with 500 µl glass beads and vortexed 3 times for 10 minutes each time, with at least 1 minute on ice between. Lysate was centrifuged 13,000 rpm at 4°C for 5 minutes to clear debris, then supernatant was centrifuged at 55,000 rpm for 30 minutes. 50 µl trichloroacetic acid (TCA) was added to each tube in the fraction collector and 0.5ml of cleared lysate was separated by gel filtration chromatography (Superose 6; GE Healthcare). Collected TCA-precipitated samples were pelleted and resuspended in sample buffer for analysis by SDS-PAGE and Western Blot.

Co-immunoprecipitation

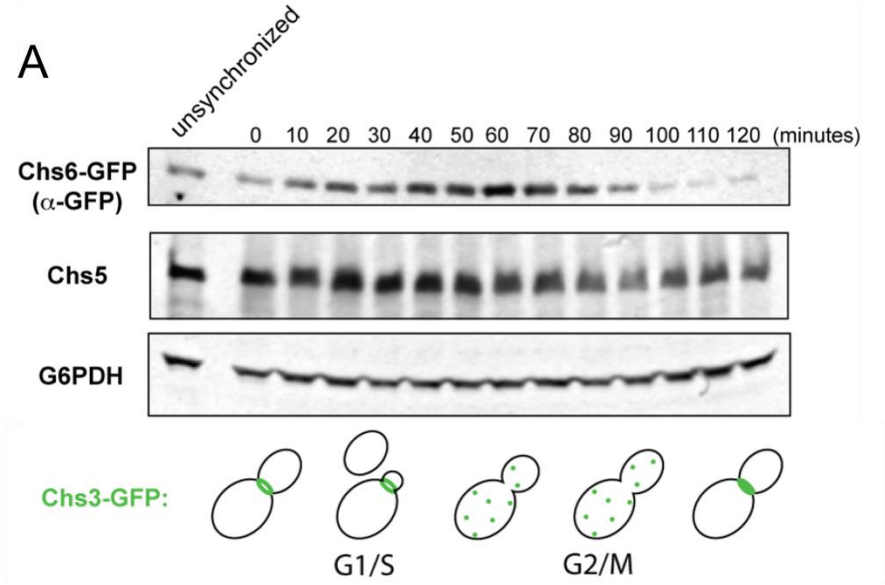
Yeast culture was grown to log phase and 25 OD600 of yeast cells were pelleted. Cells were washed with 1 ml cold TE, transferred to a 2 ml tube, and pelleted. After removing supernatant, pellet was flash frozen in liquid nitrogen (and in some cases, stored at -80°C). 1 ml lysis buffer (50 mM Tris pH 7.5, 0.2% Tween-20, 150 mM NaCl, 5 mM EDTA, 1X Protease Inhibitor (Roche), 1 mM PMSF) was added to pellet with 500 µl glass beads and sample was vortexed 3 times for 10 minutes each time, with at least 1 minute on ice between. Sample was centrifuged 13,000 rpm at 4°C for 5 minutes to clear debris. 10 µl of α-HA resin (Sigma) was added to lysate and incubated overnight. Beads were pelleted at 4,000 rpm for 1 minute and washed 3 times with 1 ml lysis buffer. 10 µl of 2x urea sample buffer was added and samples heated at 55°C for at least 15 minutes before analysis by SDS-PAGE and Western Blot.

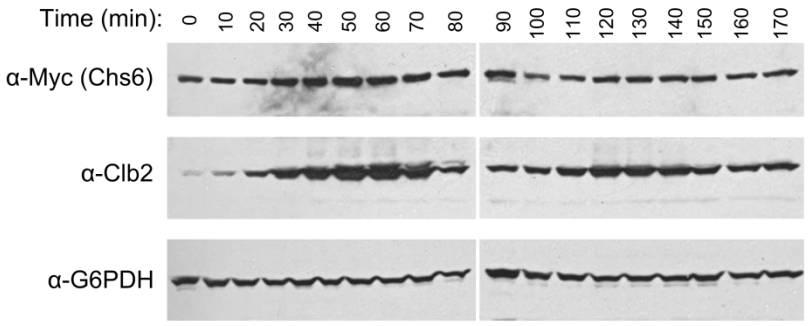
3.4. Results

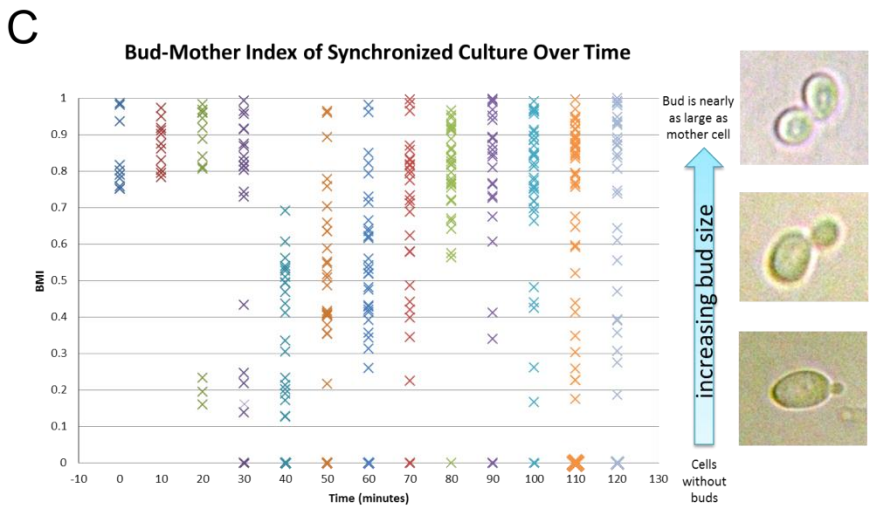
Chs6 protein level varies throughout the cell cycle.

Chs3 is transported from the TGN to the PM only at specific times in the cell cycle, but the cell cycle-dependent regulatory mechanism is unknown. To determine whether the level of exomer proteins changes throughout the cell cycle, we synchronized cultures of yeast cells, and assayed the level of proteins at time points after release from synchronization. After synchronizing cells with nocodazole, which arrests cells just before mitosis, Chs6 levels increased and then decreased over one 120-minute cell cycle, peaking around 60 minutes (Figure 3.1A). In these same samples, Chs5 levels stayed fairly constant throughout the cell cycle. When compared to the levels of the cyclin Clb2, which accumulates during G2 and is degraded upon entry into mitosis, Chs6 levels peak slightly earlier, around the beginning of G2 (Figure 3.2B).

Figure 3.2: Chs6 expression varies throughout the cell cycle. (**A**) Western blot analysis of protein levels in cells at indicated time points after release from synchronization with Nocodazole. Chs6-GFP as indicated by α-GFP blot (top), Chs5 levels from the same cell lysates (middle) and G6PDH loading control (bottom). (**B**) Chs6-Myc levels through the cell cycle compared to the cyclin Clb2 using methods as in (A). (**C**) Verification of the efficacy of Nocodazole synchronization. Bud-Mother Index is the ratio of bud length to mother length.


A
unsynchronized
0 10 20 30 40 50 60 70 80 90 100 110 120 (minutes)
Chs6-GFP
(α-GFP)
Chs5
G6PDH
Chs3-GFP:
G1/S
G2/M


B
Time (min): 0 10 20 30 40 50 60 70 80 90 100 110 120 130 140 150 160 170
α-Myc (Chs6)
α-Clb2
α-G6PDH


C
Bud-Mother Index of Synchronized Culture Over Time
BMI
Time (minutes)
Bud is nearly as large as mother cell
increasing bud size
Cells without buds

To verify that cells were progressing through the cell cycle concurrently after my synchronization procedure, I measured the Bud-Mother Index (BMI) of cells at 10 minute time points. The BMI is the ratio of the length of the bud, from bud neck to tip, to the length of the mother. The range of BMI values widened as the cell cycle progressed, indicating that cells remained well synchronized until around the end of one cell cycle (Figure 3.2C).

To determine whether this variation in expression level is a feature of all of the ChAP proteins, we examined Bud7 levels after synchronization. These levels remained constant through the cell cycle (Figure 3.3), indicating the variation is specific to certain ChAPs.

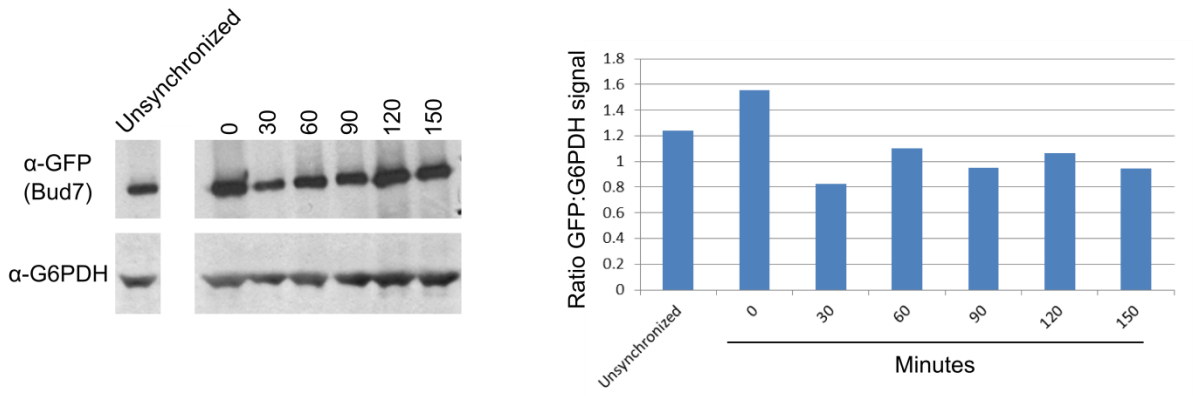


Figure 3.3: Bud7 levels remain constant throughout the cell cycle. Cell lysates at indicated times after release from nocodazole synchronization analyzed by Western blot with α-GFP to detect Bud7-GFP.

Promoter-swapping of the ChAPs does not disrupt Chs3 transport.

Since Chs6 levels change throughout the cell cycle and Bud7 levels do not, I hypothesized the variation in Chs6 levels may play a role in its function. To test this, I created a set of "promoter-swapped" plasmids in which each of the proteins Chs6, Bud7 and Bch1 were under the control of the promoter of each of the others. The plasmids expressing Chs6 were adequate to rescue CW sensitivity of a *chs6Δ* strain regardless of which promoter was driving Chs6 expression (Figure 3.4A, left). Likewise, expression of either Bud7 or Bch1 under the Chs6 promoter was able to rescue CW sensitivity in the *bch1Δ/bud7Δ* strain (Figure 3.4A, right). Chs3-GFP localization was consistent with these results, as expression of Chs6 under either of the other promoters did not prevent Chs3-GFP from reaching the bud neck or the cytokinetic septum. These results suggest the difference in expression levels of these ChAPs do not play a significant role in efficient Chs3 transport to the PM.

Chs6 promoter is necessary for efficient Chs3 transport in YPH499 strain of yeast.

While the promoter-swapping experiment did not indicate a dependence of Chs6 on its own promoter versus that of Bud7 or Bch1, it is possible that it plays only a subtle role in regulation that is necessary under different conditions. Therefore, I tested these promoter-swapped constructs in several other common laboratory strains of yeast. The SEY6211 strain of yeast is the one used throughout this dissertation. The BY4742 strain is most well known as the background of the ResGen gene deletion collection. Neither of these strains showed any defect in Chs3 transport when Chs6 was paired with the Bud7 promoter. In the YPH499 strain, however, there was a significant increase in CW resistance (Figure 3.5A). The steady-state expression of Chs6 was not significantly different under its own promoter or that of Bud7, in either the

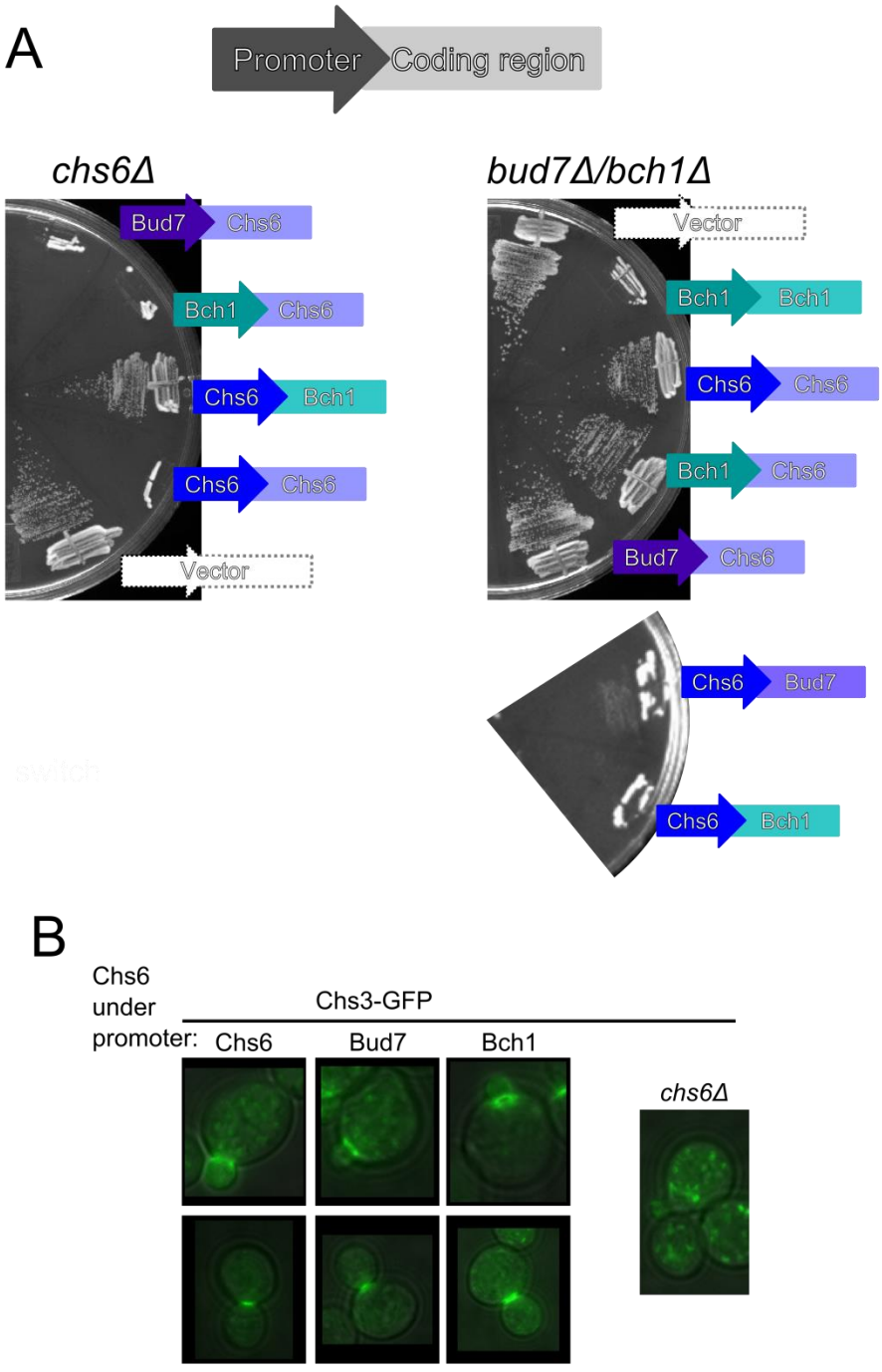


Figure 3.4: Promoter-swapping of ChAP proteins does not lead to Chs3 mislocalization. (**A**) Strains with indicated ChAP protein coding genes deleted, expressing the promoter-swapped ChAP from a plasmid, were grown on 50µg/ml CW at 30°C for two days before imaging. (**B**) Chs3-GFP localization at the neck of small buds (top) or septum during cytokinesis (bottom) with promoter-swapped Chs6 protein expressed from plasmids as in (A). GFP image is overlayed with DIC image to show cell boundaries.

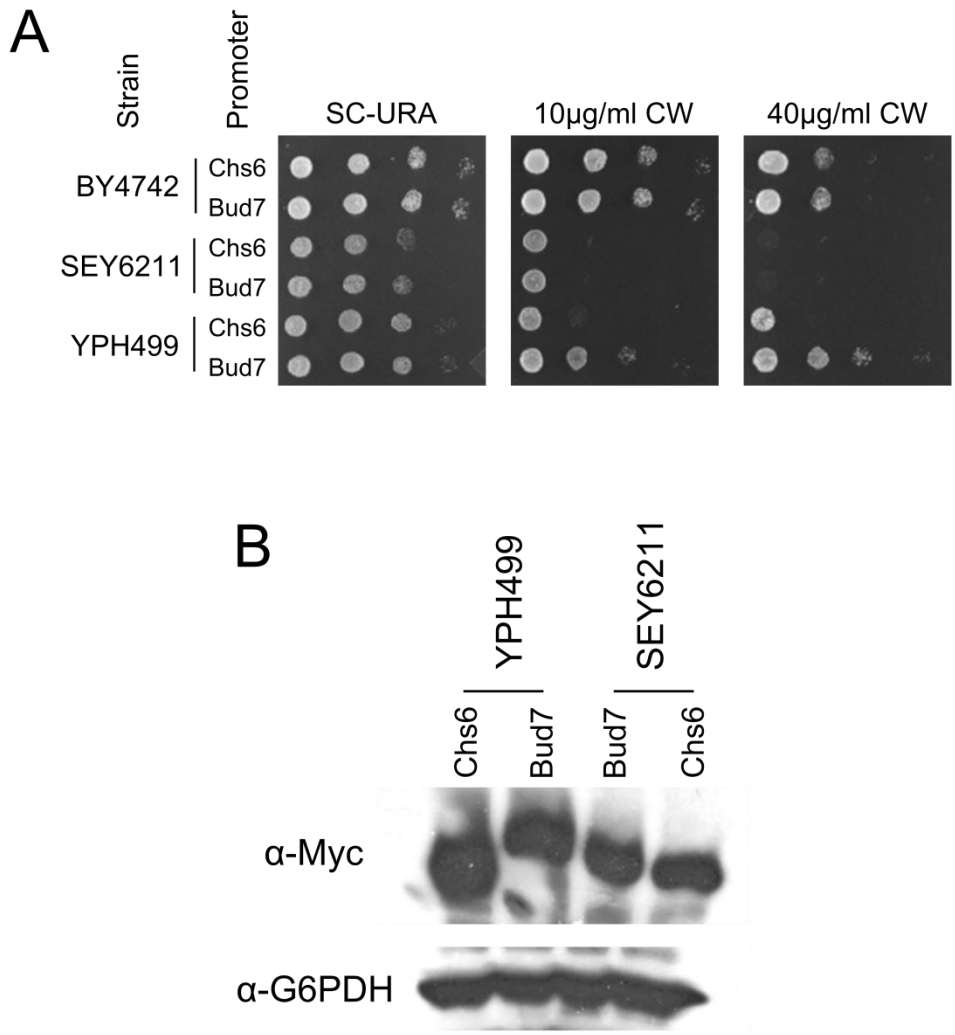


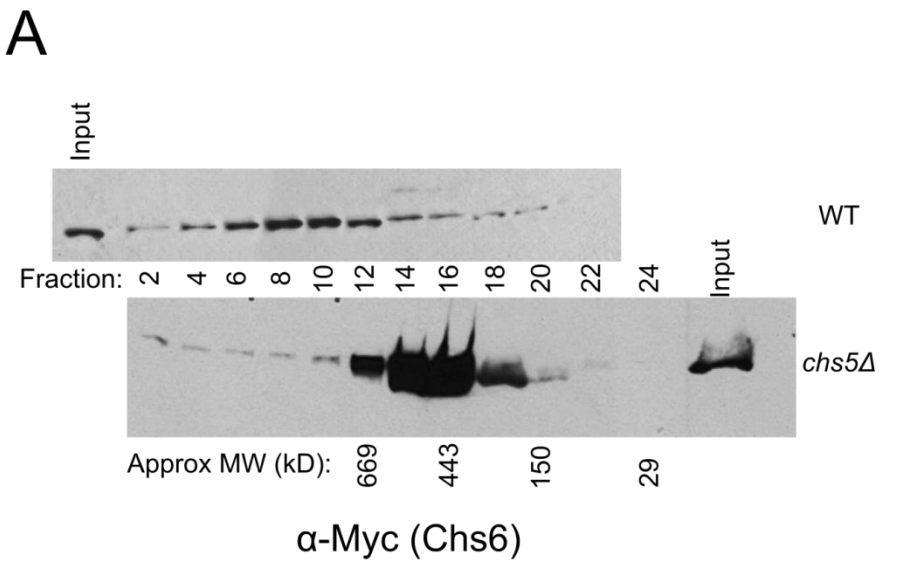
Figure 3.5: Chs6 promoter is important for efficient transport in YPH499 yeast strain. (**A**) Indicated strains with CHS6 gene deleted, expressing the promoter-swapped Chs6 from a plasmid, were grown on 50µg/ml CW at 30°C for two days before imaging. (**B**) Expression of Chs6-Myc under its own promoter or that of Bud7 in each yeast strain, as assayed by α-Myc blot. Loading control is shown at bottom.

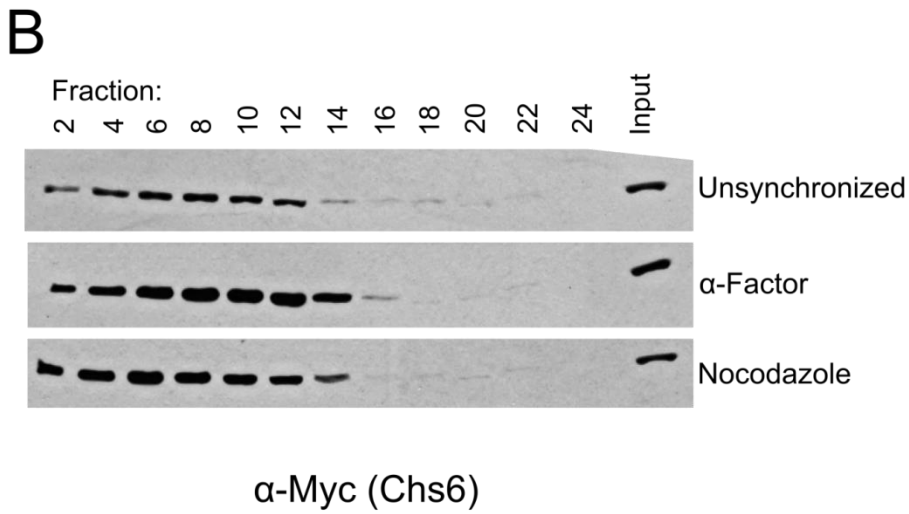
SEY6211 or the YPH499 strain (Figure 3.5B). Therefore, the difference in Chs3 transport in the YPH499 strain must be due to a different factor, such as the variation in expression throughout the cell cycle.

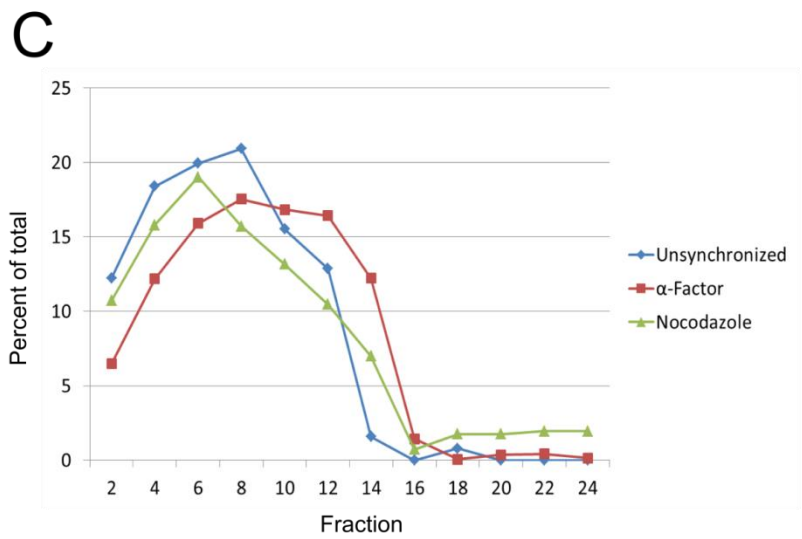
Most Chs6 is present in Chs5-dependent complexes.

Chs6 levels vary throughout the cell cycle, but the total protein level is not necessarily correlated with the number of functional Chs6-containing exomer complexes. To determine what proportion of Chs6 is in a complex with Chs5 at any given time, I used gel filtration chromatography. Cell lysates were centrifuged at high speed to separate lipids, then run on a gel filtration column to separate by size, and the resulting fractions analyzed by Western blot. In otherwise wild type cells, myc-tagged Chs6 was mostly in high molecular weight complexes, eluting even earlier than a 669 kD molecular weight marker (Figure 3.6A). An exomer complex composed of two copies of Chs5 and two copies of Chs6-Myc has a molecular weight of about 350 kD. The difference between the expected molecular weight and the apparent molecular weight observed on the column could be because Chs5 tends to elute at higher apparent molecular weights, likely because of its large, unstructured C-terminal portion. Gel filtration is quite sensitive to the shape of a protein, as long extended shapes cannot sample as much of the space within the resin as compact, globular shapes can. It is also possible I am seeing multiple exomer complexes interacting with each other, or there are other accessory proteins binding that have not yet been identified. To test whether these Chs6-containing complexes involved Chs5 as well, I performed the same experiment in a *chs5Δ* strain. Under this condition, the Chs6 signal shifted to later eluting fractions (Figure 3.6A) indicating it was in smaller species, although still larger than expected for monomeric Chs6. This indicates Chs5 is required for the formation of

Figure 3.6: Most Chs6 protein is present in Chs5-dependent complexes. (**A**) Fractions from gel filtration chromatography of cell lysates were analyzed by SDS-PAGE and Western blotting with α-Myc. Experiment was performed in otherwise WT cells (top) and *chs5Δ* cells (bottom). Fractions in which MW standards normally elute are indicated at bottom. (**B**) Sizing of Chs6-containing complexes in unsynchronized cells as performed in (A) (top), and in populations synchronized with α-Factor (middle) or nocodazole (bottom). (**C**) Quantification of Western blot in (B).


A
Input
WT
Fraction: 2 4 6 8 10 12 14 16 18 20 22 24 Input
chs5Δ
Approx MW (kD): 669 443 150 29
α-Myc (Chs6)


B
Fraction:
2 4 6 8 10 12 14 16 18 20 22 24 Input
Unsynchronized
α-Factor
Nocodazole
α-Myc (Chs6)


C
Percent of total
25
20
15
10
5
0
2 4 6 8 10 12 14 16 18 20 22 24
Fraction
Unsynchronized
α-Factor
Nocodazole

the exomer complexes seen in wild type cells, consistent with previous reports (Sanchatjate & Schekman, 2006).

To examine whether Chs6 incorporation into a larger complex varies throughout the cell cycle, I synchronized cells at two different points in the cell cycle and sized the complexes as before. Nocodazole arrests cells at the G2/M transition, producing cells with very large buds, while α-factor induces a mating response in mating type "a" cells, arresting them before bud formation occurs. There was no significant difference in Chs6 distribution throughout the sizing fractions whether the cultures were unsynchronized or synchronized with either method (Figure 3.6B,C).

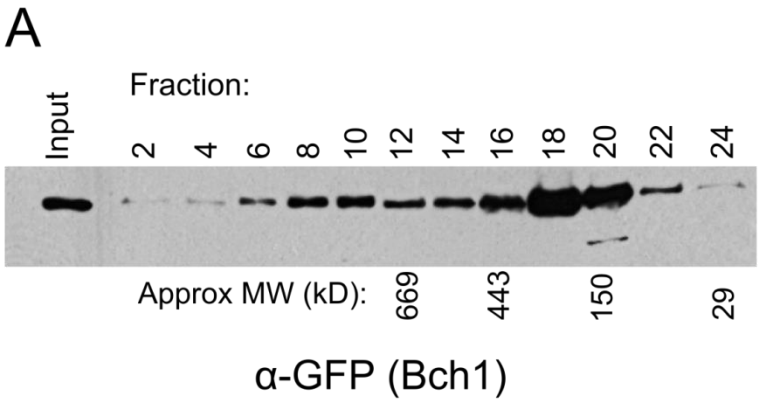
Bch1 incorporation into complexes increases upon synchronization.

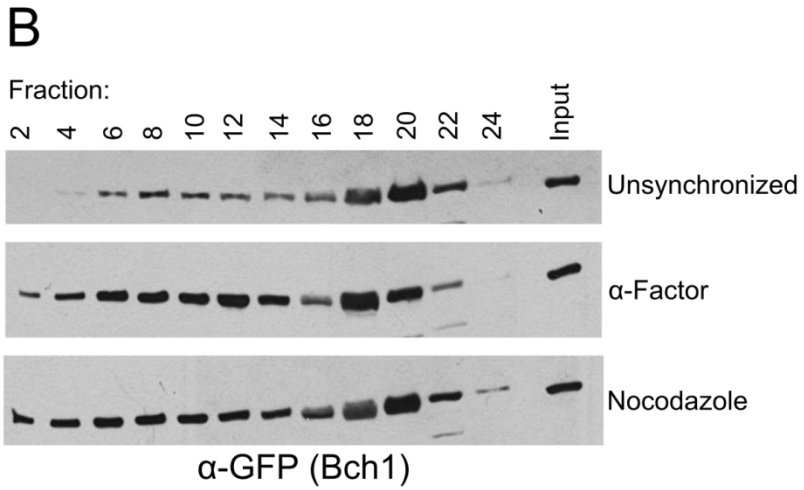
Unlike Chs6, which eluted mostly in high molecular weight complexes, Bch1 eluted from the column in lower molecular weight fractions (Figure 3.7A). However, when synchronized with nocodazole or α-factor, some of the Bch1 shifts into an apparent higher molecular weight complexes (Figure 3.7B,C). Incorporation into complexes when synchronized with α-factor could be due to the need for Bch1 in complexes to transport Fus1 to the schmoo tip. There may be another exomer cargo requiring Bch1 for its transport that is required just before or during mitosis, causing the incorporation into complexes when synchronized with nocodazole.

Chs5 incorporation into complexes increases slightly upon synchronization.

Chs5 incorporation into complexes was more difficult to assay because there is some background signal from the α-Chs5 antibody (Figure 3.8A, bottom) and many degradation products of Chs5 (Figure 3.8A, top). The long, flexible C-terminal portion of Chs5 may be easily

Figure 3.7: Bch1 is incorporated into higher molecular weight complexes upon synchronization. (**A**) Fractions from gel filtration chromatography of cell lysates were analyzed by SDS-PAGE and Western blotting with α-GFP for Bch1-GFP. (**B**) Sizing of Bch1-containing complexes in unsynchronized cells as performed in (A) (top), and in populations synchronized with α-Factor (middle) or nocodazole (bottom). (**C**) Quantification of Western blot in (B).


A
Fraction:
Input
2
4
6
8
10
12
14
16
18
20
22
24
Approx MW (kD):
669
443
150
29
α-GFP (Bch1)


B
Fraction:
2
4
6
8
10
12
14
16
18
20
22
24
Input
Unsynchronized
α-Factor
Nocodazole
α-GFP (Bch1)

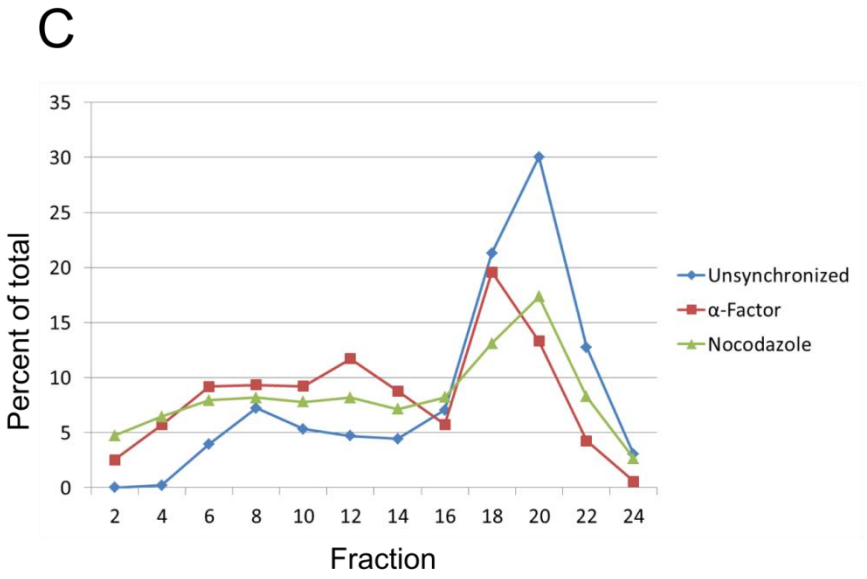
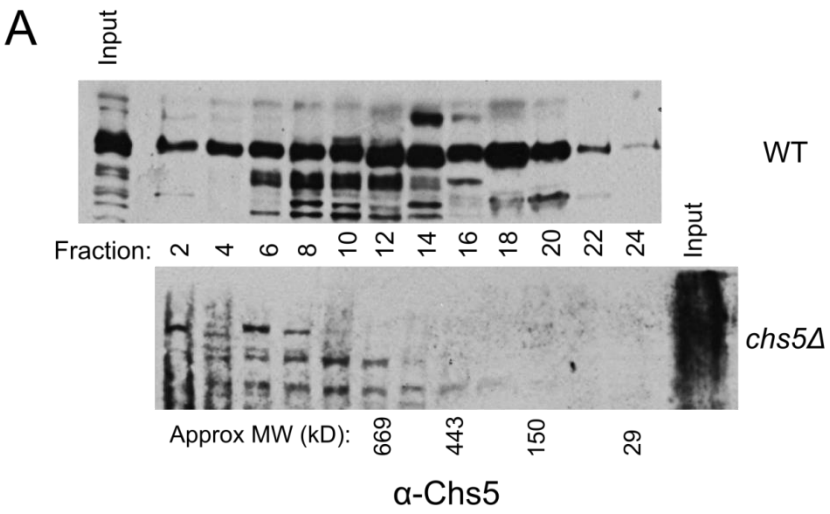
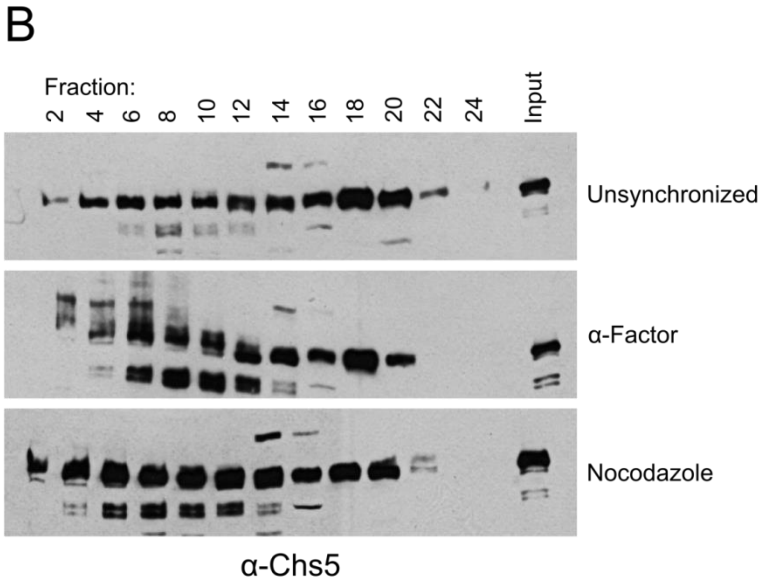
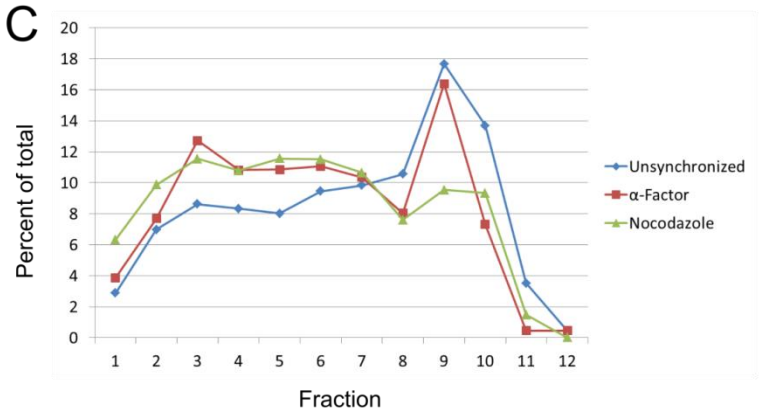

C
Percent of total
35
30
25
20
15
10
5
0
2
4
6
8
10
12
14
16
18
20
22
24
Fraction
Unsynchronized
α-Factor
Nocodazole

Figure 3.8: Chs5 incorporation into exomer complexes. (**A**) Fractions from gel filtration chromatography of cell lysates were analyzed by SDS-PAGE and Western blotting with α-Chs5. (**B**) Sizing of Chs5-containing complexes in unsynchronized cells as performed in (A) (top), and in populations synchronized with α-Factor (middle) or nocodazole (bottom). (**C**) Quantification of Western blot in (B).


A
Input
WT
Fraction: 2 4 6 8 10 12 14 16 18 20 22 24 Input
chs5Δ
Approx MW (kD): 669 443 150 29
α-Chs5


B
Fraction:
2 4 6 8 10 12 14 16 18 20 22 24 Input
Unsynchronized
α-Factor
Nocodazole
α-Chs5


C
Percent of total
20
18
16
14
12
10
8
6
4
2
0
1 2 3 4 5 6 7 8 9 10 11 12
Fraction
Unsynchronized
α-Factor
Nocodazole

cleaved by proteases, despite the protease inhibitors included in the buffers used. Chs5 appeared in most fractions, indicating it can be present in many different sizes of complexes. Upon synchronization with α-factor there was a very slight shift of Chs5 toward larger complexes, and this shift was more significant when synchronized with nocodazole (Figure 3.8B). As Bch1 incorporation into complexes increased in synchronized cells (Figure 3.7B), it is logical that more Chs5 would be incorporated into complexes and more exomer complexes may form under these conditions.

Chs6 mutations that disrupt Chs3 trafficking.

Before our lab had solved a structure of an exomer complex (Paczkowski et al., 2012), I made a number of mutations in Chs6 based on conservation among all ChAPs, and conservation among only Ch6 homologues. The purpose of these experiments was to determine which residues were important for binding Chs5, binding cargo, recruitment by the small GTPase Arf1, or membrane interaction. Most of these mutations did not disrupt Chs3 function as assayed by CW resistance or Chs3-GFP localization (see Chapter 4: Table 4.4 and Figure 4.4F).

The first mutation that did disrupt Chs3 transport, as assayed by CW resistance, was F590A/Y594A/D596K, referred to as Chs6(FYD)-Myc (Figure 3.9A). This construct was designed by the author but constructed by an undergraduate researcher, Jodie Chang. This mutated protein was present in cells at levels equal to the wild type (Figure 3.9B), indicating the disruption is not due to decreased expression or stability. By co-immunoprecipitating wild type or mutant Chs6-Myc with Chs5, I determined that Chs6(FYD)-Myc was unable to interact with Chs5. This suggests the defect in Chs3 transport may be due to the inability of Chs6(FYD)-Myc to be incorporated into exomer complexes. Examining the location of these residues within the

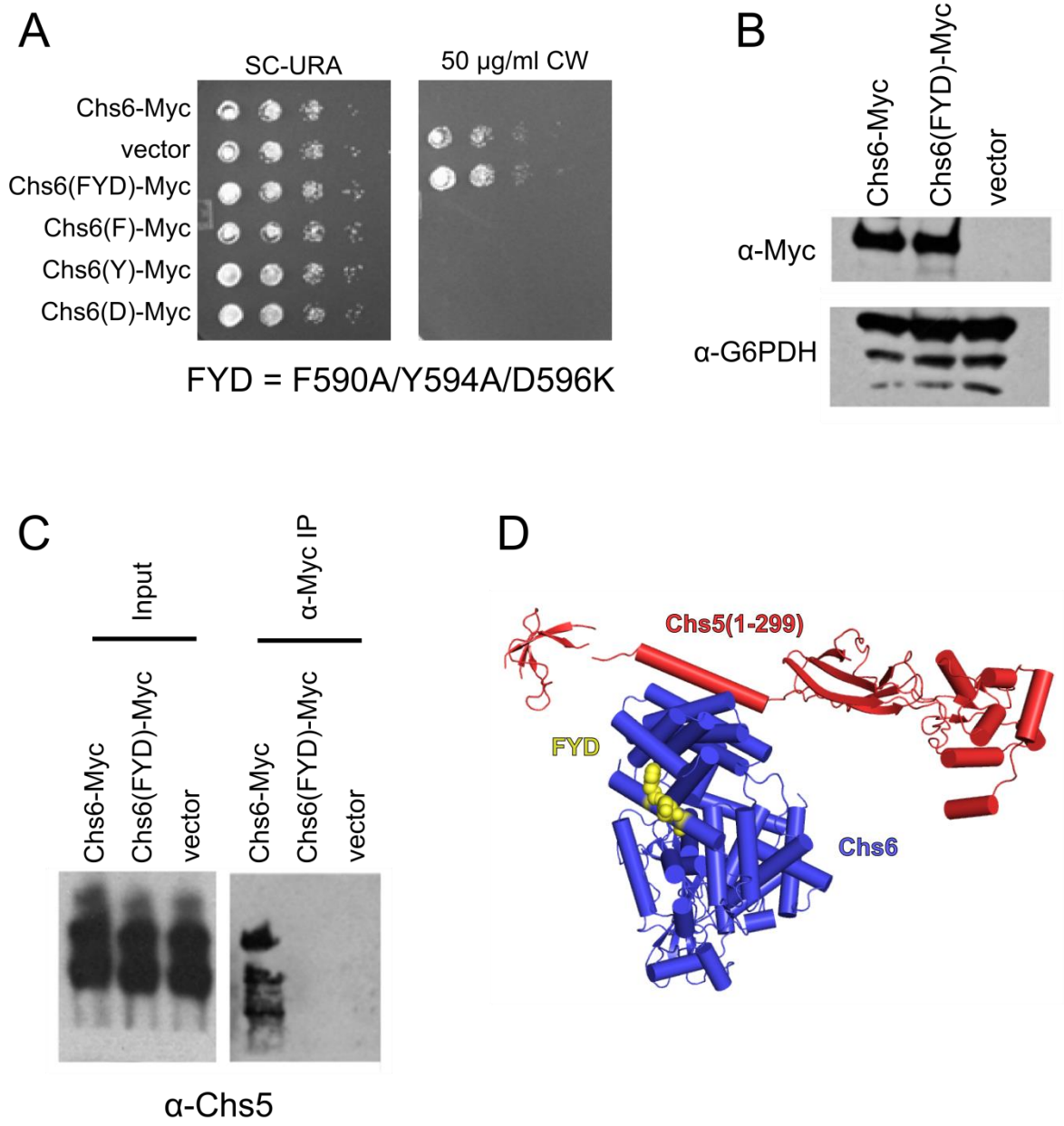


Figure 3.9: A mutation in Chs6 disrupts interaction with Chs5. (**A**) Plasmids expressing Chs6-Myc with the indicated residues mutated were transformed into *chs6Δ* strain which was grown on CW plates for 2 days before imaging. Cloning to create these plasmids was performed by undergraduate researcher Jodie Chang. (**B**) Total protein level of WT and FYD mutant Chs6-Myc constructs. This experiment was conducted by J.C. (**C**) Co-immunoprecipitation using α-Myc to pull down Chs6-Myc and α-Chs5 to detect interacting Chs5. (**D**) Structure of Chs5(1-299)/Chs6 (PDB ID: 4GNS)(Paczkowski et al., 2012) with mutated residues shown as yellow spheres.

structure of exomer, it is not obvious why these would disrupt the interaction with Chs5, since

the residues are not particularly close to the interaction site. However, the bundle of α-helices

between the mutated site and the Chs5 interaction interface are quite tightly packed, and a disruption of the structure may be propagated through these helices to the interaction site.

Another mutation that disrupted Chs3 transport to the PM, as shown by lack of Chs3-GFP localization to the bud neck, was Chs6(RRDD)-Myc (Figure 3.10A). This mutation consisted of the following residue substitutions: R146E/R148E/D150K/D152K. The level of Chs6(RRDD)-Myc in cells was equivalent to the wild type protein (Figure 3.10B). Co-immunoprecipitation with Chs5 showed that Chs6(RRDD)-Myc is able to interact nearly as well as the wild-type protein. We now know that these residues are in the β-sheet on a surface of Chs6 opposite where it interacts with Chs5. The newest data from our lab indicates this is very close to the surface of Chs6 that interacts with membranes (Paczkowski and Fromme, in press), suggesting it may interfere with exomer membrane binding.

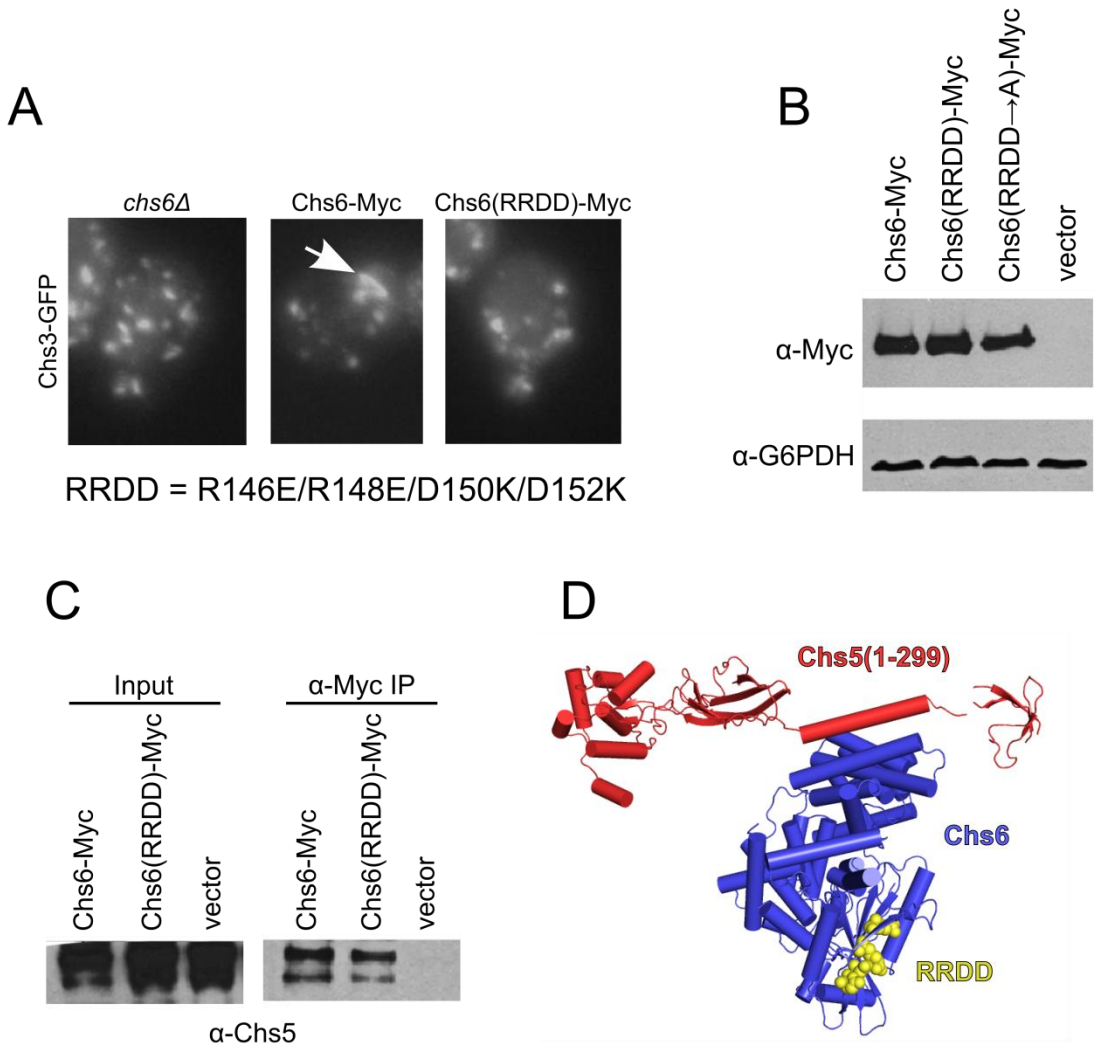


Figure 3.10: A mutation in Chs6 disrupts Chs3 transport. (**A**) Chs3-GFP localization in a *chs6Δ* strain containing plasmids expressing WT or "RRDD" mutant Chs6-Myc. (**B**) Total protein level of WT, RRDD, and a mutant in which the four residues were mutated to alanine instead of a charge reversal. (**C**) Co-immunoprecipitation using α-Myc to pull down Chs6-Myc and α-Chs5 to detect interacting Chs5. (**D**) Structure of Chs5(1-299)/Chs6 (PDB ID: 4GNS)(Paczkowski et al., 2012) with mutated residues shown as yellow spheres.

3.5. Discussion

I have shown that the levels of Chs6 protein vary throughout the cell cycle, while levels of Bud7 do not. However, when Chs6 was placed under the control of the Bud7 promoter, and therefore likely to have a constant rate of expression, it did not affect Chs3 trafficking in the strain of yeast used elsewhere throughout this study. If variations in Chs6 level were to correlate to variations in exomer complex level, it would mean that most of the Chs6 protein at any time was present in an exomer complex. This was shown to be true, whereas Bch1 was shown to be mostly present in monomeric form. Also discussed here were two mutations in Chs6 that perturbed Chs3 trafficking – one that disrupts the interaction of Chs6 with Chs5, and another that we now know is likely to affect membrane association. This ability to evaluate mutations made years ago in the context of our exomer structure can lead to new conclusions about their activity, and perhaps the mutations will be useful in future studies.

The variation in protein level of Chs6 during the cell cycle did not have an effect on Chs3 transport under the normal laboratory conditions we used. In an alternative strain of yeast, however, putting Chs6 expression under the control of the Bud7 promoter did increase CW resistance. This suggests there are conditions under which the varied expression level is important. In addition to differences in strain background, external conditions could also have an effect. For instance, the cell wall protects yeast from osmotic pressure, and it is possible that under mild osmotic stress, a more careful regulation of Chs6 expression level is necessary for cells to thrive.

Unlike the two Chs6 mutations described here, most mutations that I have made in Chs6 have not had an effect on Chs3 transport, as discussed further in Chapter 4. It is possible some of these mutations may have had an effect if the yeast strain were altered in other ways. Since Chs3

transport is also disrupted when both the *BUD7* and *BCH1* genes are deleted, it is possible that deletion of just one of these genes would sensitize cells to Chs6 mutations that did not have an effect in wild type cells.

3.6. Acknowledgements

I would like to thank Jodie Chang for the experiments she performed to contribute to this project, as indicated. I am grateful for protocols and advice from the Smolka and Emr labs, and thank the Emr lab and Bret Judson for use of the microscope and technical assistance with microscopy.

3.7. References

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CHAPTER 4

Distinct N-terminal regions of the exomer secretory vesicle cargo Chs3 regulate its trafficking itinerary[2]

4.1. Abstract

Cells transport integral membrane proteins between organelles by sorting them into vesicles. Cargo adaptors act to recognize sorting signals in transmembrane cargos and to interact with coat complexes that aid in vesicle biogenesis. No coat proteins have yet been identified that generate secretory vesicles from the *trans*-Golgi network (TGN) to the plasma membrane, but the exomer complex has been identified as a cargo adaptor complex that mediates transport of several proteins in this pathway. Chs3, the most well-studied exomer cargo, cycles between the TGN and the plasma membrane in synchrony with the cell cycle, providing an opportunity to study regulation of proteins that cycle in response to signaling. Here we show that different segments of the Chs3 N-terminus mediate distinct trafficking steps. Residues 10-27, known to mediate retention, also appear to play a role in internalization. Residues 28-52 are involved in transport to the plasma membrane and recycling out of endosomes to prevent degradation in the vacuole. We also present the crystal structure of residues 10-27 bound to the exomer complex, suggesting different cargo adaptors could compete for binding to this segment, providing a potential mechanism for regulation.

[2] This work was reproduced from a submitted manuscript: Weiskoff A.M. and Fromme J.C. **Distinct N-terminal regions of the exomer secretory vesicle cargo Chs3 regulate its trafficking itinerary.** Submitted to *Frontiers in Cell and Developmental Biology*, April 2014. Minor formatting modifications have been made.

4.2. Introduction

Eukaryotic cells must transport transmembrane proteins between different subcellular compartments, often in response to specific signals or conditions. This transport is mediated by coat complexes, which help to form the shape of transport vesicles (Bonifacino & Glick, 2004). These coat complexes also contain or interact with adaptor proteins that recognize sorting signals in the cytosolic domains of cargo proteins to sort them into the vesicles.

No coat complexes are known to mediate transport directly from the *trans*-Golgi network (TGN) to the apical plasma membrane (PM) of polarized cells, which corresponds to secretory vesicles in the model organism *Saccharomyces cerevisiae* (budding yeast). Therefore, it remains poorly understood how cells regulate this trafficking step. One of the many transmembrane proteins that follow this route of transport in yeast is the chitin synthase enzyme Chs3. Chs3 cycles between the TGN and the cell surface in a cell cycle dependent manner (Chuang, 1996; Zanolari et al., 2011; Ziman et al., 1998). This localization pattern is reminiscent of other proteins for which localization is regulated by signaling, such as the human Glut4 glucose transporter (Bryant et al., 2002). Chs3 is localized to the bud neck (junction of mother and daughter cells) through its interaction with its activator Chs4, which binds the septin-interacting protein Bni4 (DeMarini, 1997; Reyes et al., 2007).

The transport of Chs3 to the cell surface requires the exomer complex, which acts as a cargo adaptor for Chs3 and other cargos. Exomer consists of the core protein Chs5, and four paralagous adaptor proteins called Chs5-Arf1-binding Proteins (ChAPs): Chs6, Bud7, Bch1, and Bch2. Deletion of the *CHS5* or *CHS6* genes, or simultaneous deletion of the *BUD7* and *BCH1* genes, prevents Chs3 transport to the cell surface. (Santos & Snyder, 1997; Sanchatjate & Schekman, 2006; Trautwein et al., 2006; Starr et al., 2012; Wang et al., 2006)

Two other proteins have been identified as exomer cargos: Fus1, a protein involved in mating, and Pin2, a prion-like domain-containing protein. Transport of Pin2, unlike Chs3, requires Bch1 or Bch2, and requires the C-terminus of Pin2, which has little similarity to any part of Chs3 (Ritz et al., 2014) Fus1 has a sorting signal not found in Chs3 and requires Bch1 and Bud7 for transport (Barfield, 2009). This suggests that exomer recognizes multiple motifs, possibly through interaction with different subsets of ChAPs.

Retention of Chs3 at the TGN requires AP-1, an adaptor protein complex that mediates trafficking between the TGN and endosomes (Valdivia et al., 2002). Disruption of the AP-1 complex partially rescues the phenotype of an exomer deletion, due to escape of Chs3 to the cell surface when both exomer and AP-1 pathways are blocked. Disruption of the Gga1/2 clathrin adaptors has a similar effect (Copic et al., 2007).

It was recently reported that both the exomer dependent transport and the AP-1 dependent retention of Chs3 are mediated by a motif near the N-terminus of the protein, $_{19}$DEESLL$_{24}$ (Starr et al., 2012). An interaction between exomer and the C-terminus of Chs3 was also found to be required for its transport out of the TGN (Rockenbauch et al., 2012).

In this study we examine the interaction between Chs3 and exomer. We find that different regions of Chs3 play different roles in balancing Chs3 traffic to and away from the PM. We also present a crystal structure of a portion of the Chs3 N-terminus bound to the exomer complex. We propose a role for the N-terminus in regulating both transport and retention of Chs3 by facilitating competition between the protein complexes required for these two processes.

4.3. Materials and methods

All yeast strains used in this study are listed in Table 4.1, and plasmids used in this study are listed in Table 4.2.

Microscopy

Cells were grown to log phase (OD_{600} ~ 0.5) in synthetic dropout media, and imaged on a DeltaVision RT wide-field deconvolution microscope (Applied Precision). Images were deconvolved in SoftWoRx 3.5.0 software (Applied Precision) and min/max light levels adjusted for clarity in ImageJ (Abramoff et al., 2004) with levels kept consistent within each experiment.

Exomer purification

Recombinant "core" exomer complex (Chs5 residues 1-77 and Chs6-6xHis) was purified as described for the Chs5(1-299)/Chs6-6xHis construct (Paczkowski et al., 2012). Protein was concentrated to ~25 mg/ml for crystallography, or 5 mg/ml for the interaction assay.

Interaction assay

GST-Chs3 fragment constructs were constructed in the pGEX-2T vector (GE Healthcare) and transformed into Rosetta2 (DE3) *E. coli* cells (Novagen) for expression. 1L culture was grown to ~3 OD_{600} in TB media at 37°C, temperature lowered to 18°C, then expression induced with 240 uM IPTG. After overnight expression, cells were harvested by centrifugation, resuspended in 50 ml PBS buffer with 1 mM DTT, and lysed by sonication. GST fusion proteins were isolated by adding 100 µl equilibrated glutathione resin (G-Biosciences) to 5 ml cleared lysate and incubating with rotation at least 2 hours at 4°C. Resin was washed 3 times with 1 ml

PBS+DTT and resuspended in 500 µl PBS+DTT. 10 µl of 5 mg/ml exomer protein was added and mixture was incubated ~1 hour at 4°C. Resin was washed 3 times with 1 ml PBS+DTT and analyzed by SDS-PAGE and Western blot with anti-6xHis antibody (Covance).

Crystallography

The Chs5(1-77)/Chs6-6xHis exomer complex was co-crystallized with Chs3 peptides (Genscript, 95% purity) using the hanging drop vapor diffusion method. The peptides were resuspended in the precipitant solution (0.3 M ammonium sulfate, 0.1 M citric acid pH 4.0) to a concentration of 100 µM. 1 µl of the peptide solution was mixed with 1 µl of 25 mg/ml exomer, resulting in a molar ratio of 3.85:1 peptide:exomer, and this drop was placed on a cover slip above the precipitant solution. Hexagonal plate-shaped crystals appeared after 5 to 7 days. Crystals were cryoprotected in 0.3 M ammonium sulfate, 0.1 M citric acid pH 4.0, 30% glycerol, and 100 µM peptide. Diffraction data were collected at CHESS (Cornell High Energy Synchrotron Source) beamline A1 and processed using HKL-2000 (Otwinowski & Minor, 1997). The structure was solved by molecular replacement with Phaser in the PHENIX software suite (Adams et al., 2010) using residues 1-77 of Chs5 and all of Chs6 from the Chs5(1-299)/Chs6 exomer complex structure (PDB: 4GNS; Paczkowski et al., 2012). Density for the Chs3 peptide was clearly visible in initial difference maps (Figure 4.7A). The model was refined by manual adjustment in Coot (Emsley et al., 2010) and refinement in PHENIX. Our software is maintained by SBGrid (Morin et al., 2013).

Table 4.1. Yeast strains used in Chapter 4.

Name	Genotype	Source
SEY6210	MATα his3-Δ200 leu2-3,112 lys2-801 trp1-Δ901 ura3-52 suc2-Δ9	(Robinson & Klionsky, 1988)
SEY6210.1	MATa his3-Δ200 leu2-3,112 lys2-801 trp1-Δ901 ura3-52 suc2-Δ9	(Robinson & Klionsky, 1988)
CFY1328	SEY6210 Sec7-Mars::TRP1 chs3Δ::NatMX	This study
CFY1331	SEY6210 Sec7-Mars::TRP1 chs3Δ::NatMX apl2Δ::KanMX	This study
CFY209	SEY6210.1 chs3Δ::KanMX	This study
CFY247	SEY6210.1 chs5Δ::KanMX	(Paczkowski et al., 2012)
CFY267	SEY6210.1 apl2Δ::KANMX	This study
CFY1863	SEY6210 Sec7-Mars::TRP1 chs3Δ::NatMX chs5Δ::His3	This study
CFY1864	SEY6210 Sec7-Mars::TRP1 chs3Δ::NatMX apl2Δ::KANMX chs5Δ::His3	This study

Table 4.2. Plasmids used in Chapter 4.

Name	Description/Protein expressed	Vector Backbone	Source
pRS416	Centromeric URA3 plasmid		(Sikorski & Hieter, 1989)
pRB259	Chs3-GFP	pRS416	Schekman Lab (unpublished)
pAS114	Chs3(Δ10-27)-GFP	pRS416	This study
pAS118	Chs3(13-15→AAA)-GFP	pRS416	This study
pAS119	Chs3(16-18→AAA)-GFP	pRS416	This study
pAS120	Chs3(19-21→AAA)-GFP	pRS416	This study
pAS121	Chs3(22-24→AAA)-GFP	pRS416	This study
pAS122	Chs3(25-27→AAA)-GFP	pRS416	This study
pAS123	Chs3(22,26 S→A)-GFP	pRS416	This study
pAS124	Chs3(22,26 S→D)-GFP	pRS416	This study
pAS125	Chs3(10-12→AAA)-GFP	pRS416	This study
pAS140	Chs3(W1145*)-GFP	pRS416	This study
pAS163	Chs3-GFP(Δ2-52)-GFP	pRS416	This study
pAS165	Chs3(28-30→AAA)-GFP	pRS416	This study
pAS166	Chs3(31-33→AAA)-GFP	pRS416	This study
pAS167	Chs3(35-37→AAA)-GFP	pRS416	This study
pAS168	Chs3(38-40→AAA)-GFP	pRS416	This study
pAS169	Chs3(41-43→AAA)-GFP	pRS416	This study
pAS170	Chs3(44-46→AAA)-GFP	pRS416	This study
pAS171	Chs3(47-49→AAA)-GFP	pRS416	This study
pAS172	Chs3(50-52→AAA)-GFP	pRS416	This study
pAS186	Chs3(Δ28-52)-GFP	pRS416	This study
pAS188	Chs3(Δ2-9)-GFP	pRS416	This study
pGEX-2T	GST gene fusion expression vector		GE Healthcare
pAS137	GST-Chs3(10-27)	pGEX-2T	This study
pAS138	GST-Chs3(1105-1165)	pGEX-2T	This study
pAS151	GST-Chs3(1-52)	pGEX-2T	This study
pAS154	GST-Chs3(1-52, Δ10-27)	pGEX-2T	This study
pAS176	GST-Chs3(1-52, 41-43→AAA)	pGEX-2T	This study
pAS187	GST-Chs3(1-27)	pGEX-2T	This study
pAS189	GST-Chs3(1-52, Δ2-9)	pGEX-2T	This study
pAS190	GST-Chs3(28-52)	pGEX-2T	This study
pETDuet-1	T7-promoter driven expression plasmid		Novagen
pBCR402	Chs5(1-77)/Chs6-6xHis	pETDuet-1	(Richardson & Fromme, 2013)

4.3. Results

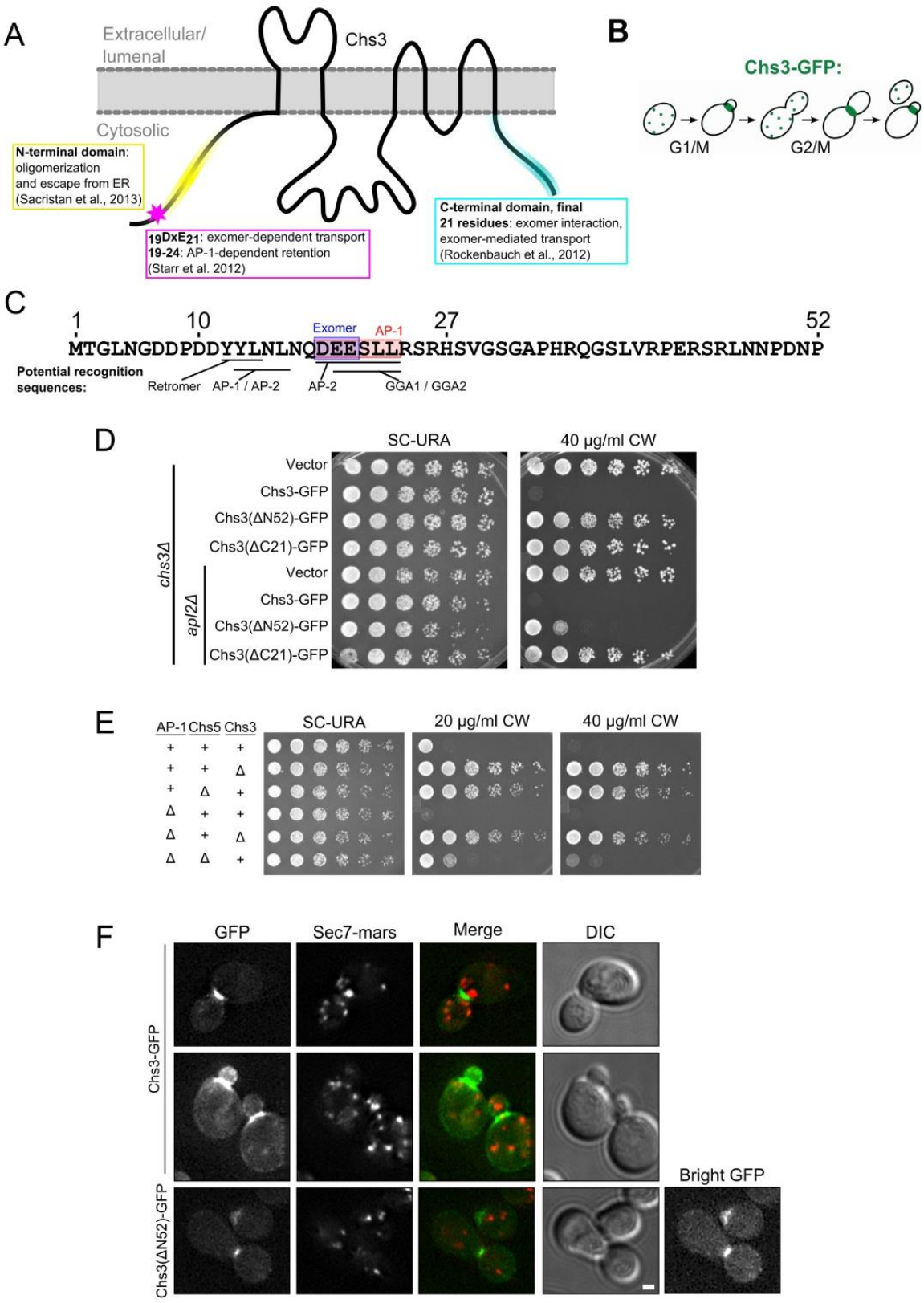
The N-terminus of Chs3 is important for function and localization

Chs3 is a polytopic membrane protein with both termini exposed to the cytoplasm (Figure 4.1A). The cell cycle dependent localization of Chs3 (Figure 4.1B) depends upon its transit through multiple trafficking pathways. Several potential sorting signals reside in the N-terminus of Chs3 (Figure 4.1C).

To assess the importance of the N- and C-termini of Chs3 in its trafficking, we transformed truncated forms of the Chs3 protein into a strain in which Chs3 had been deleted. Chs3 transport defects were assayed by resistance to calcofluor white (CW), which indicates decreased levels of chitin in the cell wall. Increased resistance to CW arises through inactivation or mislocalization of Chs3. All mutations were made in a Chs3-GFP plasmid, which was found to be functional by its ability to rescue normal CW sensitivity levels. Deleting either residues 2-52 (ΔN52) or 1145-1165(ΔC21) of Chs3 caused significant CW resistance (Figure 4.1D).

Disrupting the AP-1 clathrin adaptor complex impairs Chs3 retention within the cell, and therefore will partially rescue mutants that are compromised specifically in TGN to PM trafficking (Figure 4.1E) (Valdivia et al., 2002). CW sensitivity of a strain containing Chs3(ΔN52)-GFP was rescued by deletion of the AP-1 component *APL2* (Figure 4.1D), suggesting this truncation disrupts transport to the PM. In contrast, CW sensitivity of the Chs3(ΔC21)-GFP mutant was not rescued by the AP-1 disruption (Figure 4.1D). This suggests an additional unknown role for the C-terminus of Chs3 in trafficking or activation, in addition to its identified role interacting with the exomer complex (Rockenbauch et al., 2012). Together, these results indicate that both the N-terminus and C-terminus of Chs3 are important for its trafficking, though they play different roles.

Figure 4.1: The N-terminus of Chs3 is important for its function and localization. (**A**) The topology of Chs3 (Sacristan et al., 2013) and regions known to be important for its trafficking. (**B**) The itinerary of Chs3 throughout the cell cycle. (**C**) The N-terminus of Chs3 with sites known to be required for adaptor protein interaction (shaded rectangles) and predicted sorting signals for other cargo adaptors. (**D**) Chs3-GFP mutant plasmids were transformed into both a *chs3*Δ *SEC7-Mars* strain and a *chs3Δ SEC7-Mars apl2Δ* strain and a tenfold dilution series was plated on indicated media. Plates were imaged after 2 days at 30°C. (**E**) Strains with indicated phenotypes were plated on indicated concentrations of CW. All strains are genomic *chs3Δ*. Chs3 "+" strains contain Chs3-GFP plasmid, and other strains contain empty expression vector to support growth on SC-URA. (**F**) Chs3-GFP and Sec7-Mars localization in *chs3Δ* cells. Wild type cells showing bud neck localization in small buds (first row) and at cytokinesis (second row) are represented. Chs3(ΔN52)-GFP intensity at bud neck is very low, so a "bright GFP" image with light levels scaled to increase visibility is included. Scale bar, 1μm.


A
Extracellular/
lumenal
Chs3
Cytosolic
N-terminal domain:
oligomerization
and escape from ER
(Sacristan et al., 2013)
19DxE21: exomer-dependent transport
19-24: AP-1-dependent retention
(Starr et al. 2012)
C-terminal domain, final
21 residues: exomer interaction,
exomer-mediated transport
(Rockenbauch et al., 2012)
B
Chs3-GFP:
G1/M
G2/M
C
1
10
27
52
Exomer
AP-1
MTGLNGDDPDDYYLNLNQDEESLLRSRHSVGSGAPHRQGSLVRPERSRLNNPDNP
Potential recognition sequences:
Retromer
AP-1 / AP-2
AP-2
GGA1 / GGA2
D
SC-URA
40 µg/ml CW
Vector
Chs3-GFP
Chs3(ΔN52)-GFP
Chs3(ΔC21)-GFP
Vector
Chs3-GFP
Chs3(ΔN52)-GFP
Chs3(ΔC21)-GFP
chs3Δ
apl2Δ
E
AP-1 Chs5 Chs3
SC-URA
20 µg/ml CW
40 µg/ml CW
F
GFP
Sec7-mars
Merge
DIC
Bright GFP
Chs3-GFP
Chs3(ΔN52)-GFP

We examined the localization of the N-terminally truncated Chs3-GFP relative to that of the wild type Chs3-GFP. While Chs3 is seen at the bud neck in small buds and at the septum during cytokinesis in both the mutant and wild type strains (Figure 4.1F), the fluorescence intensity at these structures was lower for the Chs3(ΔN52)-GFP than the wild type. Surprisingly, there are fewer visible internal punctae of GFP containing the truncated protein, indicating an additional defect in retention at the TGN. To determine if this mutant protein has reduced expression relative to the wild-type, we performed α-GFP Western blot analysis and observed that the level of expression appears similar to that of the wild-type protein (Figure 4.2). Deletion of residues 1-63 of Chs3 was previously reported to result in Chs3 being retained in the ER (Sacristan et al., 2013), but we did not observe any ER-retention of the ΔN52 mutant. Overall, residues 1-52 appear to play a role in efficient transport to the bud neck, but their deletion does not completely prevent transport to the PM.

Distinct regions of the Chs3 N-terminus mediate different trafficking pathways

Since the deletion of residues 2-52 may remove multiple sorting signals (Figure 4.1C), shorter segments within residues 1-52 (Figure 4.3A) were deleted to more precisely map the function of the N-terminus. Deletions comprising residues 2-9, 10-27, or 28-52 all conferred some resistance to CW (Figure 4.3B). Deletion of residues 2-9 had the mildest phenotype. Deletion of residues 10-27, which contain residues reported to interact with exomer and AP-1 (Starr et al., 2012), had a moderate phenotype. The deletion of residues 28-52 had the greatest effect, nearly equivalent to that of a strain lacking Chs3. These results suggest that residues 28-52 are the most important in this region for the transport of Chs3 to the PM.

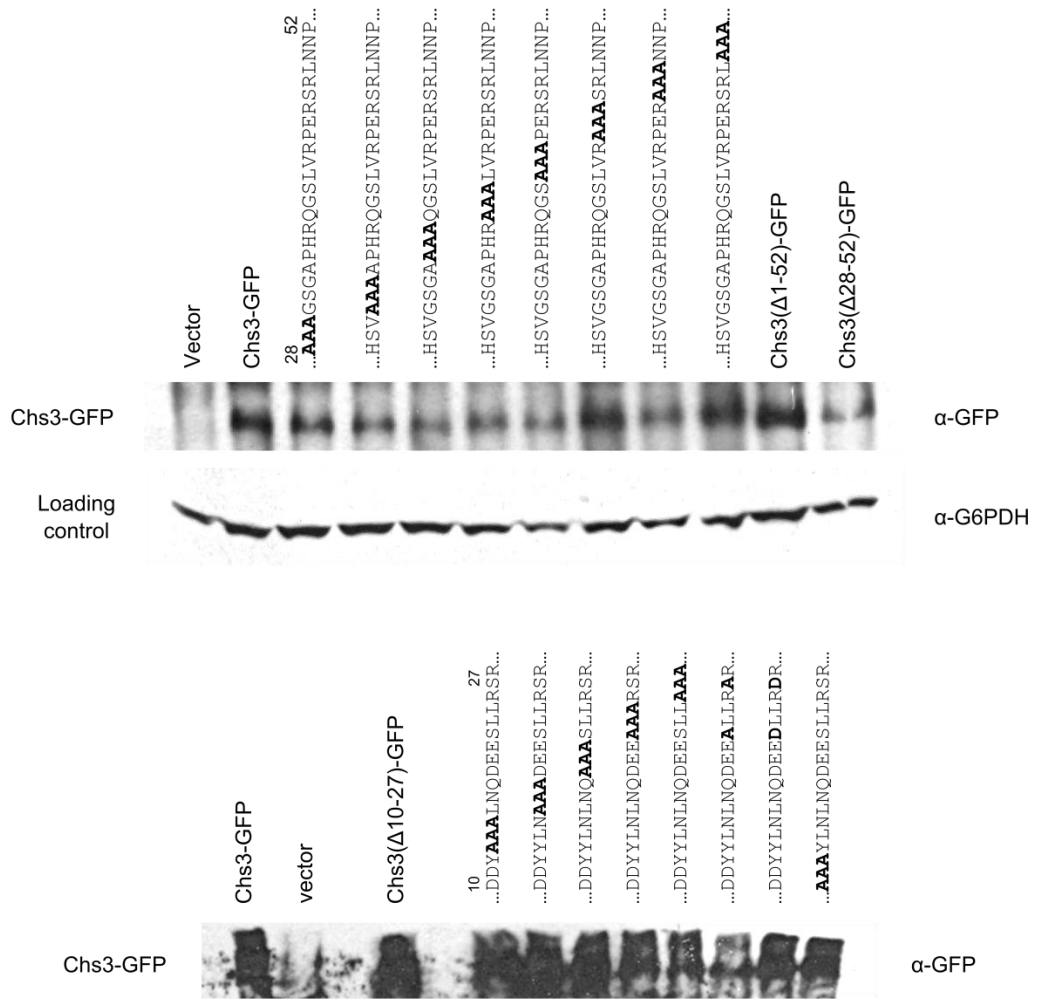


Figure 4.2. Expression of Chs3-GFP constructs. Expression levels of mutated proteins were compared by α-GFP Western blot.

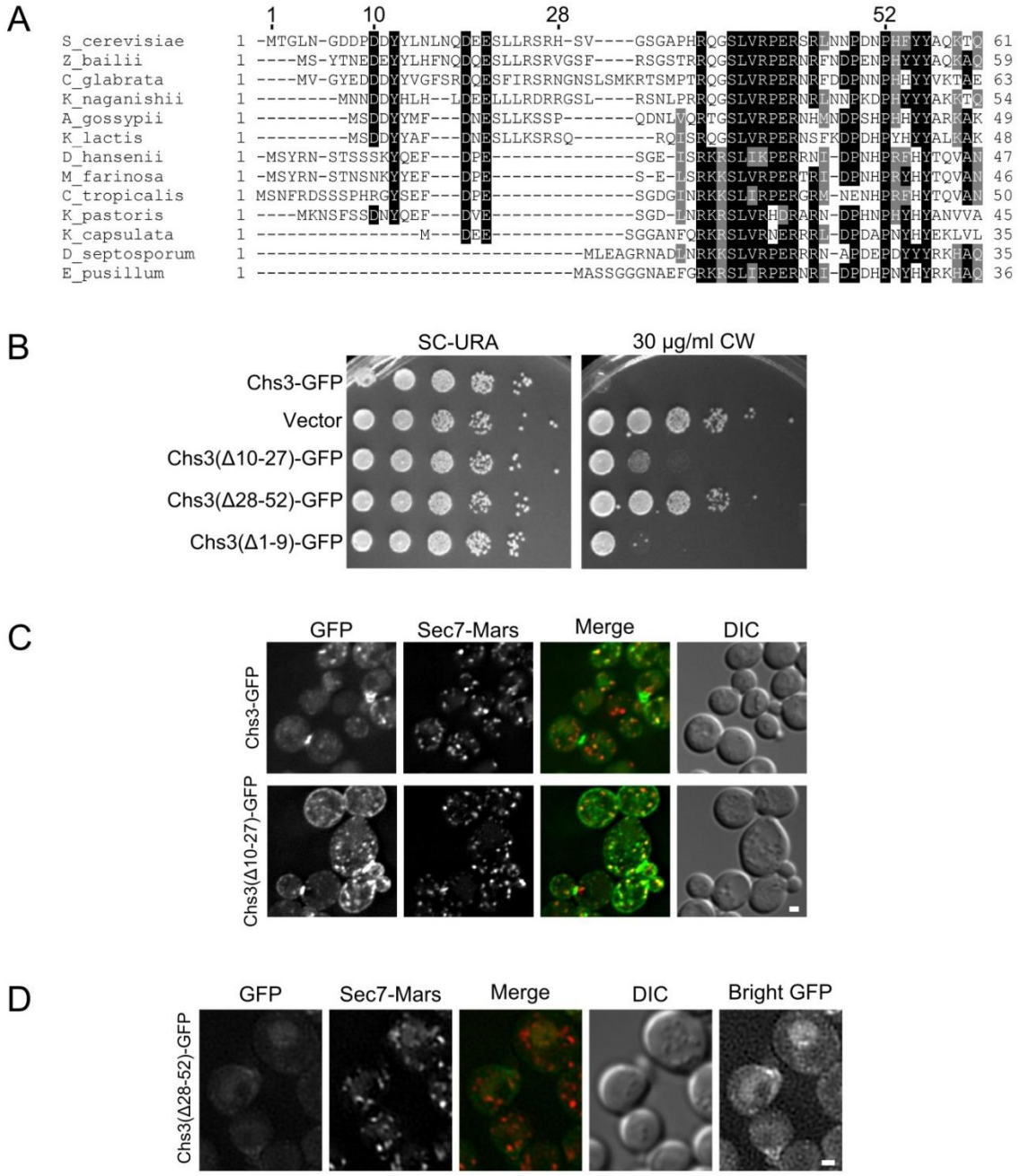


Figure 4.3: Residues 10-27 and 28-52 are important for distinct trafficking steps. (**A**) Multiple sequence alignment of several Chs3 homologues. Residues are shaded to highlight similarity (gray) or identity (black). (**B**) The indicated plasmids were transformed into both a *chs3Δ SEC7-Mars* strain and a *chs3Δ SEC7-Mars apl2Δ* strain and plated on indicated media. Plates were imaged after 2 days at 30°C. (**C**) Chs3-GFP and Sec7-Mars localization in *chs3Δ* cells for WT Chs3-GFP and Chs3(Δ10-27)-GFP, at equivalent light levels. Scale bar, 1µm. (**D**) Chs3(Δ28-52)-GFP localization examined as in (C). Additional "Bright GFP" image with light levels scaled to improve visibility is presented.

The Chs3(Δ10-27)-GFP protein localized to small buds and the primary septum as well as the wild type (Figure 4.3C). However, it also mislocalized to large portions of the PM, especially in non-budded and large-budded cells. This suggests a defect in internalization by endocytosis. Chs3 enzymatic activity requires activation by Chs4, which is held at the bud neck by an indirect interaction with septins (DeMarini, 1997). Therefore, the mutant Chs3 mislocalized throughout the PM is unlikely to be actively producing chitin, likely causing the increased CW resistance we observed.

The Chs3(Δ28-52)-GFP mutant exhibited a different pattern of localization (Figure 4.3D). It localized to the bud neck and septum, but with lower fluorescence intensity than the wild type protein. We also observed GFP in the vacuole, and less localization to TGN punctae. The inability to divert endocytosed Chs3(Δ28-52)-GFP back to the TGN and away from the vacuole could explain the lower level of this protein in cells (Figure 4.2).

Mutation of residues 19-21 leads to increased PM localization

To identify the residues within the 10-27 segment most important for its function, alanine scanning mutagenesis was performed, with groups of three sequential amino acids mutated to alanine. In addition, the two serine residues were mutated, as serine is a potential phosphorylation target. The serines were mutated to either alanine or aspartate, to prevent or mimic phosphorylation, respectively. Two of these mutants had a mild CW resistance phenotype: $_{19}$DEE$_{21}$→AAA, and S24D/S26D (Figure 4.4A).

Since a role for residues 19-21 in exomer-mediated transport is consistent with previous findings (Starr et al., 2012), we also looked at the localization of this mutant protein (Figure 4.4B). Its pattern of localization appeared the same as that of the deletion of residues 10-27

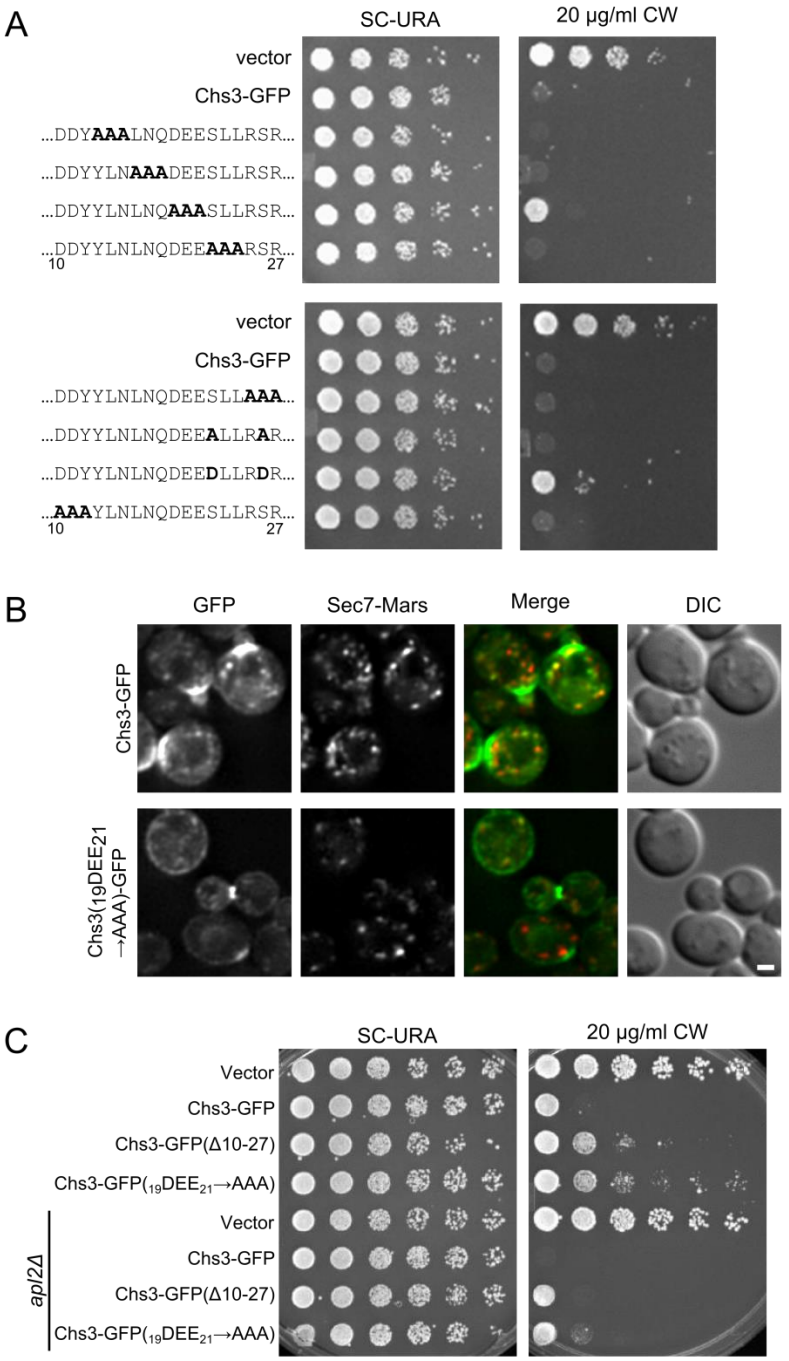


Figure 4.4: Mutation of Chs3 residues 19-21 causes a defect in internalization. (**A**) Chs3-GFP plasmids containing alanine scanning mutants were transformed into a *chs3Δ SEC7-Mars* strain and plated on indicated concentrations of CW. Plates were imaged after 2 days at 30°C. (**B**) GFP and Sec7-Mars localization in *chs3Δ* cells for WT Chs3-GFP and Chs3($_{19}$DEE$_{21}$→AAA)-GFP, at equivalent light levels. Scale bar, 1µm. (**C**) Chs3(Δ10-27)-GFP and Chs3($_{19}$DEE$_{21}$→AAA)-GFP were introduced into the *chs3Δ* and *chs3Δapl2Δ* strains and plated on indicated media. Plates were imaged after 2 days at 30°C.

(Figure 4.3C): normal localization at the bud neck, and mislocalization along the entire PM. This suggests that residues 19-21 are important for internalization and possibly retention of Chs3. Previously these residues were shown to be important for the interaction between Chs3 and AP-1 (Starr et al., 2012), and it is possible they are also important for the interaction of Chs3 with AP-2 during endocytosis, as AP-1 and AP-2 recognize similar sorting signals (Bonifacino & Traub, 2003).

We next determined whether disruption of AP-1 function would rescue the CW sensitivity of the Chs3(Δ10-27)-GFP or Chs3($_{19}$DEE$_{21}$→AAA)-GFP mutants. AP-1 disruption did increase the CW sensitivity of both mutants (Figure 4.4C). This result indicates that AP-1 contributes to retention of the Chs3($_{19}$DEE$_{21}$→AAA)-GFP mutant, and implies AP-1 can still interact with this mutant despite disruption of this motif.

Mutation of residues 41-43 leads to decreased PM localization

We used alanine scanning mutagenesis to determine the most important portions of the segment containing Chs3 residues 28-52. The mutation of residues 41-43 had the strongest CW resistance, equivalent to that of the ΔN52 truncation (Figure 4.5A). Mutations in residues immediately preceding or following these residues (38-40 and 44-46) had more modest CW resistance phenotypes. This indicates a region centered around residues 41-43 is required for normal transport of Chs3. Importantly, this region is highly conserved (Figure 4.3A). The $_{41}$LVR$_{43}$→AAA mutation also affects Chs3 localization, with decreased levels at the bud neck and the septum (Figure 4.5B). This localization defect was slightly less severe than deleting residues 28-52. This could indicate the residues around 41-43 are still able to facilitate transport even when 41-43 are mutated, perhaps by interacting with exomer.

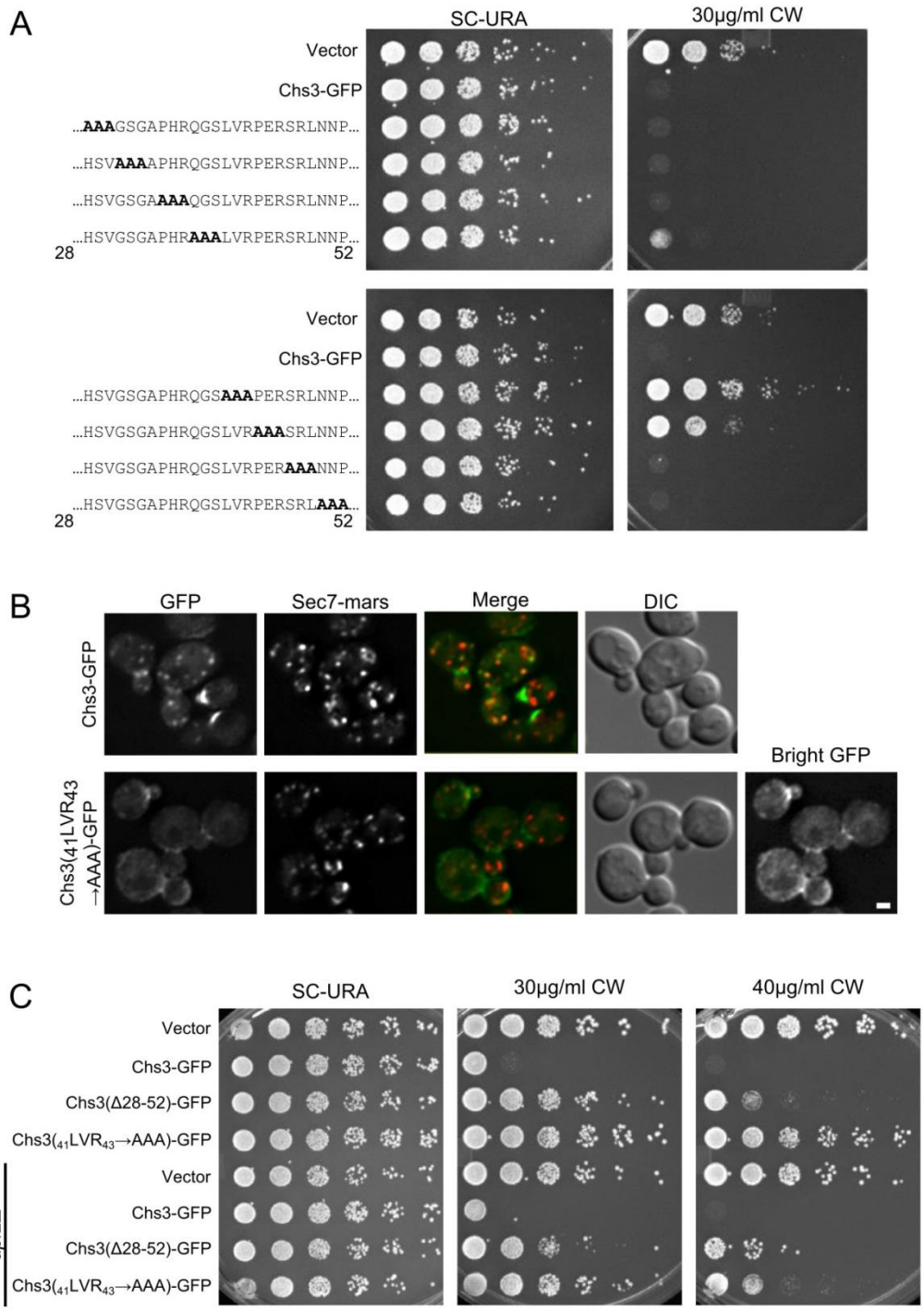


Figure 4.5: Deletion of Chs3 residues 41-43 has a strong defect in transport. (**A**) Chs3-GFP plasmids containing alanine scanning mutants were transformed into a *chs3Δ SEC7-Mars* strain and plated on indicated concentrations of CW. Plates were imaged after 2 days at 30°C. (**B**) GFP and Sec7-Mars localization in *chs3Δ* cells for WT Chs3-GFP and Chs3($_{41}$LVR$_{43}$→AAA)-GFP, at equivalent light levels. Scale bar, 1µm. (**C**) Chs3(Δ28-52)-GFP and Chs3($_{41}$LVR$_{43}$→AAA)-GFP were introduced into the *chs3Δ* and *chs3Δapl2Δ* strains and plated on indicated media. Plates

were imaged after 2 days at 30°C.

Surprisingly, the Chs3($_{41}$LVR$_{43}$→AAA)-GFP mutant was even more resistant to CW than the Chs3(Δ28-52)-GFP mutant (Figure 4.5C). This may suggest a more drastic local structural rearrangement when these residues are mutated to alanine versus what occurs when the entire segment is deleted. Alternatively, more than one trafficking pathway may be disrupted by deletion of the entire segment. The Chs3($_{41}$LVR$_{43}$→AAA)-GFP mutant was partially rescued by the *apl2* deletion (Figure 4.5C), indicating it is still able to interact with AP-1 and suggesting a role for this region in transport to the PM. In contrast, the CW sensitivity of the Chs3(Δ28-52)-GFP mutant was not rescued by *apl2* deletion. This suggests this mutant is not efficiently retained by AP-1, and is consistent with our observation that this mutant is degraded in the vacuole (Figure 4.3D).

Residues 28-52 interact with exomer more strongly than residues 10-27 or the C-terminus

We created an *in vitro* assay to detect the direct interaction of Chs3 fragments with exomer. GST-fusion proteins were immobilized on glutathione resin, which was incubated with exomer complex. Residues 1-52 were able to interact with exomer (Figure 4.6A). Residues 10-27 did not interact with exomer in this assay, and deleting residues 10-27 from the residue 1-52 segment only decreased exomer binding by about half. Together, these indicate residues 10-27 are neither necessary nor sufficient for interaction in this assay. Residues 28-52 were able to interact with exomer even more strongly than residues 1-52 (Figure 4.6B).

A segment at the C-terminus of Chs3 was unable to interact with exomer in this assay (Figure 4.6C), despite previously published evidence that it does interact with exomer (Rockenbauch et al., 2012). The previous report detected an interaction with exomer from cell lysates, rather than with purified exomer as used in our assay, so it is possible that the interaction

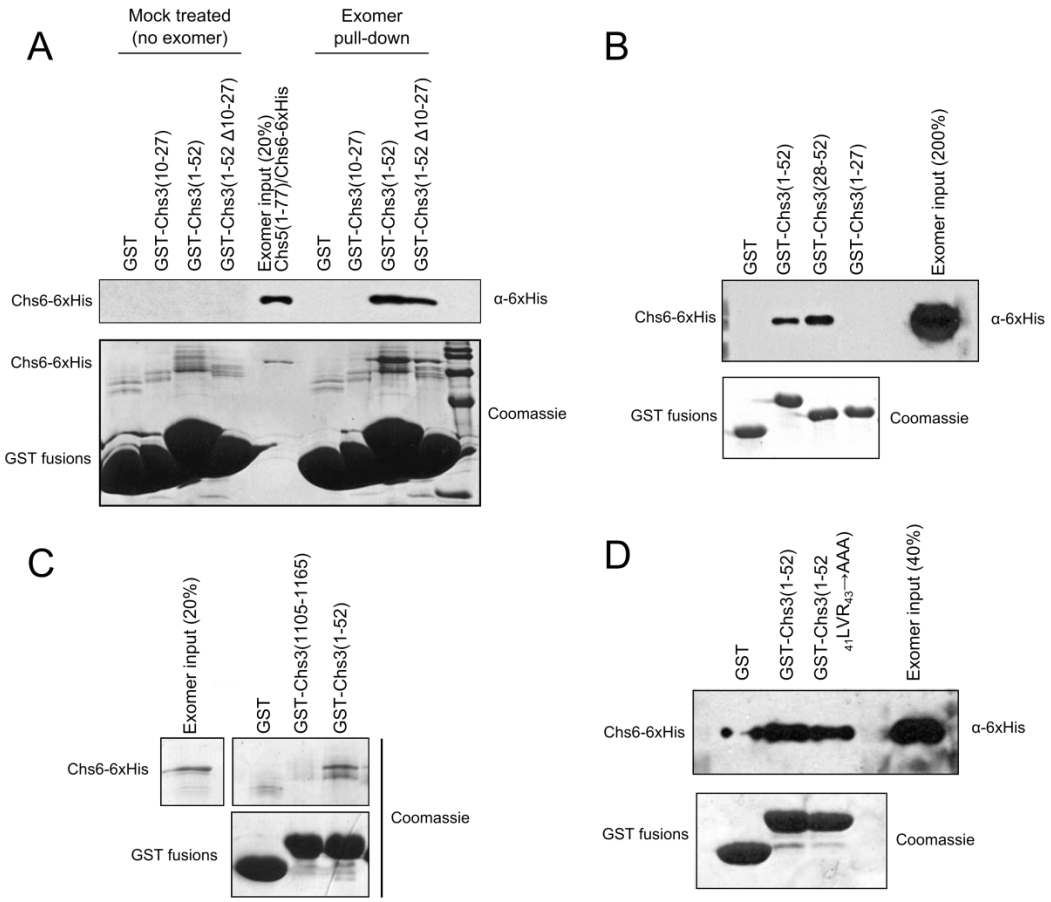


Figure 4.6: Residues 28-52 interact with exomer in vitro. (**A**) *In vitro* interaction assay to measure ability of GST-tagged Chs3 fragments to interact with purified exomer complex. Exomer was detected by α-6xHis antibody which recognized His-tagged Chs6. GST-tagged constructs were detected by Coomassie. In the "mock treated" samples, the final exomer purification buffer was added in place of exomer. (**B**) Chs3 fragment pull-down assay as described in (A) comparing the two halves of the Chs3(1-52) segment. (**C**) Assay as described in (A) to compare interaction of N- and C-termini, but with bound exomer detected by Coomassie staining. (**D**) Assay as described in (A) to analyze effect of $_{41}$LVR$_{43}$→AAA mutation on interaction.

requires another binding partner or a post-translational modification to occur. Alternatively, a more complete Chs5 protein may be required for the interaction, since the exomer complex used here contained only residues 1-77 of Chs5. This truncated Chs5 is not fully functional, but it contains all the structural components necessary to homodimerize and interact with ChAP proteins (Paczkowski et al., 2012), which should allow the exomer complex to interact normally with cargo *in vitro*. Alternatively, post-translational modification of exomer or Chs3 may regulate the interaction.

Since the $_{19}$DEE$_{21}$→AAA mutation had such a strong effect on Chs3 trafficking, we tested its effect on the *in vitro* interaction between Chs3(1-52) and purified exomer. Surprisingly, this mutated fragment interacted as well as the wild-type GST-Chs3(1-52) fragment (Figure 4.6D). The assay may not be sensitive enough to observe a slight reduction in binding affinity, which could strongly affect Chs3 trafficking *in vivo*.

Residues 10-27 interact with exomer based on structural analysis

Obtaining a crystal structure of exomer bound to its sorting signal within Chs3 would elucidate both the location of binding within Chs6, and the structure of that segment of Chs3. Therefore, we attempted to co-crystallize exomer with several peptides from the N- and C-termini of Chs3. Most crystals diffracted well but did not contain the peptide. After screening many crystals, several were found that contained the peptide matching residues 10-27. This peptide formed an alpha-helical structure, and bound to a region on the surface of Chs6 roughly opposite where Chs5 binds (Figure 4.7A, B; Table 4.3). This observed helix is 14 residues in length, and we found residues 12-25 to be the best fit to the density. This indicates residues 10-27 can interact directly with exomer at high concentrations, despite the lack of binding seen in

Figure 4.7: Exomer co-crystalizes with Chs3 residues 10-27. (**A**) 2Fo-Fc map showing electron density around the Chs3 peptide (ball-and-stick modeled) contoured at 2.5σ. (**B**) Model of an exomer complex containing Chs5(1-77) (red), Chs6-6xHis (blue) and a peptide of Chs3 residues 10-27 (yellow, only residues 12-25 are visible). Model was created using Chs5(1-77)/Bch1 structure (PDB ID: 4IN3) (Richardson & Fromme, 2013) as a template, replacing each Bch1 subunit with the structure of Chs6 and peptide from this study. (**C,D**) Close-up views of the interaction of the Chs3 peptide with Chs6. (**E**) Chs6 colored by conservation within Chs6 and Bch2 ChAP proteins, white to blue representing low to high conservation. (**F**) Structure rotated 90° from (A), showing location of Chs6 mutations that had no effect on Chs3 trafficking (green spheres), as listed in Table 4.2.This panel also depicts the crystallographic asymmetric unit.

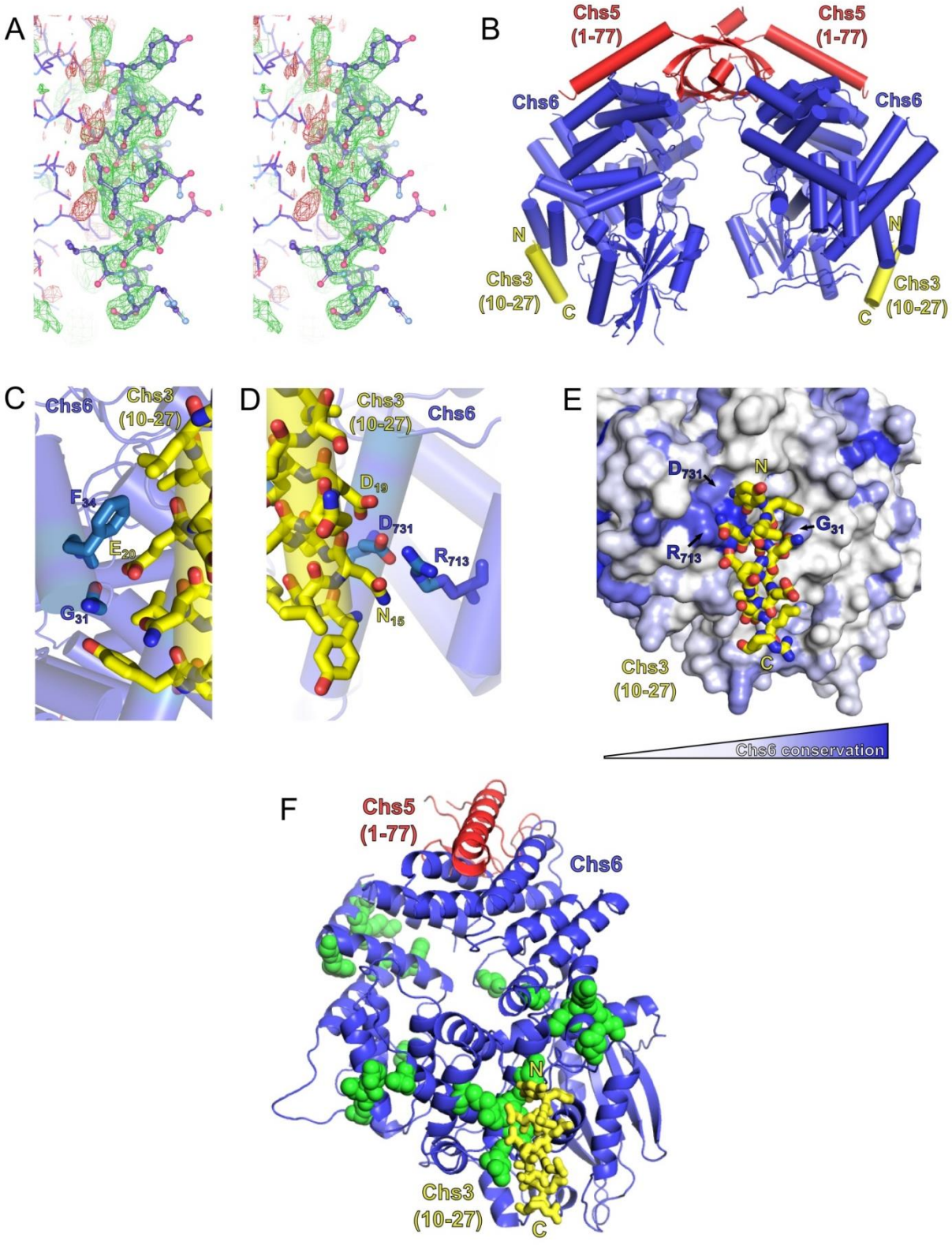

A
B
Chs5
(1-77)
Chs5
(1-77)
Chs6
Chs6
N
N
Chs3
(10-27)
C
C
Chs3
(10-27)
C
Chs6
Chs3
(10-27)
F34
E20
G31
D
Chs3
(10-27)
Chs6
D19
D731
R713
N15
E
D731
N
G31
R713
Chs3
(10-27)
C
Chs6 conservation
F
Chs5
(1-77)
Chs6
N
Chs3
(10-27)
C

Table 4.3. Crystallographic statistics. Values in parentheses represent the highest resolution shell.

Wavelength (Å)	0.9767
Resolution range (Å)	44.65 - 2.59 (2.683 - 2.59)
Space group	P 63 2 2
Unit cell	a = 218.613 Å, b = 218.613 Å, c = 137.848 Å α = 90°, β = 90°, γ = 120°
Total reflections	59,962
Unique reflections	59936 (5730)
Multiplicity	12.2 (10.0)
Completeness (%)	99.00 (96.33)
$< I > / < \sigma(I) >$	19.45 (5.06)
Wilson B-factor	50.18
R_{sym}	0.090 (0.717)
R_{cryst}	0.1796 (0.2202)
R_{free}	0.2107 (0.2534)
Number of atoms	6100
Protein (755 residues)	6008
Water	92
RMS (bonds) (Å)	0.006
RMS (angles) (°)	1.09
Ramachandran favored (%)	94
Ramachandran outliers (%)	1.5
Clash score	7.88
Average B-factor	68.20
Protein	68.40
Water	55.60

our in vitro assay (Figure 4.6B). It is possible other peptides that interact more strongly with exomer disrupted crystal contacts or destabilized the structure, leading to their exclusion from the crystals. This is supported by our observation that when a peptide containing residues 1-52 of Chs3 was added to the cryoprotectant solution, the exomer crystals dissolved. We also note that no additional electron density was visible when crystals were soaked with a peptide corresponding to residues 1128-1165 of the Chs3 C-terminus.

The Chs3 peptide makes direct contact with several residues on the surface of Chs6 (Figure 4.7C-E). Chs3 residue D19 appears to participate in a hydrogen bonding network with Chs6 residues D731 and R713. Chs3 residue E20 appears to hydrogen bond with the main chain carbonyl oxygen of Chs6 residue G31. Both of these Chs3 residues, D19 and E20, are part of the conserved DEESLL sequence that was previously found to be important for interaction with AP-1 (Starr et al., 2012) and that we found to be important for endocytosis of Chs3.

Based on the crystal structure, several mutations were made in the Chs6 protein in an attempt to disrupt the interaction (Figure 4.7F, Table 4.4). These mutated proteins, in addition to many mutations made previously based on sequence conservation (Figure 4.7E), were still able to mediate transport of Chs3. This supports our hypothesis that residues 10-27 are not required for transport of Chs3 to the PM, but rather play a more important role in endocytosis of Chs3. Nevertheless, the structure reveals how this clathrin adaptor motif can be bound by exomer, perhaps providing a basis for competition between adaptors at the TGN.

Table 4.4. Many Chs6 mutations have no phenotype. The indicated residues were mutated in a Chs6-Myc plasmid and transformed into a *chs6Δ* strain. The cells showed no CW resistance. Mutations in italics were made based on the crystal structure in (6A).

Plasmid name	Residues mutated
Mutations made before crystal structure:	
pAS62	Wild-type Chs6-Myc
pJC1	G540A W541A
pJC2	R548E F552A
pJC3	C582A W585A D587K
pAS70	S237A
pAS71	S253A
pAS72	T516A
pAS73	S612A
pAS92	C216A K217D K218D
Mutations based on crystal structure:	
pAS109	R713A
pAS110	D724A
pAS111	V728D
pAS112	D731A
pAS115	R713A V728D
pAS116	R713A D731A
pAS117	D724A D731A
pAS126	V728W
pAS127	A735W
pAS128	V728R
pAS129	A735R
pAS130	V728E
pAS131	A735E
pAS152	V728R A735R
pAS153	V728E A735E
pAS180	K210A
pAS183	R637A, T638A
pAS184	D672A

4.5. Discussion

We have shown the importance of the N-terminal 52 residues of Chs3 for its transport, as deletion of these residues decreases the amount of Chs3 at the bud neck as well as TGN punctae, and the mutant cells are CW resistant. We also endeavored to more precisely identify sorting signals within this region that interact with exomer and potentially other cargo adaptor proteins. Residues 10-27 contribute to internalization, while residues 28-52 contribute to retrieval of Chs3 from the endosomal system and to exomer-mediated transport to the PM, and are sufficient to interact with exomer *in vitro*. While residues 10-27 did not interact in our *in vitro* pull-down assay, they did interact with exomer in the crystals, suggesting they can contribute to the interaction, though perhaps with low affinity.

The itinerary of Chs3 involves transport through several pathways in the cell (Table 4.5, Figure 4.8). The first step after synthesis of Chs3 is exit from the ER, which requires Chs7 (Trilla, 1999). This process requires the N-terminus of Chs3, since truncation of the first 126 residues prevents ER exit (Sacristan et al., 2013), but Chs3(ΔN52)-GFP exits the ER normally indicating the required residues are located between 52 and 126. After exit from the ER and transit through the Golgi, Chs3 can be transported to the PM by two pathways. The first is exomer-mediated, and requires both the N- and C-termini of Chs3. Our results indicated residues 28-52 are particularly important for this step. The second pathway is not exomer-mediated, and can allow normal levels of Chs3 to reach the PM when retention is disrupted. It is unknown whether this is mediated by any cargo adaptors or requires sorting signals, or whether it is a non-specific inclusion of Chs3 into secretory vesicles. Chs3 endocytosis is mediated by the AP-2 clathrin adaptor. The mislocalization of Chs3(Δ10-27)-GFP and Chs3($_{19}$DEE$_{21}$→AAA)-GFP to the PM suggests AP-2 may bind the DEESLL motif at residues 19-24. Therefore, AP-1, AP-2,

Table 4.5. Summary of Chs3 mutations analyzed in this study and their effect on trafficking.

Mutant	CW growth phenotype	CW sensitivity rescued by *apl2Δ*	Localization phenotype	Likely pathways disrupted
chs3Δ	++++	No	N/A	N/A
ΔN52	++++	Strongly	Weak bud neck, few punctae	TGN→PM (exomer) TGN→Endosomes (AP-1, Gga1/2) Endosomes→TGN (Retromer, Snx4/41/42)
$_{19}$DEE$_{21}$ →AAA	+	Moderately	Normal bud neck and punctae, additional at PM	Endocytosis (AP-2) TGN→Endosomes (AP-1, Gga1/2)
Δ10-27	++	Moderately	Normal bud neck and punctae, additional at PM	Endocytosis (AP-2) TGN→Endosomes (AP-1, Gga1/2)
Δ28-52	+++	No	Weak bud neck, few punctae, vacuole	TGN→PM (exomer) Endosomes→TGN (Retromer, Snx4/41/42)
$_{41}$LVR$_{43}$ →AAA	++++	Moderately	Weak bud neck, few punctae	TGN→PM (exomer)
ΔC21	++++	No	TGN punctae only (Rockenbauch et al., 2012)	TGN→PM (exomer)

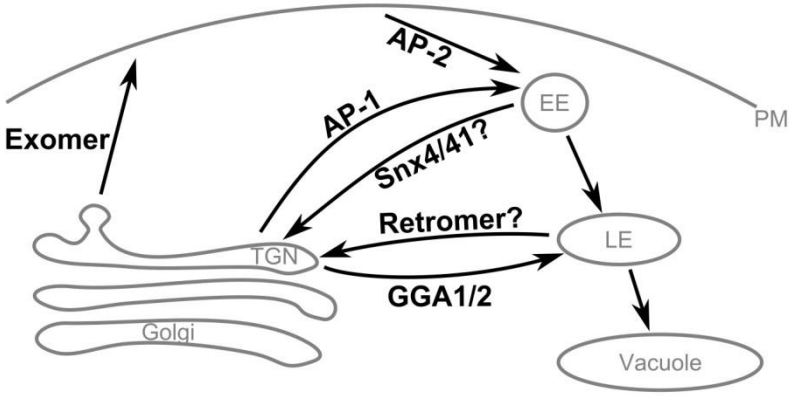


Figure 4.8: Summary of Chs3 trafficking pathways. Chs3 is transported through several intracellular trafficking pathways throughout the cell cycle. As the yeast TGN is not a stable compartment, retention of cargo at the TGN appears to require traffic to endosomes by the AP-1 and Gga1/2 clathrin adaptors and subsequent retrieval back to the TGN, likely via the retromer and/or SNX4/41/42 pathways.

and exomer likely bind to this motif during different stages of Chs3 trafficking. Interestingly, the conformation of this motif when bound to Chs6 is alpha-helical, in contrast to the conformation (D/E)XXXLL motifs adopt when bound to AP-1 and AP-2 (Kelly et al., 2008; Jackson et al., 2010). In those interactions, the (D/E)XXXLL motif is in an extended conformation.

The TGN in yeast is a transient compartment that acts to sort proteins before distributing them to other locations within the cell. Therefore, retention of a protein at the TGN is not a static storage process, but a dynamic process of tubular and vesicular transport between the TGN and the endosomal system. Chs3 requires AP-1 and Gga1/2 to transport it into the endosomal system. AP-1 binds Chs3 residues 19-24, but also must interact elsewhere in Chs3, since AP-1 deletion can rescue the CW sensitivity even when residues 1-52 are deleted. Two complexes that mediate transport from the endosomal system to the TGN are the retromer complex and the Snx4/41/42 complex. One or both of these complexes could be required for the retrieval of Chs3 back to the

TGN. One of the complexes involved in this step may bind to residues 28-52 of Chs3, as we saw mutations in this region resulted in mislocalization to the vacuole.

Our results suggest a competition of cargo adaptors for overlapping or closely adjacent sorting signals could be used to regulate and balance the pathways required for Chs3 transport. This form of regulation is unlikely to be specific to Chs3, since another exomer cargo, Pin2, contains a potential AP-1 sorting signal within the segment required for exomer-mediated transport (Ritz et al., 2014). An analogous mechanism has been demonstrated for the binding of the myosin V motor to different cargos, although the situation is reversed, in which multiple cargos compete for binding the same site on the motor (Eves et al., 2012).

This complex set of overlapping sorting signals and redundant transport pathways could allow for careful control of Chs3 localization throughout the cell cycle and efficient changes in response to signals. It is possible there are proteins in many organisms that use competitive binding of cargo adaptors to cycle between the cell surface and internal compartments.

4.6. Acknowledgement

We thank Jon Paczkowski for assistance with exomer complex crystallography and reading the manuscript. We thank Brian Richardson for helpful discussions and reading the manuscript. We thank Trevor Starr and Randy Schekman, and Anne Spang and Cesar Roncero, for sharing unpublished data. We thank Robyn Barfield for plasmid pRB259. We thank the Emr lab for use of their microscope. We thank the staff of the CHESS for the use of beamline A1 and their technical assistance. CHESS is supported by the National Science Foundation and the National Institutes of Health/National Institute of General Medical Sciences under NSF award DMR-0936384, using the Macromolecular Diffraction at CHESS (MacCHESS) facility, which is

supported by award GM-103485 from the National Institute of General Medical Sciences, National Institutes of Health. This work was funded by startup funds to JCF from Cornell University, and by NIH Training Grant (T32GM07273) awards to AMW.

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CHAPTER 5

Conclusions and future directions

Even in an organism as seemingly simple as yeast, membrane trafficking requires dozens of proteins. These proteins control a few major pathways, and many more shortcuts and detours. No coat proteins have been identified that mediate transport directly from the TGN to the PM. While exomer may or may not be adequate to deform membranes into vesicles, it appears to be acting as the adaptor to sort its cargo into secretory vesicles at the TGN. Thus far exomer is the only cargo adaptor known to sort cargo into secretory vesicles. Therefore, understanding the regulation of exomer-mediated transport contributes to knowledge of the secretory pathway in general, and could be applicable to other adaptor protiens. In addition, the cycling of Chs3 throughout the cell cycle is a model that could provide information applicable to other proteins that cycle.

In Chapter 2, I examined the structure and function of Chs5. I determined the minimal functional fragment, which allowed my collaborators in the lab to create constructs for crystallization. I also identified interactions with the FBE domain of Chs5. This included a nucleotide-independent interaction with the GTPase Arf1, and a surprising interaction with the retromer complex. However interesting the FBE domain may be, it remains puzzling why over half of Chs5 is dispensable for function. The C-terminus contains PEST motifs, which are usually involved in degradation of proteins (Rechsteiner & Rogers, 1996; García-Alai et al., 2006). It would be interesting to determine whether the C-terminus of Chs5 affects its degradation, which could be assayed by a pulse-chase experiment. While cells do not require the C-terminus of Chs5 under normal laboratory conditions, it would be worthwhile to test for a

requirement under other conditions. Conditions that challenge the cell wall – such as temperature, pH, or osmotic stress – would be a good starting point as they may highlight a need for careful regulation of Chs3 transport. There may be other regulatory elements in Chs5 as well. There are some predicted phosphorylation sites in the C-terminus of Chs5 that might regulate its function either positively or negatively.

Some of the interactions between Chs5 and other proteins also present new areas for study. The nucleotide independent interaction of the FBE domain with Arf1 has now been explained by the Exomer-Arf1 structure (Paczkowski and Fromme, in press) but the interactions with Chs2 and retromer will require more experiments. Chs2 does not require exomer for transport, so its interaction with Chs5 is puzzling. The interaction of exomer and retromer presents a possible model where exomer acts as an adaptor for retromer to aid in recycling of Chs3 out of the endosomal system. Finding mutations that disrupt the interaction of Chs5 and retromer would allow testing of this hypothesis.

My focus shifted to the ChAPs and their regulation in Chapter 3. Chs6 is the ChAP required for transport of Chs3, so I explored how Chs6 might be regulated differently than the other ChAPs. I discovered a difference in Chs6 expression throughout the cell cycle, but it was only required for Chs3 transport in one yeast strain background. It is possible other conditions would also show a requirement for Chs6 to be expressed under its own promoter. The conditions listed previously that would stress the cell wall would be interesting ones to test. However, it is possible the variation in Chs6 levels is not required in these cases, and other mechanisms are regulating Chs6. Post-translational modifications of Chs6, such as phosphorylation, have not yet been explored and may play a role in linking Chs3 transport to the cell cycle.

In Chapter 4, I focus on sequences within Chs3 and their effect on transport. This began as an effort to characterize the interaction between Chs3 and Chs6. It was soon apparent it would be difficult to separate interactions with exomer and interactions with other transport proteins, as transport of Chs3 is complex. I identified a region of Chs3 sufficient for interaction with exomer *in vitro*, Chs3(28-52), but it has only a modest effect on Chs3-GFP localization. A sequence in Chs3 that was bound in the crystal structure I obtained, Chs3(10-27), was neither necessary nor sufficient for an interaction with exomer, but seems to have a role in endocytosis.

There are many experiments that could be done to begin unraveling these and the other complicated results that were described. For example, co-crystalizing exomer with the Chs3(28-52) fragment could define the interaction site on Chs6. My attempts to co-crystalize exomer with Chs3(1-52) proved unsuccessful, but perhaps a shorter fragment of Chs3 would be less destabilizing in crystals. Another interesting question is whether other exomer cargo also bind in the same region on their corresponding ChAP proteins. Since the targeting motif in Fus1 has already been identified (Barfield et al., 2009), a peptide with this sequence could be co-crystallized with an exomer complex containing either Bch1 or Bud7.

Other general questions exist about exomer function and regulation. For instance, is exomer acting as a traditional coat adaptor complex, and recruiting a coat to deform membranes? Other research in our lab has found a role for the ChAP proteins in deforming membranes which, along with the membrane deformation due to Arf1 inserting its amphipathic helix, could cause a vesicle to bud. This would require clustering of the exomer complexes. Are there accessory proteins that link multiple exomer complexes together, or do the exomer components themselves interact?

Hopefully the insights gained by the study of exomer-mediated transport can be applied to other transport processes in yeast as well as in other organisms. There are still many mysteries surrounding the generation of secretory vesicles in all types of eukaryotes. In mammals, the secretory system supports cell polarization, releases important materials like hormones and digestive enzymes, and sends neurotransmitters between neurons. Knowing more about how cells control these important processes will be of interest not just to cell biologists, but also to the medical community.

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