INTRACELLULAR TRAFFICKING OF A MODEL POLYTOPIC MEMBRANE PROTEIN IN *SACCHAROMYCES CEREVISIAE*

by

Sai Tejasvi Dharwada

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Abstract

Polytopic membrane protein synthesis involves translation followed by translocation across the lipid bilayer at the ER. Here, various chaperones, together with enzymes that add post and co-translational modifications, help the protein achieve a final three-dimensional structure. General and substrate-specific chaperones prevent toxic aggregation of proteins by shielding and preventing interaction between non-native species. Terminally misfolded proteins are destroyed by the quality control machinery of the cell and the amino acids are recycled for further use.

In the following study, we used Chitin synthase III (Chs3) of *Saccharomyces cerevisiae* as a model to dissect the complexities involved in polytopic membrane protein synthesis at the ER. Previous genetic screens from our lab have revealed a novel regulator of Chs3 trafficking called Pfa4, a DHHC enzyme required for Chs3 palmitoylation at the ER. At the ER, Chs3 also requires Chs7, a dedicated chaperone for folding and assembly. We identified a novel secondary role for Chs7 in Chs3 trafficking as a co-factor required for Chs3 function at the plasma membrane. Our study also examined the role of palmitoylation in Chs3 trafficking. Palmitoylation of Chs3 is required for its efficient interaction with Chs7, in addition to folding and ER export. A genome-wide screen also identified the Ubp3/Bre5 deubiquitination complex as a regulator of non-lipidated Chs3 degradation at the ER. The discovery that dedicated chaperones can take on additional roles and that palmitoylation can influence chaperone-client interactions could provide insights into the workings of the protein folding machinery at the ER.

Preface

Chapter 2

A version of chapter 2 is in preparation for submission. Dharwada ST, Padmanabhan N, Choi C, Schluter C, Davey M, Conibear E (2015). The chaperone Chs7 forms a stable complex with Chs3 and promotes its activity at the cell surface. Nirmala Padmanabhan and I are co-first authors. We designed all of the experiments. Nirmala Padmanabhan and Michael Davey performed all of the CW activity assays, protease shaving experiments, and part of the microscopy. I performed the rest of the experiments and analyzed the data. Cayetana Schluter and Michael Davey performed the majority of the strain constructions. I wrote the manuscript for publication with the assistance of Elizabeth Conibear.

Chapter 3

A version of chapter 3 is in preparation for submission. Dharwada ST, Padmanabhan N, Lam KKY, Pagant S, Lin DT, Roth A, Davis NG, Miller EA, Conibear E (2015). Quality control of palmitoylation-deficient Chs3 at the ER and Golgi. Nirmala Padmanabhan and I are co-first authors. We designed all the experiments and did all the data analysis. Amy Roth, from the Davis lab, performed palmitoylation assays and Silvere Pagant, from the Miller lab, performed *in vitro* COPII budding experiments. Karen Lam performed the genome-wide screen and a majority of the ground work required for the project. David Lin assisted with microscopy. Michael Davey performed strain construction. Nirmala Padmanabhan assisted with the ubiquitination assay, protease shaving assay and part of the microscopy. I performed Co-immunoprecipitation experiments and Blue-Native Page experiments, a part of the microscopy and analyzed the data. I also prepared the manuscript for submission with the assistance of Elizabeth Conibear.

In chapters 2 and 3 I shall use “we” consistently to reflect co-authors that contributed to the studies.

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List of Abbreviations

AAP- Amino acid permeases
AP – Adaptor protein
APT – Acyl protein thioesterase
ATP – Adenosine triphosphate
BN-PAGE – Blue native polyacrylamide gel electrophoresis
CNX – Calnexin
COP – Coatmer protein
CRT- Calreticulin
CW – Calcofluor white
DHHC – Aspartate-histidine-histidine-cysteine
DIC – Differential interface contrast
DSP- Dithiobis [succinimidyl] propionate
ER – Endoplasmic reticulum
ERAD - endoplasmic reticulum associated degradation
GDP- Guanosine diphosphate
GFP – Green fluorescent protein
GTP - Guanosine triphosphate
HA- Hemagglutinin
HSP – Heat shock protein
kD – Kilo Dalton
LDL- Low density lipoprotein
LRP- Low density lipoprotein receptor
M_r – Relative molecular mass
OD_{600} - optical density at 600nm
PAT – Palmitoyl acyl transferase
PDI – protein disulphide isomerase
PM – Plasma membrane
RFP – Red fluorescent protein
SDS-PAGE - sodium dodecyl sulfate polyacrylamide gel electrophoresis
SNARE - Soluble N-ethylmaleimide-sensitive factor attachment protein receptors
TMDs – Transmembrane domains
YPD - yeast extract peptone dextrose

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I would like to thank my research supervisor Dr. Elizabeth Conibear for her guidance and mentorship throughout my degree. Thank you, Liz for being a patient teacher and for believing in my abilities even when I didn't. Being in your lab has been a great learning experience for me. I would also like to thank my committee members Dr. Chris Lowen and Dr. Stefan Taubert for their advice on my project and for being plain awesome!

I would like to thank all the members of the Conibear lab past and present for all their love, moral support, help and constructive criticism. I wouldn't trade you for the world. Okay, maybe for a billion dollars. First, I would like to thank Michael Davey and Nirmala Padmanabhan for their technical support, advice and support on both the studies. Thank you, Bjorn, Lauren, Shawn and Kathleen for being fantastic friends and lab buddies. And David - Thank you for letting me rant now and then about life and science. Thank you, for lending your ear and your shoulder during the tough times. Finally, I would like to thank Phoebe Lu (Kobor Lab) for being a ray of sunshine during the gloomy days.

Dedication

I dedicate this thesis to my wonderful husband and my parents. Thank you for all the compromises you had to make over the last few years so I can have a future. Words cannot describe the love and respect I have for the three of you.

Also, to my Uncle (Santosh). You filled my life with love and happiness. Even though you are long gone, not a day goes by when I don't miss you. You will always be in my heart.

I have frequently been questioned, especially by women, of how I could reconcile family life with a scientific career. Well, it has not been easy. - Madame Marie Curie.

Chapter 1: Introduction

1.1 Biosynthesis of membrane proteins – overview

Biosynthesis of membrane proteins is thought to occur in two stages- translocation and membrane insertion (Popot and Engelman, 1990). In the first stage, membrane proteins partially translocate co-translationally into the lipid bilayer. This partial translocation involves insertion of selective residues into the lipid membrane. It has been proposed that membrane proteins possess a so-called "insertion code" that dictates the residues that are inserted into the membrane vs. those that are released (Hessa et al., 2005). The second stage involves re-orientation of the TMDs and oligomerization of subunits to form a three-dimensional native structure (Bowie, 2005). Figure 1.1 shows the three steps involved in the synthesis of polytopic membrane proteins. The endoplasmic reticulum harbors the translocation machinery that targets and inserts proteins across the lipid bilayer (Walter et al., 1984). The signal recognition particle mediates transfer of proteins at the translocon (Sec61 complex in yeast) by binding to signal sequences (Deshaies and Schekman, 1987). Membrane protein translocation and insertion are tightly linked to events such as protein folding, modification and assembly that contribute to the overall maturation of the protein (Schnell and Hebert, 2003). Once the protein is correctly folded and verified by the quality control machinery, it is packed into transport vesicles for further trafficking (Dobson, 2003).

1.1.1 Protein folding in the ER

Protein folding is a complex phenomenon that depends on the fundamental amino acid sequence of the protein as well as various accessory factors. This process is further complicated for membrane proteins since they are exposed to diverse cellular environments such as cytosol, ER lipid membrane, and the ER lumen. *In vitro* experiments have shown that the final three-dimensional structure of a protein is one that supports the lowest energy configuration. Given that there are a number of possible conformations for a protein to choose from, it is thought they evolved to fold efficiently and in a manner that supports their function. However, achieving one particular functional conformation is a narrow possibility and when misfolded these proteins are destined for degradation (Dobson, 2004; Dobson et al., 1998; Tartaglia et al., 2007). Misfolded proteins are prone to aggregation which causes additional functional disturbance. Misfolded

proteins result in either a loss-of-function phenotype due to degradation or a gain-of-function phenotype due to toxic aggregation (McClellan et al., 2005). Aggregated proteins have been implicated in several diseases such as cancer and cystic fibrosis (Soto, 2003). Figure 1.1 shows the overall process of membrane protein biosynthesis at the ER.

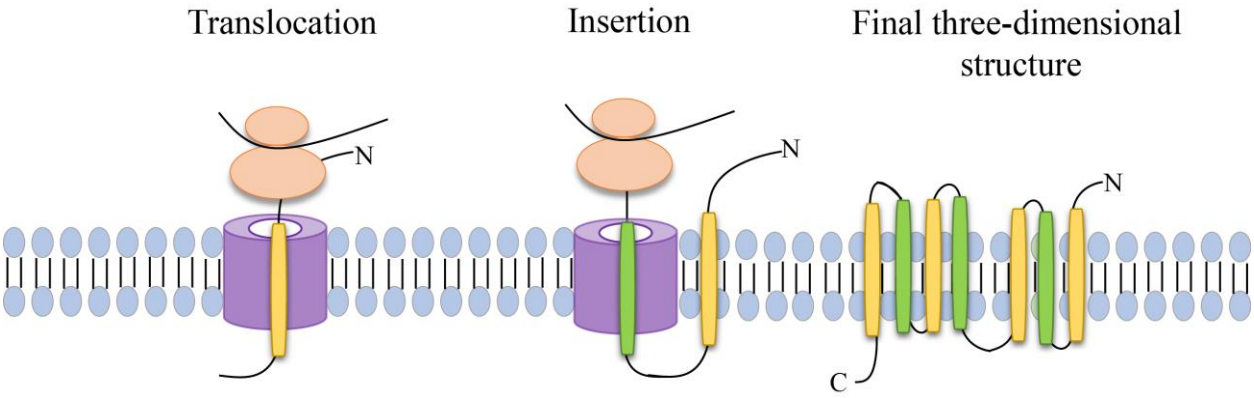

Figure 1.1 Biosynthesis of polytopic membrane proteins
Polytopic membrane proteins are co-translationally translocated across the translocon followed by membrane insertion, folding and modification to attain a final three-dimensional structure.

1.1.2 Chaperones

Since proper folding of membrane proteins is so crucial, a strict quality control process is employed by the cell to ensure the health of the proteome (Chen et al., 2011). Central to this machinery is set of factors called molecular chaperones. Although previous *in vitro* experiments concluded that the native conformation of a protein depends on only the amino acid sequence, it became apparent that complex multi-subunit proteins needed the assistance of accessory factors called chaperones to fold and function (Buchner, 1996). Molecular chaperones are defined as a set of unrelated proteins that aid in the folding and assembly but are not a part of the final structure of a protein. Although chaperones are not exclusively ER proteins, the majority of them do reside there. Chaperones promote folding of a protein and if unable to do so, target the protein for destruction via the ubiquitin-proteasome pathway or via sequestration of non-native species (Chen et al., 2011; McClellan et al., 2005). Sequestration helps alleviate the burden on the proteasome and reduce inheritance of aggregated proteins. Chaperone function and expression are often regulated by ER and cellular stress pathways (Kaufman, 1999).

Chaperones are thought to aid folding by binding to exposed structural features that are typically buried in native species and shielding them to prevent intermolecular aggregation (Ellis and van der Vies, 1991; Hartl and Hayer-Hartl, 2002; Rapoport et al., 2004). Traditionally, chaperones have been distinguished from folding catalysts such as protein disulphide isomerase (PDI) which accelerate the rate of folding, and molecular escorts such as Rab escort protein 1(REP1), which is required for membrane targeting of Rab proteins (Alexandrov et al., 1994). It is now widely accepted that chaperones have secondary overlapping functions, and they serve as both folding catalysts and molecular escorts and are required for surface targeting and function of substrates. For example, NinaA (Neither inactivation nor afterpotential A) acts as an escort chaperone for Rh1 opsin protein. In the absence of NinaA, Rh1 is retained in the ER due to aggregation. NinaA also co-localizes with Rh1 throughout the secretory cycle and could be required for the function of Rh1 (Colley et al., 1991).

Apart from serving as folding catalysts and escorts, certain chaperones can "untangle" and refold aggregated proteins. The Yeast AAA^+ ATPase Hsp104 helps disaggregate proteins to restore their folding and function. Chaperones can also associate with other chaperones to form a network such as small heat shock proteins (sHsps) which exist as large dynamic complexes (Haslbeck et al., 2005). Recently it has also been shown that chaperones can directly regulate the degradation of misfolded proteins via ubiquitination. CHIP (Carboxy-terminal Hsp interacting protein), an E3 ubiquitin ligase, physically interacts with Hsp70 and Hsp90 chaperones to mediate ubiquitination and destruction of non-native species (Cyr et al., 2002). These examples highlight the diversity of chaperone functions and their role in maintaining cellular homeostasis. Chaperones can also be divided based on their substrate specificity into general chaperones and substrate or client specific chaperones. Figure 1.2 highlights the various functions of chaperone proteins in a cell.

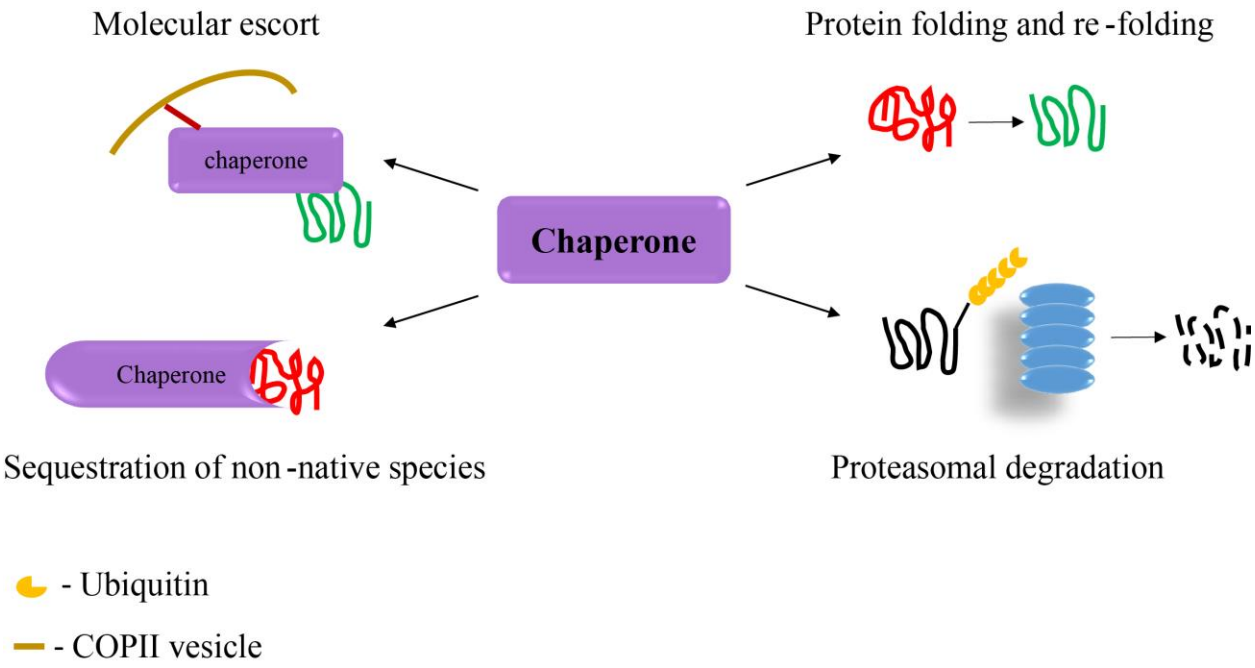

Figure 1.2 Functions of chaperone proteins
Chaperone proteins assist in folding and re-folding of proteins, and act as molecular escorts to help proteins exit the ER in COPII vesicles. In the case of terminally misfolded proteins, chaperones can either direct them to proteasomal degradation or sequester the proteins to prevent inheritance of toxic species and aggregation.

1.1.2.1 General chaperones

General or 'public' chaperones assist in the folding of most newly synthesized proteins in the ER. Their substrates show no obvious sequence or structural similarity. However, some proteins are harder to fold and require the assistance of specific or 'private' chaperones. The next few paragraphs will describe a few well-studied examples of general chaperones. BiP (Immunoglobulin binding protein and yeast Kar2), a member of the Hsp70 family, is a principal regulator of protein folding in the ER. Apart from folding, BiP also regulates efficient translocation of proteins through the ER translocon (Lyman and Schekman, 1995). It binds to aliphatic residues that are usually hidden in correctly folded species through its C-terminal domain and attempts to stabilize them by preventing aggregation (Flynn et al., 1991). It consists of an ATP binding N-terminal domain, which upon hydrolysis to ADP dictates substrate release and binding. BiP has been shown to associate with other chaperones such as Hsp90 family members and calnexin to extend its repertoire of substrates. For example in *S.cerevisiae* Sec63, a member of the Hsp40 family of proteins, binds to BiP and enhances its rate of ATP hydrolysis

resulting in the ADP form which binds substrate proteins better than the ATP-bound form (Corsi and Schekman, 1997). Similar to many chaperone systems, the expression of BiP is upregulated as a part of the unfolded protein response (UPR) in yeast and mammalian cells (Gething, 1999). Another well-studied chaperone system is the calnexin/calreticulin (CNX/CTR) complex required for the folding and quality control of glycoproteins. CNX/CTR complex competes with other chaperone systems such as BiP for binding to substrate proteins. The location of N-glycan residues on the substrate proteins dictates the choice of chaperone systems. The closer the glycans are to the N-terminus of the protein the higher their affinity for the CLX/CTR complex (Molinari and Helenius, 2000).

Hsp90 acts as a chaperone for important substrates that are involved in cellular signal transduction, growth control, and cell survival. Unlike most chaperones Hsp90 mostly resides in the cytosol but some forms of Hsp90 also reside in the ER. It binds to the substrate in a 1:1 or 2:1 ratio and suppresses aggregation of nascent polypeptide chains. Hsp90 substrate binding and release also depend on ATP/ADP cycles (Whitesell and Lindquist, 2005; Wiech et al., 1992). Many co-chaperones such as Sgt1 (Catlett and Kaplan, 2006), UNC-45 (*C.elegans*) (Barral et al., 2002), and immunophilin-related cofactor XAP2 interact with Hsp90 (Meyer and Perdew, 1999) and modulate its substrate specificity and biochemical activities. Therefore, although general chaperones don't exhibit the substrate specificity of 'client specific' chaperones, they do show some amount of substrate preference mainly due to binding of other co-factors.

1.1.2.2 Client/substrate specific chaperones

Some proteins require complex structural assembly and depend on secondary chaperones for folding and stability. These secondary chaperones are usually highly substrate specific and act on either a single protein or a class of proteins (Ellgaard et al., 1999). The following section will highlight the functional importance of a few selected examples. Collagen has an elaborate biosynthesis pathway and requires the assistance of as many as six general chaperones such as PDI and CLX/CTR. As a second tier of quality control collagen needs the help of specific chaperones such as Hsp47. Hsp47 is predicted to prevent ER retention and degradation of the collagen fibers by shielding them and giving them enough time to mature. Hsp47 is absolutely

critical for collagen maturation and secretion and could play additional roles in its trafficking (Hendershot and Bulleid, 2000). Absence of Hsp47 has been implicated in severe forms of Osteogenesis Imperfecta (Christiansen et al., 2010).

B-cell receptor-associated protein 31 (BAP31) acts to transport cellubrevin specifically out of the ER to the Golgi compartment. In the absence of BAP31, cellubrevin is ER-retained. BAP31 is predicted to serve as a sorting chaperone and interacts with cellubrevin via transmembrane domains. BAP31 actively incorporates cellubrevin into COPII vesicles for further trafficking. BAP31 is thought to leave the ER along with cellubrevin and is retrieved back to the ER (Annaert et al., 1997). Similar escort activity has also been reported for yeast Vma21, which exits the ER along with the vacuolar H^+ ATPase pump and is retrieved back to the ER via COPI vesicles. In the absence of Vma21, the H^+ ATPase pump is ER retained and aggregated (Hill and Stevens, 1994). Mesd (*Mice*) or Boca (*Drosophila*), an ER resident protein, serves as a specific chaperone for WNT co-receptors LRP5 and LRP6. In the absence of Mesd, LRP6 forms intramolecular disulfide bonds and no longer exists as a monomer. Mesd is thought to facilitate proper localization of LRP6 on the plasma membrane by ensuring proper ER export of LRP6. Mesd might act on other members of the LDLR family as well, thus showing class specificity in its chaperone activity (Hsieh et al., 2003).

Another substrate specific chaperone that deserves a mention here is the Receptor associated protein (RAP), which acts as both a folding and an escort chaperone for the members of the low-density lipoprotein (LDL) receptor family. LDL proteins contain many tandem cysteine-rich repeats that, in the final structure of the protein, are bound together by disulphide bonds. Because of this extensive post-translational assembly, they require a dedicated chaperone to avoid misfolding and aggregation in the ER. RAP acts as a chaperone and as an escort protein at the ER to ensure proper trafficking. Additionally, it also regulates ligand binding activity of the LDL by preventing premature ligand-receptor interactions in the ER (Bu and Schwartz, 1998).

Several substrate specific chaperone systems have been studied in yeast, and some of these have been described in detail in Chapter 2 of this thesis. It is not surprising to see that cells have

dedicated chaperones to assist folding of complex proteins. Every protein is different in its requirement for achieving a final maturation state. These differences are further elaborated by co and post-translational protein modifications adding another layer of complexity to the final three-dimensional structure of a protein.

1.1.3 Post-translational modifications

Some of the most common post-translational modifications (PTM) include phosphorylation, glycosylation, fatty acylation, ubiquitination, sulfation and methylation (Mann and Jensen, 2003). Given that roughly ~ 50% of proteins in the human proteome are glycosylated, its importance cannot be stressed enough. Glycosylation affects protein function, folding, secretion, targeting, and stability. Glycosylation usually begins at the ER and is elaborated at the Golgi compartment (Wong, 2005). Phosphorylation is the covalent addition of a phosphate group to a serine, threonine or a tyrosine residue and is considered the 'bread and butter' of cellular signaling. Because phosphorylation is reversible, a protein can switch between the two states, and this often dictates the activity of a protein. It plays a role in a wide variety of cellular processes such as growth, metabolism, motility, trafficking, immunity, learning, memory and more (Ubersax and Ferrell Jr, 2007).

Sulfation is the addition of a sulphate group to a tyrosine residue of a protein. So far only secreted and transmembrane proteins in multicellular eukaryotes are subject to this PTM. It has been shown to play a role in protein-protein interactions involved in cell adhesion and chemokine signaling (Kehoe and Bertozzi, 2000). Ubiquitination, which is the covalent addition of ubiquitin to lysine residues of proteins, is generally thought of as a signal for protein degradation by the proteasomal system (Wilkinson, 2000). Misfolded membrane proteins are tagged with poly-ubiquitin in the ER and transported to the cytoplasm for destruction. However, multi-ubiquitination or monoubiquitination of proteins plays a role in endocytic cycling, sorting into the multivesicular bodies (MVB), functional regulation, signalling, replication, protein-protein interactions and transcription (Mukhopadhyay and Riezman, 2007). Disturbance in ubiquitination/deubiquitination cycles causes diseases such as cancer, Liddle's syndrome,

Parkinson's disease and many more. Fatty acylation, also known as lipidation, will be the focus of the next section.

1.1.3.1 Lipid modifications

Proteins can be covalently modified by a wide variety of lipids that include fatty acids such as palmitate and myristate, isoprenoids, cholesterol, and glycolipids. *N*-myristoylation is the irreversible addition of a 14 carbon saturated fatty acid to the N-terminal glycine residue of a protein by an amide bond. Myristoylation can be both co and post-translational. An enzyme known as *N*-myristoyl transferase (NMT) catalyzes the transfer of a myristoyl group from myristoyl CoA to the substrate. There is an absolute requirement for a glycine for myristoylation and the general sequence requirements are Met-Gly-X-X-X-Ser/Thr. It has been shown that in yeast residue 6 (Ser/Thr) is essential for substrate recognition by NMT (Resh and Resh, 1999; Towler et al., 1987). Several important proteins are myristoylated including protein kinases and phosphatases, GTP-binding proteins, viral proteins such as Gag proteins of HIV-1, and cytoskeletal-bound proteins. *N*-myristoylation is necessary for stabilizing the three-dimensional structure of a protein, membrane binding, and targeting. Mutation of the glycine residue to an alanine residue completely abolishes myristoylation and decreases the membrane binding of the protein and disrupts its sub-cellular localization. For example, Yeast Gpa1 (Song et al., 1996) and HIV-1 Gag proteins are mislocalized to the cytosol when not myristoylated (Hermida-Matsumoto and Resh, 2000). Myristoylation most commonly occurs in combination with palmitoylation.

Prenylation is a post-translational covalent addition of either farnesyl (15-carbon) or geranylgeranyl (20-carbon) to the C-terminal cysteine residues of a protein. Three enzymes catalyze the addition of the farnesyl or geranylgeranyl groups to the substrates: farnesyltransferase and geranylgeranyltransferase type I and type II. Prenylated proteins contain a CaaX motif, and this dictates the type of prenylation a protein can undergo. If the X is a Ser, Met or Glu, proteins are farnesylated, and when the X is a leucine, geranyl-geranyl is added to the proteins (Zhang and Casey, 1996). Prenylation plays a significant role in protein localization, function, and protein-protein interactions. For example absence of Rab prenylation

leads to Hermansky-Pudlak syndrome (HPS). HPS is characterized by immunodeficiency, hypopigmentation and abnormal bleeding (Pereira-Leal et al., 2001). Farnesyl transferase inhibitors have been developed as therapeutic agents to treat cancer since farnesylation of Rab proteins is essential for their membrane targeting. Even though they are not effective for the treatment of cancer, they have been used in the treatment of Progeria caused by a mutant form of lamin A protein called progerin. Wild type lamin A is farnesylated and at a later stage the farnesyl group is cleaved from the protein. The resulting mature protein is incorporated into the nuclear membrane. Progerin, which is a truncated form of lamin A, is also farnesylated but the farnesyl group cannot be cleaved and progerin cannot be incorporated efficiently into the nuclear membrane (Hennekam, 2006). This leads to abnormal nuclear membrane structure and premature aging in children suffering from progeria (Resh, 2013)

Very little is known about cholesterol addition to proteins as a post-translational modification. One important protein that is modified by cholesterol is Sonic Hedgehog. Cholesterol modification is thought to play a role in targeting Sonic Hedgehog to so-called lipid raft microdomains within the membrane. Sonic Hedgehog signaling plays an imperative role in early development, and its importance can be understood by studying diseases such as Smith-Lemli-Opitz Syndrome (SLOS). SLOS patients have a deficiency in cholesterol biosynthesis and hence Hedgehog proteins are not modified by cholesterol in these patients leading to various developmental disorders (Kelley and Hennekam, 2000; Mann and Beachy, 2000; McMahon, 2000). The next section will focus entirely on palmitoylation and its significance in various aspects of protein trafficking inside the cell.

1.1.3.2 Palmitoylation

Palmitoylation is the post-translational addition of palmitic acid, a 16 carbon saturated fatty acid moiety, to the cysteine residues of a protein. There are two types of palmitoylation – *S*-palmitoylation, which is a reversible thioester linkage of palmitic acid also known as *S*-acylation, and *N*-palmitoylation, which is the attachment of palmitic acid through a more permanent amide linkage to the N-terminal cysteine residues of a protein (Linder and Deschenes, 2007). Secreted morphogen Sonic Hedgehog is one protein known to be *N*-palmitoylated (Pepinsky et al., 1998).

The rest of the section will focus on *S*-palmitoylation. *S*-palmitoylation is unique because a) the cysteine residues that are palmitoylated are not specified by a particular sequence of amino acids and b) *S*-palmitoylation is reversible (Munday and López, 2007). Although a specific sequence is not required for palmitoylation, Roth et al. (2006) found that for SNARE proteins the presence of a Cys residue near the TMDs is an excellent indicator of palmitoylation. Many proteins undergo palmitoylation/depalmitoylation cycles as a part of dynamic regulation (Resh, 2013; See Figure 1.3 for a summary). Many lipoproteins carry dual lipid modifications such as *N*-myristoylation and palmitoylation. Since most lipid modifications are stable, addition of a palmitate group offers another regulatory step in the folding and trafficking of these proteins. For example, N-Ras is farnesylated and *S*-palmitoylated. *S*-palmitoylation undergoes rapid turnover. *S*-palmitoylation is likely required for the proper membrane targeting of N-Ras. It has been proposed that for proteins that undergo sequential lipid modifications *N*-myristoylation or prenylation could happen first followed by *S*-palmitoylation. Myristylation and prenylation offer weak binding of the protein to the membrane and bring the protein in close proximity to enzymes that can add a palmitate group and increase the membrane attachment of the protein significantly. This has been described as the 'kinetic membrane trapping' model. (Shahinian and Silvius, 1995). Fyn, a tyrosine protein kinase, undergoes dual lipidation (co-translational *N*-myristoylation followed by post-translational *S*-palmitoylation) and this is necessary for rapid membrane targeting and for a strong interaction with the lipid bilayer (Van't Hof and Resh, 1997).

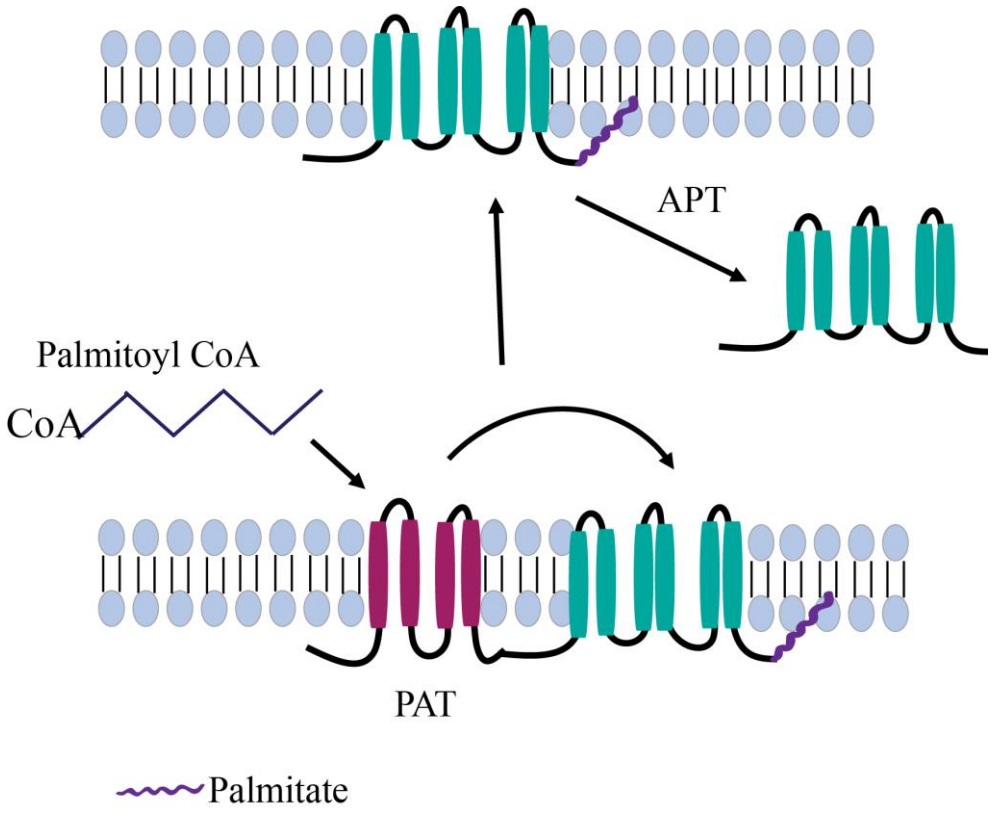

Figure 1.3 Palmitoylation and depalmitoylation of a membrane protein
S-Palmitoylation is the only reversible lipid modification, and hence proteins undergo cycles of palmitoylation and depalmitoylation which controls their localization and activity. Therefore, *S*-palmitoylation has been compared to phosphorylation.

Previously, methods to detect protein palmitoylation involved the incorporation of radioactively labeled palmitate or palmitate analogues into cells (Resh, 2006). Recently, a few alternative procedures have been developed to detect protein palmitoylation in the cell. One of these methods is the acyl-biotin exchange assay. In this method, proteins are first treated with N-ethylmaleimide to block all the free reactive thiol groups. This is followed by treatment with ammonium hydroxide to cleave thioester bonds on cysteines not blocked by NEM. Biotin now occupies the free thiol group, and this can be detected after affinity purification of biotinylated proteins using standard western blot analysis (Wan et al., 2007). Another recent approach is the use of the biorthogonal probe 17-octodecanoic acid (17-ODYA) to label palmitoylated proteins. 17-ODYA is metabolically incorporated into the proteins and can be detected by a click chemistry reaction (Cu (I) catalyzed azide-alkyne [3+2] cycloaddition reaction) to biotin,

fluorescent or other commercially available chemical tags. These proteins can then be detected by western blot or fluorescence microscopy (Hannoush and Arenas-Ramirez, 2009; Martin and Cravatt, 2009; Yap et al., 2010).

1.1.3.3 Palmitoyl acyltransferases (PATs) and acyl protein thioesterases (APTs)

The dynamic nature of palmitoylation means it is often compared to other post-translational modifications that are reversible such as phosphorylation. Just as phosphorylation is regulated by kinases and phosphatases, palmitoylation is regulated by the palmitoyl acyltransferases (PATs), which catalyze the addition of the palmitate group to proteins from palmitoyl-CoA and acyl protein thioesterases (APTs), which catalyze the removal of palmitate group from the proteins. Initially palmitoylation was believed to be autocatalytic. Later, PAT enzymes were first identified in yeast and after that were reported in several model organisms (Planey and Zacharias, 2009). PATs contain a conserved DHHC (Asp-His-His-Cys) sequence at the active site. A total of 7 PAT enzymes in *Saccharomyces cerevisiae*, 23 PAT enzymes in humans and *Drosophila melanogaster*, 16 in *Caenorhabditis elegans* and 31 in *Arabidopsis thaliana* have been identified to date (Mitchell et al., 2006). PAT enzymes are 4-6 transmembrane domain proteins that are localized to the ER, the Golgi or the plasma membrane. During the reaction the palmitate group is first added to the cysteine of the DHHC group (autopalmitoylation), which results in an acyl-enzyme intermediate, followed by a transfer to the cysteine residue of the protein, in what is known as the two-step transfer mechanism (Jennings and Linder, 2012). Therefore, mutation of the active Cys residue in the DHHC motif to an alanine or serine abolishes the PAT activity of the enzyme (Roth et al., 2002). Interestingly there is a lot of overlap in PAT enzyme substrate specificity. For example Pfa4, a yeast PAT enzyme, shows specificity for multi transmembrane domain proteins. It palmitoylates the chitin synthase Chs3 (Lam et al., 2006), and the amino acid permeases Tat1, Agp1 and Gap1 (Roth et al., 2006). DHHC enzymes also show specificity for the acyl-CoA chain used for the transfer of palmitate to substrates. DHHC2 has been shown to prefer chains longer than 14 carbons whereas DHHC3 prefers 14 and 16 carbon length acyl-CoA chains (Jennings and Linder, 2012). DHHC enzyme mutations are involved in pathologies such as cancer and neurological diseases such as Huntington's disease (Ohno et al., 2012). These diseases result from lack of palmitoylation of

client proteins such as Ras, which mislocalizes to various other compartments and engages in inappropriate cellular interactions.

Depalmitoylation of substrates, on the other hand, is catalyzed by acyl protein thioesterases (APTs). APT1 depalmitoylates a variety of substrates including H-Ras, and N-Ras. *In vitro* assays have also expanded the APT1 substrates to SNAP-23 (Flaumenhaft et al., 2007), viral glycoproteins (Veit and Schmidt, 2001) and GAP43 (Tomatis et al., 2010). Siegel et al. (2009) have shown that APT1 depalmitoylation of $G_{\alpha 13}$ is essential for dendritic spine enlargement, and in the absence of APT1 Ras proteins mislocalize to the cytosol (Dekker et al., 2010). Two other APTs – APT2 (66% similarity to APT1) and APTL1 (33% similarity to APT1) – have also been described in the literature (Conibear and Davis, 2010). Together, PATs and APTs are involved in the dynamic regulation of palmitoylation necessary for their localization and function.

1.1.3.4 Significance of protein palmitoylation

Membrane targeting and localization: Protein palmitoylation aids the association of several soluble and membrane proteins with the lipid bilayer. As mentioned earlier, in dually lipidated proteins palmitoylation happens after prenylation or myristoylation and this dual lipidation serves to strengthen and prolong the protein membrane interaction (Conibear and Davis, 2010). Examples of proteins that are dually lipidated include non-receptor tyrosine kinases (NRTK), A kinase anchoring proteins, AKAP 18 and AKAP 15, and in yeast Vac8, a protein involved in cytoplasm to vacuolar targeting of cargo (Dunphy and Linder, 1998). Palmitoylation also directs proteins to individual lipid microdomains identified as 'lipid rafts'. Lipid rafts are membrane domains rich in sphingolipids, cholesterol and phospholipids enriched with saturated fatty acids (Simons and Toomre, 2000). For example, palmitoylated Src kinases associate specifically with lipid raft domains. Similarly, palmitoylation of T cell receptors CD4 and CD8 enriches the proteins in lipid raft domains. This is essential, since CD8 and Lck kinase exclusively interact in these lipid microdomains. Therefore, by targeting proteins to specific subcellular locations palmitoylation specifies membrane association, interaction with other proteins and function (Arcaro et al., 2000). For example, when the site of palmitoylation is mutated in the G-protein coupled receptor α_z, the protein is still myristoylated but mislocalizes to other intracellular

membranes including the plasma membrane (Morales et al., 1998). Nitric oxide synthase (eNOS) requires palmitoylation for proper targeting to the caveolae and optimal function. Palmitoylation deficient eNOS produces less NO compared to WT eNOS (Liu et al., 1996).

Intracellular trafficking of proteins: At the ER palmitoylation of proteins stabilizes them in the thin lipid bilayer of the ER. Palmitoylation is thought to induce tilting of proteins with long transmembrane domains and prevent hydrophobic mismatch between the membrane and the protein (Joseph and Nagaraj, 1995). For example LRP6, a part of the Wnt canonical pathway, has a 23-residue long TMD and palmitoylation is thought to induce tilting and stabilize the protein promoting its ER exit. Palmitoylation is also believed to prevent degradation of proteins at the ER and in other subcellular locations in the cell. For example, palmitoylation of Cys^{34} of the cytoplasmic tail of the cation dependent mannose-6-phosphate receptor is required for proper recycling of the protein from Golgi to the endosomes and when not palmitoylated it is mistrafficked to the lysosome and degraded (Schweizer et al., 1996). Palmitoylation of GluR2 receptor (a subunit of the AMPAR) requires palmitoylation for proper ER export and to avoid lysosomal degradation (Yang et al., 2009). Palmitoylation profoundly influences trafficking of proteins to the plasma membrane. For example, palmitoylation of PSD-95 and GAD65 by HIP14 is essential for proper membrane targeting of these proteins (Huang et al., 2004). Similarly, palmitoylation of Chemokine receptor CCR5 and P2X7 (Gonnord et al., 2009), an ATP gated cationic channel, is necessary for their transport to the cell surface. Finally, palmitoylation of Chs3 in yeast by Pfa4, which is the focus of Chapter III, is required to prevent aggregation and ER retention of Chs3 (Lam et al., 2006).

Protein –protein interactions: Palmitoylation is also needed for some proteins to interact with other proteins. One of the best-studied examples is CD81, a member of the tetraspanin superfamily of membrane proteins. CD81 is involved in cell signaling and activation of B cells. CD81 is localized to lipid rafts and is palmitoylated at the PM. This is essential for its stability at the PM and its interaction with other membrane proteins such as CD9 and EWI-2 (Glu-Trp-Isl [EWI] motif containing protein 2) (Delandre et al., 2009). Similarly, palmitoylation of β2 adrenergic receptor is absolutely required for its interaction with β arrestin 2 and

phosphodiesterase 4D enzymes to regulate cAMP degradation. This association between β2 adrenergic receptor and phosphodiesterase 4D is necessary for proper subcellular localization of PED 4D (Liu et al., 2012).

1.1.4 ER-associated degradation (ERAD)

As discussed in the sections above, protein folding is a complex process that requires the assistance of chaperone proteins and post-translational modifications. Proteins that fail to achieve a native confirmation must be degraded to prevent aggregation at the ER (Kostova and Wolf, 2003). The ERAD machinery selects misfolded proteins for degradation. It has been suggested that chaperones distinguish misfolded, correctly folded and terminally misfolded proteins and direct them to the degradation machinery (Römisch, 2005). Misfolded soluble proteins are transported back into the cytosol from the ER and Sec61 has been suggested as a key player in this reverse translocation. Membrane protein degradation, however, is more complicated than that of soluble proteins (Johnson and Van Waes, 1999). E1 and E2 enzymes, such as Ubc6 and Ubc7, transfer the ubiquitin to E3 ligases, such as Hrd1 or substrate-E3 complex, depending on the E3 ligase involved. E3 ligases also define the substrate specificity of the ubiquitination system (Callis, 2014). In yeast, there are three kinds of ERAD response pathways depending on the particular lesion of the misfolded protein. The ERAD-L pathway consists of the Hrd1 ubiquitin ligases and governs the degradation of proteins with luminal lesions. The ERAD-M pathway consists of Hrd1 and Hrd3, and this regulates the degradation of proteins with transmembrane lesions. The ERAD-C pathway consists of Doa1p and governs the degradation of proteins with misfolded cytosolic domains (Carvalho et al., 2006). The 26S proteasome machinery then binds, unfolds and degrades the substrate (Kostova and Wolf, 2003). Proteins are recognized by the COPII machinery and exported to Golgi. It is thought that misfolded proteins cannot be identified by COPII since the COPII export signal could be hidden. For example yeast, ATPase Pma1, when misfolded, is bound by Eps1, a membrane-bound chaperone and excluded from the COPII transport vesicles (Wang and Chang, 1999). In some cases, misfolded proteins can escape the ERAD and go to the Golgi. However, they are recognized as misfolded and sent back to the ER via COPI mediated retrieval for degradation. This was demonstrated in yeast using the fusion protein KHN (Simian virus 5 hemagglutinin-neuraminidase fused with cleavable

signal sequence from yeast Kar2). KHN is misfolded and inactive when expressed in yeast cells. Misfolded KHN is transported to the Golgi compartment and is retrieved back in COPI vesicles to the ER for degradation (Vashist et al., 2001).

1.1.5 Protein misfolding and human disease

Aggregation disorders: As discussed previously, misfolded proteins form aggregates that result in inclusion bodies and are inherently toxic. These aggregates are classified as 'gain of function' proteins. It is thought that aggregated proteins display amino acid sequences that are otherwise hidden in native proteins and can engage in inappropriate interactions with other proteins and signaling molecules (Bucciantini et al., 2002). Protein aggregation is considered as the primary cause of many neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, Huntington's disease, Amyotrophic lateral sclerosis (ALS) and prion diseases such as Kuru, Creutzfeldt–Jakob disease (CJD) and fatal familial insomnia. Protein aggregation in these conditions is a result of overproduction of the protein leading to the formation of plaques or protein deposits that cannot be cleared efficiently by the ERAD machinery. Oxidation and cellular aging also contribute to the disease phenotype. One of the therapeutic strategies suggested for relieving protein aggregation is the enhancement of cellular defense mechanisms. One drug that has been tested is geldanamycin, but unfortunately it is very toxic and is unable to cross the blood-brain barrier, but other such drugs could be developed. Other strategies include small molecules that can bind and alter protein structure to prevent aggregation (Ross and Poirier, 2004).

Disorders caused by degradation of misfolded proteins: On the other end of the spectrum degradation of misfolded proteins causes a 'loss of function' phenotype that can also be detrimental to cellular health. Cystic fibrosis, familial hypercholesterolemia, diabetes mellitus, polycystic kidney diseases, Tay-Sachs diseases are all examples of disorders where misfolded proteins are targeted for degradation (Hebert and Molinari, 2007). In cystic fibrosis, the cystic fibrosis transmembrane conductance regulator (CFTR) is misfolded due to deletion of phenylalanine at position 508 (ΔF508) in the nucleotide-binding domain in ~70% of the patients. This variant of CFTR is unable to reach the Golgi and is not glycosylated as in the WT protein.

ΔF508 is in fact degraded due to ER retention (Cheng et al., 1990). Chemical chaperones and small molecules that can refold misfolded CFTR or block degradation of the protein can be potent therapeutic agents in the treatment of this disease. Given that protein folding plays such a central role in proper functioning of cells, understanding the fundamental principles of protein folding and trafficking is central to developing therapeutic strategies for many diseases. My research focuses on examining the intracellular trafficking of a model polytopic membrane protein called chitin synthase III in *Saccharomyces cerevisiae*, one of the most versatile model organisms in biology.

1.2 Chitin synthase III (Chs3)

Chitin synthase III (Chs3), a predicted six transmembrane domain protein is responsible for the synthesis of most of the chitin in the yeast cell wall. Absence of Chs3 is not lethal in yeast, but mutants show considerable resistance to calcofluor white (CW) which is a consequence of reduced amounts of cell wall chitin (Cid et al., 1995). The other two chitin synthases in yeast are Chs1 and Chs2. All three enzymes are membrane proteins and catalyze the synthesis of chitin from UDP-*N*-acetylglucosamine. The regulation of the three chitin synthases varies in the cell to ensure correct spatial and temporal expression (Chuang and Schekman, 1996). Chs1 and Chs2 share about 40% structural and sequence similarity. They also share some similarity with Chs3, particularly at the C-terminus. While Chs2 is involved in the formation of the primary septum, Chs3 deposits the chitin ring around the base of the emerging bud and Chs1 is involved in cell wall repairs and has very little activity *in vivo*.

1.2.1 Proteins involved in intracellular trafficking of Chs3

Chs3 localization and distribution depend on the environmental stress on the cell. In WT cells, Chs3 is distributed to the budneck and intracellular punctae. Chs3 requires its dedicated chaperone Chs7, for folding and ER exit. In the absence of Chs7 (Trilla et al., 1999), Chs3 is misfolded, and retained in the ER. Chs3 also requires Pfa4, a palmitoyl acyltransferase, for efficient ER export (Lam et al., 2006). Once correctly folded, Chs3 exits the ER in COPII transport vesicles and is transported to the Golgi for further modification. Chs3 transport from the Golgi to the plasma membrane requires Chs5 and Chs6, which form an exomer coat needed

for transport to the PM. Figure 1.4 highlights the factors involved in Chs3 trafficking from the ER to the PM. During heat stress, Chs3 is re-distributed from the budneck to the plasma membrane. The synthesis of Chs3 during heat stress depends on a Rho1/Pck1 signal transduction pathway. This shift in localization is governed at the level of Golgi and requires Chs5 and Chs6 (Valdivia and Schekman, 2003). At the Golgi Chs3 retention requires the adaptor protein AP-1, which cycles Chs3 between the endosomes and the Golgi (Valdivia et al., 2002).

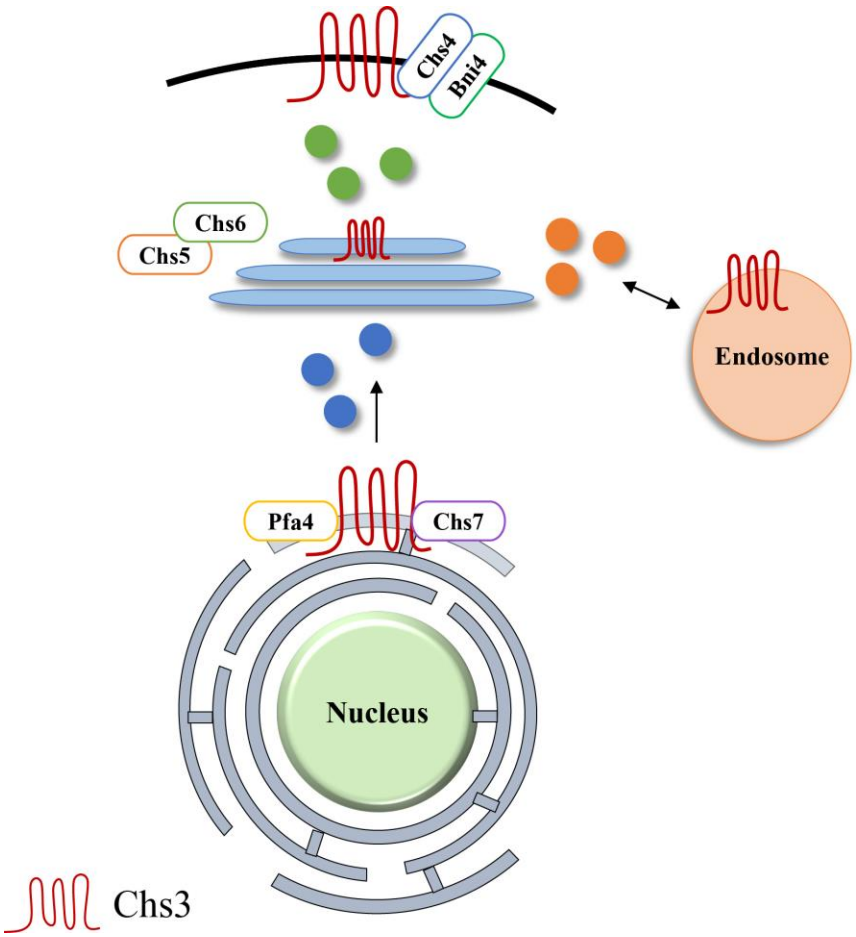

Figure 1.4 Factors involved in Chs3 trafficking
Chs7, a dedicated chaperone, and Pfa4, a DHHC PAT enzyme, aid in Chs3 folding and export at the ER. Chs5 and Chs6 form an exomer coat to transport Chs3 to the PM, where Chs4 and Bni4 regulate its activity and localization.

At the plasma membrane, Chs4 and Bni4 regulate the surface expression and distribution of Chs3. Chs4 physically binds Chs3 and is predicted to change the conformation of Chs3 from an inactive form to an active form (Ono et al., 2000). Bni4 is a scaffolding protein that interacts with Chs3 at the budneck and also with septins. Unlike in *chs4Δ* mutants, chitin levels are not dramatically altered in the absence of Bni4 (Orlean, 2012). This regulated production of Chs3 in yeast parallels the need-based transport of mammalian proteins such as GLUT4 to the cell surface. GLUT4 is a glucose transporter that is recruited to the cell surface in stimulated adipocytes and myocytes. It is thought that in the absence of stimulation (Insulin), GLUT4 is actively retained in specialized cellular compartments and is retained in an active endosomal loop. Any imbalance in this strict regulation leads to diseases such as Type II diabetes (Bryant et al., 2002).

1.3 Research objective

The sections above highlight the complexity of protein folding and maturation and the diversity of protein modifications that are required to achieve this. However, how chaperones and other ER resident proteins identify and interact with their substrates in a traffic-heavy environment such as the ER, which sorts and exports millions of cargo proteins, is yet to be fully understood. Recent advances in the field of protein folding suggest that each client and specific chaperone interaction is unique and what applies for one system does not necessarily apply to others. Some chaperones are strictly required for folding while others have numerous other functions.

For my research project, I sought to elucidate the mechanism through which chaperone proteins and PAT enzymes work together at the level of the ER to facilitate folding and export of polytopic membrane proteins. Our model protein, Chs3, needs its dedicated chaperone, Chs7, and palmitoylation by a PAT enzyme, Pfa4, to assemble into an export competent state. Recent results from our lab indicate that Chs7 is not a resident ER protein and can leave the ER and localize to the plasma membrane. Therefore, we speculate that Chs7 has additional roles in Chs3 trafficking. I hypothesize that Chs7 acts as an accessory protein and aids Chs3 function at the cell surface.

As discussed in the earlier sections, protein palmitoylation plays a vital role in protein stability, membrane association, protein-protein interactions, and trafficking. Chs3 palmitoylation could influence its stability, its trafficking and its interaction with Chs7. Chs7 and Pfa4 could act in an obligatory sequence to fold Chs3 at the ER or they could act simultaneously to fold Chs3. Chapter II focuses on the various roles of Chs7 in Chs3 trafficking, and chapter III focuses on how palmitoylation influences Chs3 trafficking and interactions and how palmitoylation deficient Chs3 is monitored and degraded at the ER.

Chapter 2: The chaperone Chs7 forms a stable complex with Chs3 and promotes its activity at the cell surface

2.1 Introduction

Polytopic membrane proteins are integrated into the endoplasmic reticulum (ER) membrane through a series of steps that include targeting of ribosome-nascent polypeptide complexes to the translocon and lateral partitioning of α-helical transmembrane spans into the lipid bilayer, followed by the chaperone-assisted folding of luminal, cytosolic and membrane-embedded protein domains (Shao and Hegde, 2011). A subset of polytopic proteins additionally requires specialist membrane-associated chaperones to achieve their fully folded state (Ellgaard et al., 1999). These dedicated chaperones are highly heterogeneous and act on a limited number of client proteins. Some may bind the transmembrane helices as they exit the translocon, thereby preventing aggregation until the substrate has attained its final topology or oligomeric state (Houck and Cyr, 2012). Client-specific chaperones may also promote ER exit by providing signals for interaction with the COPII budding machinery and thus accompany their substrate out of the ER. While some chaperones are retrieved to the ER, others are transported with their substrates to their final destination where they may play additional roles.

Currently, little is known about the precise roles of substrate-specific chaperones. Several dedicated molecular chaperones have been described in the yeast Saccharomyces cerevisiae. Loss of Shr3, Pho86, and Gsf2 causes aggregation and ER retention of their cognate substrates Gap1, Pho84, and Hxt1, respectively (Kota and Ljungdahl, 2005). A systematic study in yeast recently uncovered substrates for other ER exit chaperones including Erv14, Erv29, and Erv26 (Herzig et al., 2012). However, the mechanism by which each of these chaperones promotes folding and ER exit of their substrates may differ. Neither Shr3 nor Pho86 leave the ER (Kuehn et al., 1996; Lau et al., 2000). In contrast, both Vma21, which is required for the functional assembly and export of V-ATPase, and Gsf2 are exported from the ER with their substrates and have a KKXX signal that directs their recycling back to the ER in COPI vesicles (Hill and Stevens, 1994; Sherwood and Carlson, 1999).

Chs7 is a chaperone that is required for the folding and ER exit of the yeast chitin synthase Chs3, but is not required for folding or transport of the two other chitin synthases, Chs1 and Chs2 (Kota and Ljungdahl, 2005; Trilla et al., 1999). Although Chs7 has been described as an ER resident protein, it is not known if it engages the COPII and COPI transport machinery to recycle between the ER and Golgi. Chs3 has additional requirements for folding and transport through the secretory pathway. Palmitoylation of Chs3 by the palmitoyl transferase, Pfa4, is needed to prevent aggregation and partial retention of Chs3 in the ER (Lam et al., 2006). At the Golgi, Chs3 is recognized by components of exomer (Chs5 and Chs6); a specialized coat that regulates its export to the cell surface (Santos and Snyder, 1997; Wang et al., 2006; Ziman et al., 1998), while other factors such as Chs4 and Bni4 contribute to Chs3 activation and retention at the bud neck (Demarini et al., 1997; Ono et al., 2000). Chs3 recycles between the budneck and endosomes and is stored in intracellular vesicles until ready for use (Valdivia and Schekman, 2003).

Here, we examine how Chs7 mediates folding and export of Chs3. We find that Chs7 is not an ER resident protein as reported previously. Instead, Chs7 forms a complex with Chs3 and co-localizes with Chs3 at the cell surface and in Golgi/endosomal compartments. We show that Chs7 and Chs3 depend on each other for proper co-localization and that Chs3 requires Chs7 to function as a chitin synthase. This suggests that Chs7 is not only a Chs3-specific folding chaperone and ER exit factor, but functions as a cofactor to regulate the enzymatic activity of Chs3 at the cell surface.

2.2 Methods and materials

2.2.1 Strains, plasmids, and media

General molecular biology methods were as described (Conibear and Stevens, 2000; Conibear and Stevens, 2002). Strains and plasmids used in this study are listed in Table 2.1. ORFs were genomically tagged with sequences encoding HA, GFP or an improved version of GFP (GFP+) by PCR-based homologous recombination (Dilworth et al., 2001; Longtine et al., 1998; Scholz et al., 2000). NatR (Nourseothricin resistance) and hphR (Hygromycin B resistance) knockout strains were created by PCR-based homologous recombination using gene-specific primers to

amplify drug resistance cassettes from p4339 pCRII-TOPO:: natRMX4 plasmid (Tong et al., 2001) or pFA6-hphNT1 (Janke et al., 2004). Knockout strains were confirmed by PCR of genomic DNA. The making of temperature-sensitive *sec21-1* mutant CKY69 (MATa ura3-52 sec21-1) and its congenic wild-type CKY10 (MATa ura3-52 leu2-3,112) are described in (Gimeno et al., 1995). *CHS7* mutants were generated by site-directed mutagenesis. The split YFPN and YFPC strains were made by C-terminal PCR tagging of the indicated ORFs with YFPN sequence amplified from pHVF1CT-cln1 or YFPC amplified from pHVF2CT-cln10 (gift from Chris Loewen, UBC) to generate haploid double tagged strains. The plasmid pEL2 was constructed by cloning the *CHS3* gene into the SacI/KpnI sites of pRS316. The plasmids pTM15 (pRS313:: CHS7-GFP) and pTM16 (pRS316:: CHS7-3xHA) were a gift from Cesar Roncero.

Table 2.1 Strains and plasmids used in this study

Strain	Genotype	Source
BY4741	MATa his3-1 leu2-0 met15-0 ura3-0	Open Biosystems
CSY99	BY4741 *CHS7*-GFP^{+}::*HIS3*	This study
CSY114	BY4741 *chs3*Δ::nat^{R}, *CHS7*-GFP^{+}::*HIS3*	This study
KLY9	BY4741 *CHS3*-GFP::*HIS3*	Lam et al., 2006
KLY3	BY4741 *chs7*Δ::kan^{R}, *CHS3*-GFP::*HIS3*	Lam *et al*., 2006
CSY89	BY4741 *CHS7*-GFP^{+}::*HIS3*	This study
MDY706	BY4741 *chs7-Δ11*-GFP^{+}::*HIS3*	This study
MDY705	BY4741 *chs7-Δ14*-GFP^{+}::*HIS3*	This study
MDY704	BY4741 *chs7-Δ17*-GFP^{+}::*HIS3*	This study
MDY703	BY4741 *chs7-Δ20*-GFP^{+}:*HIS3*	This study
CSY118	BY4741 *chs7-Δ22*-GFP+::HIS3	This study
NP112	BY4741, *chs7-DxDxE*-GFP^{+}::HIS3	This study
CSY101	BY4741 *chs7-LEF*-GFP^{+}::*HIS3*	This study

Strain	Genotype	Source
CSY102	BY4741 *chs7-T293A*-GFP^{+}::*HIS3*	This study
KLY41	BY4741 *CHS3*-3HA::kan^{R}	Lam et al., 2006
MDY718	BY4741 *CHS7*-GFP^{+}::*HIS3, CHS3*-3HA*::*kan^{R}	This study
MDY707	BY4741 *chs7-LEF*-GFP^{+}::*HIS3, CHS3*-3HA*::*kan^{R}	This study
MDY708	BY4741 *chs7-T293A*-GFP^{+}::*HIS3, CHS3*-3HA*::*kan^{R}	This study
MDY709	BY4741 *chs7-Δ22*-GFP^{+}::*HIS3, CHS3*-3HA*::*kan^{R}	This study
CSY93	BY4741, *CHS7*-3HA::kan^{R}, *CHS3*-GFP^{+}::nat^{R}	This study
TDY12	BY4741 *chs3Δ*::hph^{R}, *CHS7*-3HA::kan^{R}	This study
CSCY92	BY4741 *chs7-Δ22*-3HA::kan^{R}, *CHS3*-GFP^{+}:: nat^{R}	This study
CSY154	BY4741 *chs7-LEF*-3HA::kan^{R}, *CHS3*-GFP^{+}::nat^{R}	This study
CSY155	BY4741 *chs7-T293A*-3HA::kan^{R}, *CHS3*-GFP^{+}::nat^{R}	This study
NP107	BY4741, *CHS7*-3HA::kan^{R}, *CHS3*-GFP^{+}::nat^{R}, *chs6Δ*::hph^{R}	This study
NP108	BY4741 *chs7-LEF*-3HA::kan^{R}, *CHS3*-GFP^{+}::nat^{R}, *chs6Δ*:: hph^{R}	This study
NP109	BY4741 *chs7-T293A*-3HA::kan^{R}, *CHS3*-GFP^{+}::nat^{R}, *chs6Δ*:: hph^{R}	This study
NP106	BY4741 *chs7-Δ22*-3HA::kan^{R}, *CHS3*-GFP^{+}::nat^{R}, *chs6Δ*::hph^{R}	This study
CSY3421	BY4741, *CHS7*-3HA::kan^{R}, *CHS3*-GFP^{+}::nat^{R}, *pep12*Δ::*HIS3*	This study
CSY3422	BY4741 *chs7-LEF*-3HA::kan^{R}, *CHS3*-GFP^{+}::nat^{R}, *pep12*Δ::*HIS3*	This study
CSY3423	BY4741 *chs7-T293A*-3HA::kan^{R}, *CHS3*-GFP^{+}::nat^{R}, *pep12*Δ::*HIS3*	This study
CSY3420	BY4741 *chs7-Δ22*-3HA::kan^{R}, *CHS3*-GFP^{+}::nat^{R}, *pep12*Δ::*HIS3*	This study
CSCY92	BY4741, *CHS7*-3HA::kan^{R}, *CHS3*-GFP^{+}::nat^{R}, *end4Δ*::hph^{R}	This study
CSCY87	BY4741, *chs7-LEF*-3HA::kan^{R}, *CHS3*-GFP^{+}::nat^{R}, *end4Δ*::hph^{R}	This study
CSCY89	BY4741, *chs7-T293A*-3HA::kan^{R}, *CHS3*-GFP^{+}::nat^{R}, *end4Δ*::hph^{R}	This study
CSCY91	BY4741, *chs7-Δ22*-3HA::kan^{R}, *CHS3*-GFP^{+}::nat^{R}, *end4Δ*::hph^{R}	This study

Strain	Genotype	Source
CSY101	BY4741 *chs7-LEF*-GFP^{+}::*HIS3*	This study
CSY102	BY4741 *chs7-T293A*-GFP^{+}::*HIS3*	This study
CSY118	BY4741 *chs7-Δ22*-GFP^{+}::*HIS3*	This study
CSY2895	BY4741 *CHS7*-GFP^{+}::HIS3, *CHS3*-3HA::kan^{R} *chs6Δ*::nat^{R}	This study
CSY2888	BY4741 *chs7-LEF*-GFP^{+}::HIS3, *CHS3*-3HA::kan^{R} *chs6Δ*::nat^{R}	This study
CSY2890	BY4741 *chs7-T293A*-GFP^{+}::HIS3, *CHS3*-3HA::kan^{R} *chs6Δ*::nat^{R}	This study
CSY2892	BY4741 *chs7-Δ22*-GFP^{+}::HIS3, *CHS3*-3HA::kan^{R} *chs6Δ*::nat^{R}	This study
CSY135	BY4741 *CHS7*-GFP^{+}::*HIS3, pep12*Δ::kan^{R}	This study
CSY136	BY4741 *chs7-LEF*-GFP^{+}::*HIS3, pep12*Δ::kan^{R}	This study
CSY137	BY4741 *chs7-T293A*-GFP^{+}::*HIS3, pep12*Δ::kan^{R}	This study
CSY137	BY4741 *chs7-Δ22*-GFP^{+}::*HIS3, pep12*Δ::kan^{R}	This study
CSCY62	BY4741 *CHS7*-GFP::*HIS3, end4*D::*URA3*	This study
CSCY64	BY4741 *chs7-LEF*-GFP::*HIS3, end4*Δ::*URA3*	This study
CSCY66	BY4741 *chs7-T293A*-GFP::*HIS3, end4*Δ::*URA3*	This study
CSCY68	BY4741 *chs7-Δ22*-GFP::*HIS3, end4*Δ::*URA3*	This study
KLY46	BY4741 *chs7*Δ::*HIS3, CHS3*-3HA: kan^{R}	Lam et al., 2006
CSCY97	BY4741 *CHS3-YFPN::HIS3, CHS7-YFPC::URA3*	This study
CSCY70	BY4741 *CHS3-YFPN::HIS3, chs7-Δ22-YFPC::URA3*	This study
KLY32	BY4741 *Chs3Δ::nat^{R}*	This study
CSY105	BY4741 *Chs7Δ::URAMX4*	This study
	BY4741 *chs7Δ*::kan^{R}	This study
CKY10	ura3-52 leu2-3,112	This study
NP39	CKY10 *CHS7*-GFP::kan^{R}	This study

NP40	CKY10 *sec21-1*, *CHS7*-GFP::*kan*^{R}	This study
NP41	CKY10 *CHS7*-GFP::*kan*^{R}, *chs3*Δ*::nat*^{R}	This study
NP42	CKY10 *sec21-1*, *CHS7*-GFP::*kan*^{R}, *chs3*Δ*::nat*^{R}	This study

Plasmid	**Genotype**	**Source**
pEL2	*CHS3* in pRS316	This study
pTM16	*CHS7*-3XHA in pRS316	C.Roncero
pTM15	*CHS7*-GFP in pRS316	C.Roncero
pCS30-2	*CHS7*-GFP+ in pRS316	This study
pCS40	*CHS7*-GFP+ with 1.9 Kb 5'UTR in pRS316	This study

2.2.2 Fluorescence microscopy

For fluorescence microscopy of live cells expressing GFP or YFP fusions, cells were grown to log phase at 30°C or 25°C in synthetic complete minimal media and observed directly. Cells were viewed using a 100x oil-immersion objective on a Zeiss Axioplan2 fluorescence microscope (Thornwood, NY), and images were captured with a CoolSnap camera using MetaMorph software (Universal Imaging, West Chester, PA), and adjusted using Adobe Photoshop for best visualization.

2.2.3 Co-immunoprecipitations

Co-immunoprecipitation was performed as previously described (32, 11). Briefly, 20 OD_{600} of spheroplasts were resuspended in 1 mL lysis buffer containing 1% CHAPSO, 50 mM KPO4, pH7.5, 50 mM NaCl, and a protease inhibitor cocktail. The lysate was pre-cleared with 20 µL of 75% slurry of IgG–Sepharose (GE Healthcare) for 1 h at 4°C. The pre-cleared supernatant was incubated with rabbit α-GFP (A6455; Life Technologies, Burlington, ON). Sepharose beads were washed thrice in lysis buffer, resuspended in sample buffer, and analyzed by SDS-PAGE. Co-immunoprecipitated proteins were detected by Western blotting using either mouse anti-HA antibodies (HA.11; Covance, Berkeley, CA) or anti-GFP antibodies (Clones 7.1 and 13.1; Roche

Diagnostics, Indianapolis, IN) followed by HRP-conjugated goat anti-mouse antibody (Jackson Immunoresearch Laboratories, West Grove, PA).

2.2.4 Protease accessibility

The protease accessibility assay was modified from (Chen and Davis, 2000; Schluter et al., 2008). 5 OD_{600} of log phase cells expressing Chs3-3xHA were harvested and washed in 1mL Pronase Buffer (PB) (1.4M Sorbitol, 25mM Tris-HCl pH 7.5, 10mM NaN3, 10mM NaF). Cells were resuspended in 350uL PB and incubated for 30 min at 30°C. Half was treated with a final concentration of 2mg/mL Pronase (Calbiochem-Novabiochem, La Jolla, CA) at 37°C for 1h, and the other half was mock treated with an equal volume of PB. Cells were collected at 6500rpm, resuspended in 225uL PB, and proteins were precipitated with 50uL of 100% TCA. Washed TCA pellets were resuspended in SDS-PAGE sample buffer and analyzed by Western blotting with α-HA (1:1000) or α-PGK (1:800) mAbs followed by HRP-labeled anti-mouse secondary antibody. Blots were developed with ECL and imaged on a Fluor S-Max Multi-imager.

2.2.5 BN-PAGE

20 OD600 spheroplasts were suspended in 1mL 1X Novex® NativePAGE™ Sample Buffer pH 7.2 (Life Technologies), with 1% Digitonin (Sigma-Aldrich) and Fisher® Halt Protease Inhibitor Cocktail. The protein concentration was equalized to 60μg of protein using Pierce® BCA Protein Assay Kit, and 0.5 μL of Coomassie G-250 sample additive (Life Technologies) was added to the cleared lysates just prior to loading. Proteins were detected by Western blotting using mouse anti-HA and mouse anti-GFP antibodies, followed by HRP-conjugated goat anti-mouse antibody. Blots were developed with ECL and imaged using Kodak Film Developer. Quantity One software was used for densitometry of scanned images and relative pixel values were plotted using MS excel 2010.

2.2.6 Crosslinking

Crosslinking of yeast cell lysates with DSP was performed as described (Kota and Ljungdahl, 2005) with slight modifications. DSP was added to 100ug protein in 40uL PBS. Reactions were

quenched with 40mM Tris-HCl pH 7.5 at 25°C for 30 min. Parallel samples were treated with 40mM DTT at 37°C for 30 min.

2.2.7 Calcofluor white assay

Source arrays were created by randomly placing each strain at 12 different positions of a 96-density array on a YPD plate. A Virtek automated colony arrayer (BioRad, Hercules, CA) was used to pin source arrays at 1536 density to YPD plates containing 50ug/ml Calcofluor White (Sigma), which were incubated in light-proof containers at 30C for 3 days. White-light images were acquired using an Epson 2400 flat-bed scanner, and fluorescent light images were captured with a Fluor S-Max Multi-imager (BioRad) using the 530DF60 filter and Quantity One software (Ver 4.2.1, BioRad). An in-house spot-finding program was used for densitometry of digital images. Average growth and fluorescence values were calculated for each strain using Microsoft Excel; slow growing strains were removed from the analysis.

2.3 Results

2.3.1 Chs7 localizes at the cell surface and in intracellular structures

A previous report suggested Chs7 is an ER resident protein that acts at the ER to promote the folding of Chs3 (Trilla et al., 1999). Surprisingly, we found that endogenously expressed Chs7-GFP tagged at its chromosomal location was not found in the ER at steady state but instead was present at the bud neck and intracellular puncta, similar to the distribution of Chs3-GFP (Fig 2.1 A). The previously reported ER localization of Chs7 was based on the distribution of a tagged protein expressed from a plasmid containing approximately 1 Kb of 5'UTR (Trilla et al., 1999). We found that strains containing this plasmid expressed much higher levels of Chs7-GFP compared to strains where Chs7-GFP was tagged at its chromosomal location, suggesting its ER localization could be an overexpression artifact (Fig 2.8; supplemental information). Addition of extra 930 bp upstream sequence to the plasmid-borne *CHS7* resulted in a level of expression comparable to that of the integrated reporter and restored localization to the bud neck and intracellular puncta.

We were unable to compare the co-localization of Chs7 and Chs3 directly due to the low signal of our RFP-tagged proteins. However, in strains lacking Chs3, Chs7 was no longer seen at the bud neck or intracellular puncta, but instead was present in the ER and vacuoles (Fig 2.1 A). The partial ER retention of Chs7 in a chs3 null mutant was unlikely to result from Golgi retrieval, as the temperature-sensitive COPI mutant *sec21-1* was unable to rescue it (Fig 2.1 B). The prominent vacuolar localization indicates that the bulk of Chs7 is able to exit the ER in the absence of Chs3. In contrast, Chs3 shows a strong ER localization in *chs7Δ* mutants, with no detectable vacuole or plasma membrane pool, suggesting it is unable to exit the ER in the absence of Chs7 (Fig 2.1 A). Thus, Chs7 does not entirely rely on Chs3 association for its ER exit but does require Chs3 for its subsequent trafficking and steady-state localization to the bud neck and intracellular structures.

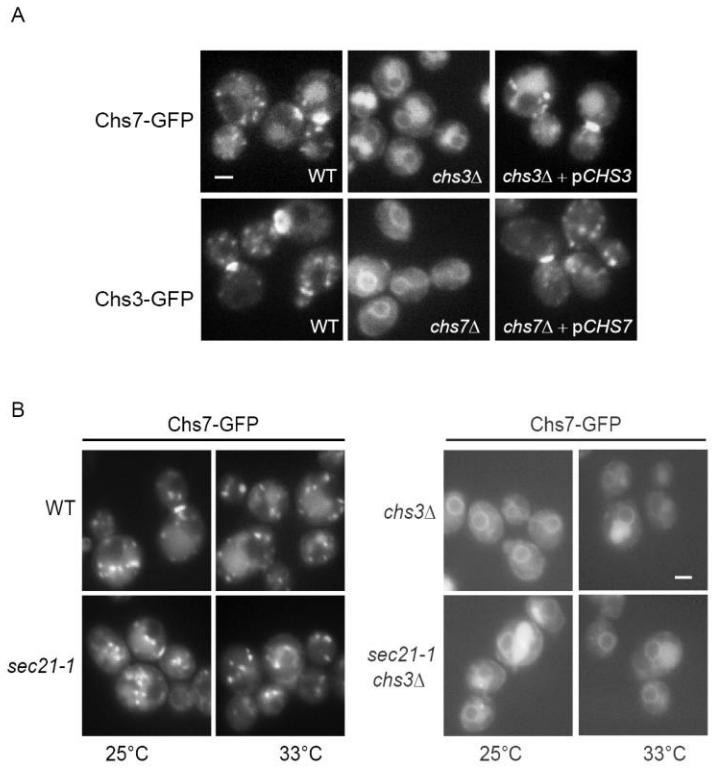

Figure 2.1 Chs7 localizes at the budneck and intracellular punctae
(A) Cells expressing genomically tagged Chs7-GFP and Chs3-GFP in the indicated strain backgrounds were observed by fluorescence microscopy. (B) Genomically tagged Chs7-GFP strains in the indicated strain backgrounds were observed by fluorescence microscopy. Scale bar, 2μM.

2.3.2 Conserved residues in the Chs7 C-terminal tail contribute to Chs3 binding

The interdependence of Chs3 and Chs7 for their steady-state localization suggests that Chs7 and Chs3 may exit the ER and be transported to the cell surface as a complex. Because Chs7 is partly competent for ER exit in the absence of Chs3, Chs7 may provide the ER export signal that allows Chs3 to enter COPII vesicles.

Chs7 is predicted to have seven membrane-spanning domains, with an extracellular N-terminus, several short cytoplasmic loops, and a C-terminal tail. The C-terminal tail contains a conserved region from 280-299, which includes a predicted di-acidic COPII binding motif DxE (DLE), whereas the last 17 residues are highly divergent among fungal species (Fig 2.2 A). To determine if the Chs7 C-terminal tail contains elements critical for ER exit, we made a series of GFP-tagged truncation mutants (Fig 2.3 A). Loss of the last 17 residues ($Chs7^{\Delta 17}$) did not affect Chs7 localization, whereas deletion of additional residues ($Chs7^{\Delta 20}$, $Chs7^{\Delta 22}$) caused a dramatic reduction in bud neck localization, an increase in vacuolar signal and detectable labeling of the ER (Fig 2.2 B).

The truncations that enhanced ER retention ablate the putative DxE exit signal. However, point mutations that specifically abolished this motif ($Chs7^{DxDxE}$; Fig 2.2 B) did not cause a comparable ER retention defect, and instead resulted in a predominantly vacuolar localization. The DxE motif may be one of the several redundant exit signals present in the Chs7 C-terminal tail. Exit signals could also be present in other regions of Chs7, although mutation of conserved residues in the largest of the intracellular loops had no effect on Chs7 localization (unpublished data). Alternatively, the DxE sequence could contribute to the folding or function of a larger conserved region that is important for the transport of Chs7 to the bud neck. We used site-directed mutagenesis to change other highly conserved residues in this region of Chs7 to alanine (Fig 2.2 A, 2.2 B). Both $Chs7^{LEF}$-GFP and $Chs7^{T293A}$-GFP mutants, similar to the $Chs7^{DxDxE}$-GFP mutant, localized primarily to vacuoles and showed greatly reduced staining of the PM, with little ER staining.

The dual ER/vacuolar localization of the most severely truncated forms of Chs7 were similar to the localization of wild-type Chs7 in a strain lacking Chs3 (Fig 2.1 A), suggesting these mutations might perturb Chs3 binding. Immunoprecipitation of wild-type Chs7-GFP resulted in efficient co-purification of Chs3-HA (Fig 2.2 C). In contrast, each of the Chs7 point mutations greatly impaired the Chs3-Chs7 interaction, and Chs7$^{\Delta 22}$-GFP showed a very low level of residual Chs3 binding. This indicates that a conserved region of the Chs7 C-terminal tail makes an important contribution to the interaction between Chs7 and Chs3.

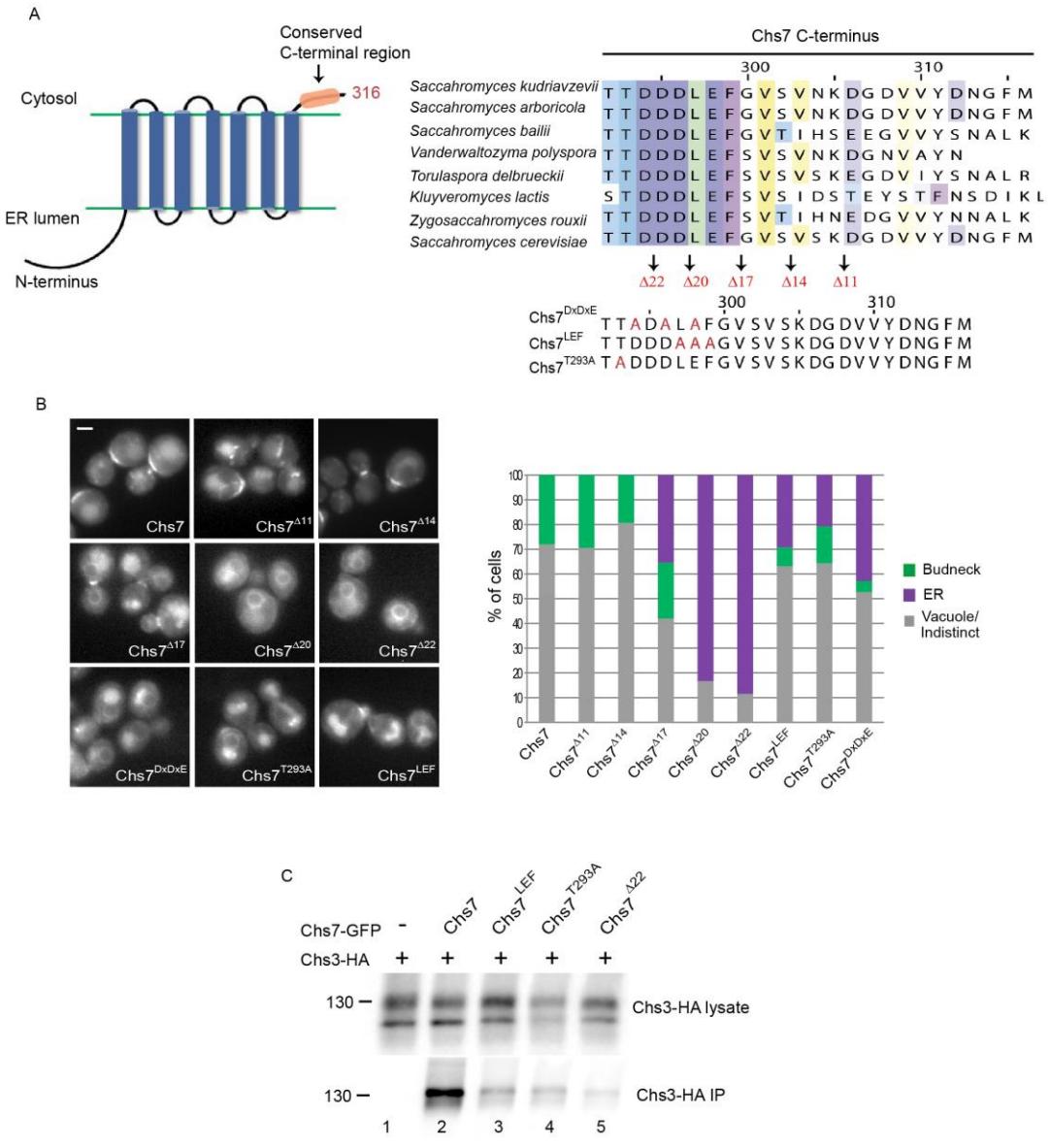

Figure 2.2 Conserved residues in the C-terminal tail of Chs7 determine its localization and binding to Chs3.
(A) Schematic showing the topology of the Chs7 protein (left) and alignment of the Chs7 C-terminal cytosolic domain from different fungal species (right). The position of truncation mutants is shown below the alignment, and residues that were changed to alanine in different point mutants is shown in red. (B) The indicated strains were genomically tagged at the C-terminus with GFP and observed by fluorescence microscopy. Right panel shows the proportion of cells in each strain with predominant ER, budneck or vacuolar localization of the indicated wild-type or mutant forms of Chs7-GFP. An average of 200 cells were counted per strain. Scale bar, 2μM. (C) Wild-type and mutant forms of Chs7-GFP were immunoprecipitated with α-GFP Ab and co-precipitating Chs3-HA was detected with α-HA mAb. Loading of the lysates relative to IP was 1:9.

2.3.3 Mutations that reduce Chs3-Chs7 complex formation do not impair folding

To estimate the proportion of cellular Chs3 and Chs7 that exists in a complex at steady state, digitonin extracts were prepared from cells co-expressing tagged forms of these proteins and resolved by Blue Native PAGE (BN-PAGE) (Fig 2.3 A,B). Complex formation was examined by comparing the co-migration of Chs3-GFP and Chs7-HA in superimposed intensity profiles (Fig 2.3 C, D). Chs3-GFP was present in a single peak in wild-type cells, whereas Chs7-HA was found in two forms: a major pool that comigrated with Chs3-GFP, and a minor, faster migrating species. The major pool of Chs7-HA was lost when *CHS3* was deleted, suggesting that a large fraction of Chs3 and Chs7 are present in a complex at steady state. All three mutant forms of Chs7-HA also showed a significant reduction of the larger co-migrating band (Fig 2.3 B), and an increase in the fast migrating band, consistent with the idea that these mutations partially ($Chs7^{LEF}$, $Chs7^{T293A}$) or largely ($Chs7^{\Delta 22}$) disrupt complex formation.

BN-PAGE was also used to determine the extent of Chs3 misfolding and aggregation caused by mutation of Chs7. Chs3-GFP extracted from *chs7Δ* cells was present in a faster migrating species and a high molecular weight smear. Unexpectedly, presence of the $Chs7^{\Delta 22}$ truncation mutant had a relatively minor effect on Chs3-GFP mobility, indicating a small degree of Chs3 misfolding and aggregation, while no misfolding was seen in strains expressing the $Chs7^{LEF}$ and $Chs7^{T293}$ point mutants (Fig 2.3 A).

We used a crosslinking assay as an alternative approach to evaluate the extent of Chs3 misfolding caused by mutation of the Chs7 tail. Chs3 aggregates are readily cross-linked at low DSP concentrations and appear as a high molecular weight smear on non-reducing gels (Kota

and Ljungdahl, 2005; Lam et al., 2006). We found that Chs3-HA was efficiently crosslinked into higher molecular weight aggregates in a *chs7* null mutant, whereas truncation of the Chs7 tail ($Chs7^{\Delta 22}$) caused an intermediate level of aggregation (Fig 2.9; supplemental information). However, in cells expressing $Chs7^{LEF}$, Chs3-HA was crosslinked only in the presence of high concentrations of DSP, similar to wild-type cells and consistent with the results of the BN-PAGE assay. The lack of Chs3 misfolding in these mutants was surprising, given that each of these mutants is strongly impaired in Chs3-Chs7 complex formation, and suggests either a small amount of Chs3-associated Chs7 is sufficient for folding or that Chs7 mutants associate transiently with Chs3 in the ER to promote folding and dissociate once folding is complete.

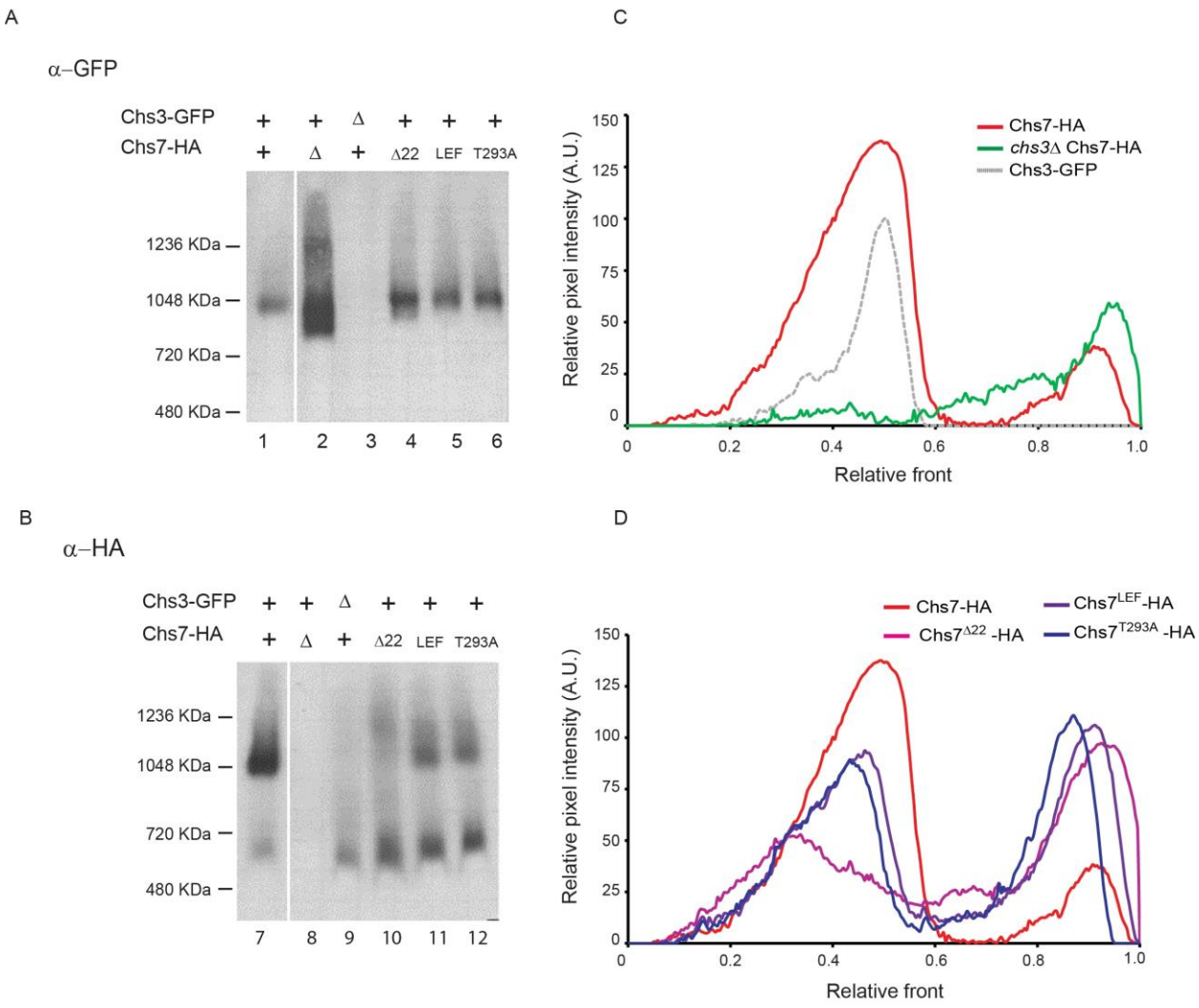

Figure 2.3 Mutations in the conserved C-terminal tail of Chs7 destabilize the Chs3-Chs7 complex. Lysates from log phase cultures of the indicated strains co-expressing genomically tagged Chs3-GFP and Chs7-HA or the indicated mutant forms were separated on parallel 3-12% Bis-Tris Native Page gels. Chs3 and Chs7 were detected using α-GFP and α-HA mAb respectively. Note that MW standards do not reflect the true molecular weight of integral membrane protein complexes in blue native gels due to variable amounts of bound lipids. (C) Densitometry plots of lanes 7 and 9 from blot shown in (B) indicate the relative migration of Chs7-HA in WT and *chs3*Δ strains. A plot of lane 1 (blot A) shows the position of Chs3-GFP. (D) Densitometry plots showing relative migration of WT and mutant forms of Chs7-HA (lanes 7, 10 11 and 12; blot B). A.U.: arbitrary units.

2.3.4 Mutation of conserved residues in the Chs7 C-terminal tail uncouples post-Golgi trafficking of Chs7 and Chs3

We next examined the effect of Chs7 tail mutants on the localization of Chs3-GFP. $Chs7^{\Delta 22}$ caused Chs3-GFP to be at least partially retained in the ER (Fig 2.4 A). Unexpectedly, point mutations that disrupted Chs3-Chs7 interactions and redirected Chs7 to the vacuole had relatively little effect on the steady-state localization of Chs3-GFP, which remained at the bud neck and in intracellular puncta. The fact that Chs3 is correctly folded and able to exit the ER suggests that the $Chs7^{LEF}$ and $Chs7^{T293}$ mutations do not affect Chs3-Chs7 interactions in the ER, nor prevent entry into COPII vesicles, but instead cause the trafficking of Chs3 and Chs7 to diverge at a post-ER transport step.

To test this interpretation, we examined the distribution of Chs3 and Chs7 in mutants lacking the exomer subunit Chs6. Chs3 contains signals to bind both AP-1 and exomer. In the absence of exomer components, Chs3 is retained intracellularly by continuous AP-1-dependent cycling between Golgi and endosomes (Starr et al., 2012; Valdivia et al., 2002). Chs7-GFP was trapped in intracellular Golgi/endosomal compartments when Chs3 was present, but not when Chs3 was absent, or when the Chs3 interaction was abrogated by mutation of the Chs7 tail (Fig 2.4 B). In contrast, Chs3-GFP was retained in intracellular puncta in exomer mutants even in cells expressing $Chs7^{LEF}$ and $Chs7^{T293}$ mutants (Fig 2.4 A). Thus, it appears that Chs3 does not require Chs7 for its normal post-Golgi sorting itinerary, whereas Chs7 relies on its association with Chs3 for recycling on the AP-1 dependent Golgi/endosomal pathway.

We used two transport blocks to determine at which point the trafficking of Chs3 and Chs7 diverged. Deletion of the endosomal syntaxin protein Pep12, which inhibits fusion of post-Golgi and endocytic vesicles with the late endosome, prevented the vacuolar transport of Chs7-GFP tail mutants. In endocytosis-defective *end4* strains, Chs7-GFP mutants showed some accumulation on the cell surface but most still reached the vacuole. In contrast, the *end4* null mutation caused cell surface accumulation of Chs3-GFP in strains expressing either wild-type or mutant forms of Chs7. This suggests that Chs3 is sorted at the Golgi and delivered directly to the PM. Taken together, these results show that Chs7 while absolutely required for the ER exit of Chs3, has little influence on its post-ER trafficking. In contrast, continued association with Chs3 is critical for Chs7 to recycle between Golgi and endosomes, and to be transported to the cell surface.

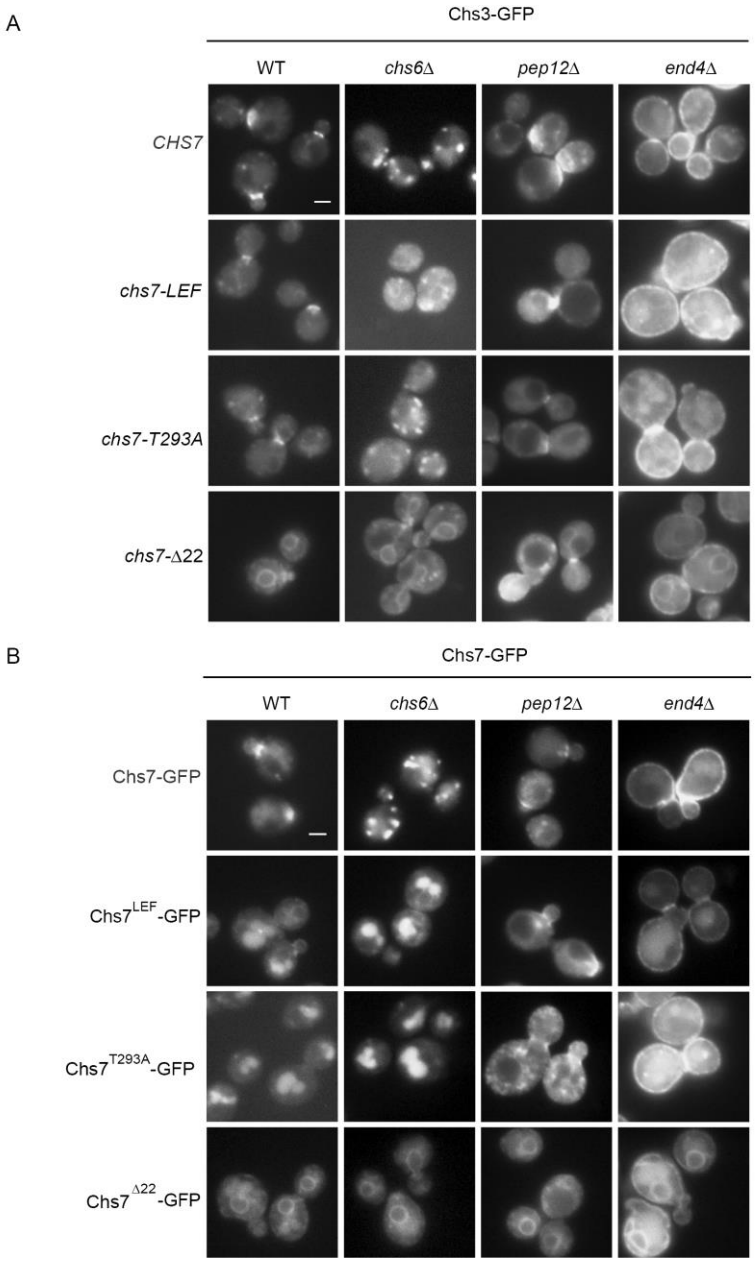

Figure 2.4 Mutation of Chs7 C-terminal tail causes Chs3 and Chs7 trafficking to diverge after ER exit

Strains co-expressing Chs3-GFP and HA-tagged wild-type and mutant forms of Chs7 in the indicated strains were observed by fluorescence microscopy. **(B)** Strains expressing wild-type and the mutant form of Chs7-GFP in the indicated strains were observed by fluorescence microscopy. All the strains were incubated at 30°C except for *end4Δ* strains, which were incubated at 25°C. Scale bar, 2μM.

2.3.5 Chs7 promotes Chs3 catalytic activity at the cell surface

Although our results suggest Chs7 is not needed for Chs3 folding or transport after ER exit, it may contribute to Chs3 function at the cell surface. To test this, a protease shaving assay was first used to quantify the amount of Chs3 exposed on the surface of each Chs7 mutant strain (Fig 2.5 A). Intact cells expressing HA-tagged Chs3 were treated with pronase, which cleaves the extracellular portion of surface-exposed Chs3-HA, releasing a C-terminal fragment that carries the HA epitope. The ratio of this C-terminal fragment to total Chs3-HA provides a measure of Chs3 levels at the cell surface (Fig 2.5 B). Using this method, approximately 21% of total Chs3-HA was found at the surface in wild-type cells, consistent with previously published estimates (Valdivia and Schekman, 2003). Chs3-HA surface levels were only slightly reduced (16% of total Chs3) in strains expressing the Chs7 point mutants. In contrast, in strains expressing the $Chs7^{\Delta 22}$ truncation mutant, which caused some misfolding and ER retention of Chs3, the amount of Chs3-HA present on the cell surface was only slightly greater that of a *chs7* null mutant.

To determine if the absence of Chs7 at the cell surface affects the enzymatic activity of Chs3, we examined steady-state chitin levels using an assay based on the binding of the fluorescent antifungal drug Calcofluor white (CW) to cell wall chitin (Burston HE, Davey M, 2008; Lam et al., 2006). As expected, the strains with the lowest fluorescence values included *chs3* and *chs7* null mutants (Fig 2.5C; 11). Chitin levels in strains expressing the $Chs7^{\Delta 22}$ truncation were not significantly higher than in *chs3* or *chs7* mutants, consistent with the low level of Chs3 present at the cell surface in these strains. Remarkably, strains expressing the $Chs7^{LEF}$ point mutant also had very low chitin levels despite having relatively normal levels of Chs3 at the cell surface. This suggests that lack of Chs7 at the cell surface does not strongly impact Chs3 transport and retention at the cell surface, but instead causes a dramatic reduction in the enzymatic activity of Chs3.

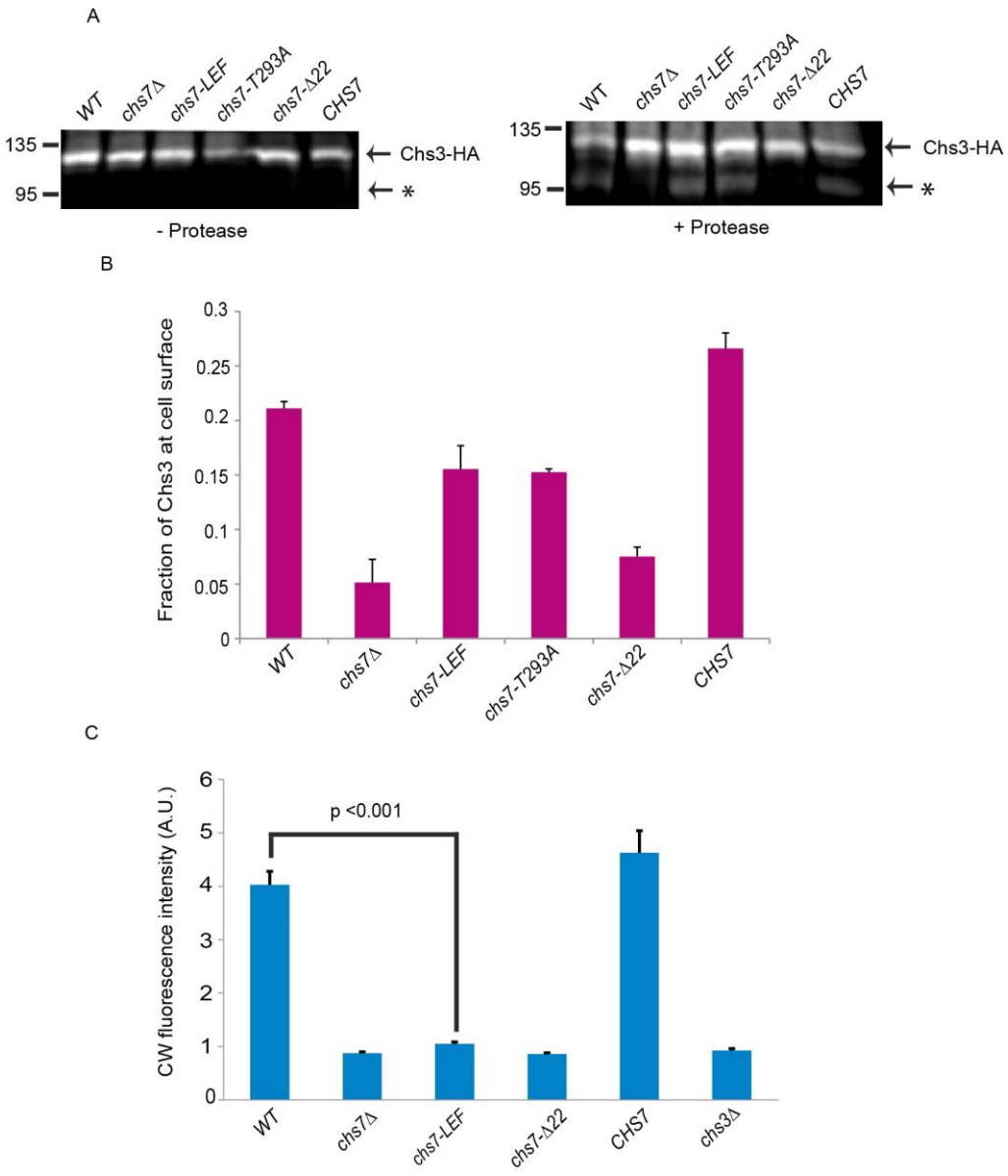

Figure 2.5 Chs7 is required at the cell surface for the chitin synthase activity of Chs3
(A) The proportion of Chs3-HA at the cell surface was determined by a protease accessibility assay in the indicated strains expressing the indicated mutant forms of Chs7. Half the cells from log phase cultures were treated with 2mg/mL of pronase enzyme (+ Protease) and the other half with buffer (- Protease). Cell lysates were separated on SDS-PAGE and subjected to immunoblotting with α-HA antibody. * indicates the position of the Chs3 fragment generated by pronase cleavage. A representative image is shown. (B) Quantitation of the protease accessibility assay shown in (A), showing the mean fraction of Chs3-HA cleaved by protease in three independent experiments (error bars represent SEM) (C) Relative chitin levels of the indicated strains were measured on yeast colony arrays grown on YPD media containing 50mg/ml CW (calcofluor white). Fluorescence of chitin-bound CW from six independent

arrays was observed using a fluoroimager and quantified by densitometry; error bars represent SEM. AU: arbitrary units.

2.3.6 Restoring the Chs7-Chs3 interaction rescues ER exit and post-Golgi trafficking

Our results suggest that dissociation of Chs3-Chs7 complexes is responsible both for missorting of Chs7 at the Golgi and for the reduced catalytic efficiency of Chs3 at the cell surface. To test this model, we sought to restore trafficking of the Chs7$^{\Delta 22}$ mutant by artificially enhancing its binding to Chs3. The bimolecular fluorescence complementation (BiFC) assay based on split-YFP proteins detects protein-protein interactions in living cells; however, the interaction is essentially irreversible once the interacting YFP fragments fold into a fluorescent protein (Hu et al., 2002). We tagged Chs3 and Chs7 with the split-YFP fragments YFP^{N} and YFP^{C}, respectively, by integration at the chromosomal locus. Strains expressing a single YFP^{N} or YFP^{C}-tagged protein showed no fluorescence, whereas a strain co-expressing both Chs3-YFP^{N} and Chs7-YFP^{C} showed fluorescence at the bud neck and in intracellular puncta similar to their endogenous localization patterns determined with GFP fusions (Fig 2.6 A, B). Interestingly, cells co-expressing Chs3-YFP^{N} and Chs7$^{\Delta 22}$-YFP^{C} exhibited punctate and bud neck fluorescence that was distinct from the ER/vacuolar localization of GFP-tagged Chs7$^{\Delta 22}$. The Chs7$^{\Delta 22}$-YFP^{C} fusion, when co-expressed with Chs3-YFP^{N}, was also retained in intracellular compartments in *chs6*Δ mutants, indicating that its post-Golgi trafficking had been restored (not shown). This supports the model that Chs7$^{\Delta 22}$ fails to bind Chs3 efficiently, and that reinforcing this association rescues the ER exit and post-Golgi trafficking of both proteins.

To determine if rescue of the Chs3-Chs7 interaction also restores catalytic activity, we used the CW binding assay to estimate chitin levels in strains where these proteins are tagged either with YFP^{N} and YFP^{C} or with HA and GFP (Fig 2.6 C). We found that cells co-expressing Chs3-YFP^{N} and Chs7$^{\Delta 22}$-YFP^{C} exhibited significantly greater fluorescence compared to cells expressing Chs3-HA and Chs7$^{\Delta 22}$-GFP. Interestingly, fusion of split YFP tags to wild-type forms of Chs3 and Chs7 also appeared to enhance Chs3 activity. Taken together, this indicates that the interaction between Chs7 and Chs3 is critical for Chs3 activity at the cell surface, and suggests that modulation of this interaction could represent a mechanism for regulating chitin production in wild-type cells.

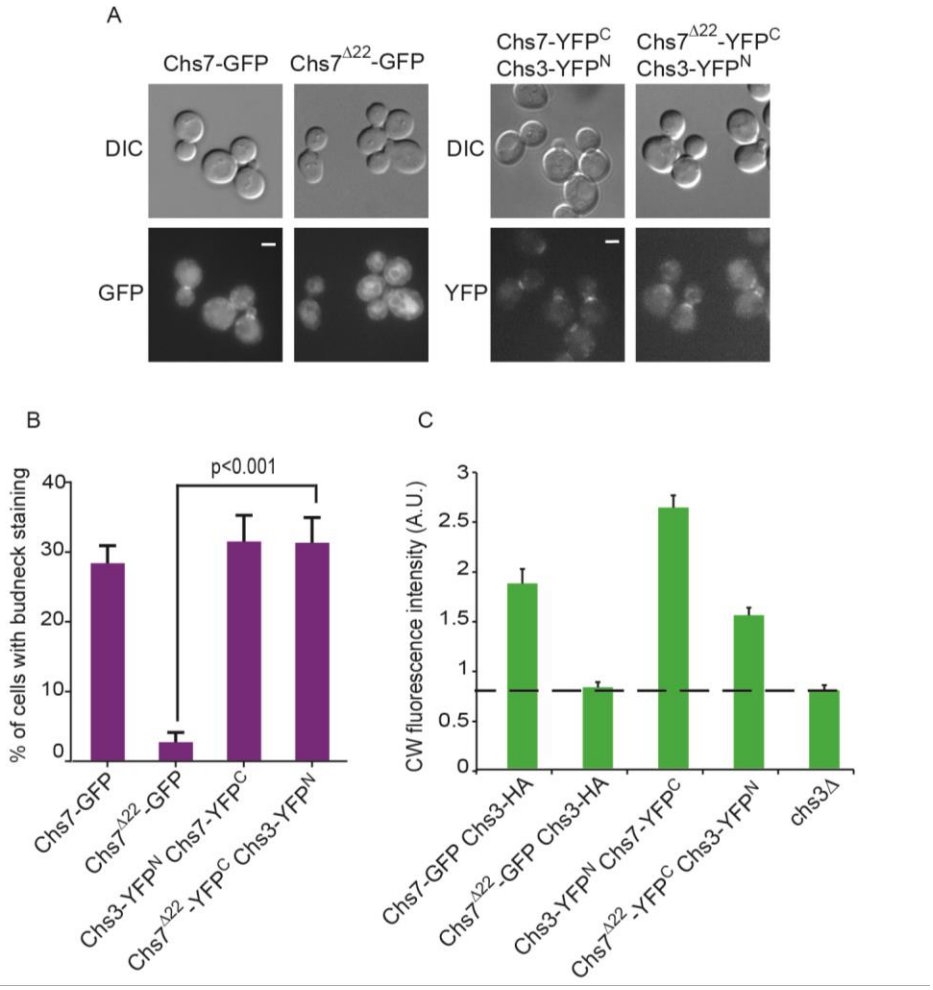

Figure 2.6 Enhancing Chs3-Chs7 interaction with split YFP fusion rescues the localization of the Chs7$^{\Delta 22}$ tail mutant and promotes function of Chs3 at the surface
(A) Log phase cultures of the indicated strains were observed by fluorescence microscopy using GFP and YFP filters. Scale bar, 2μM (B) The proportion of cells exhibiting bud neck staining was counted manually using MetaMorph software. An average of 600 cells were counted per strain (n=3; error bars represent SEM). (C) CW fluorescence values were used to assess relative chitin levels as described in Fig 5C. Mean values were plotted from six experiments; error bars represent SEM. AU: arbitrary units.

2.4 Discussion

Here, we show that Chs7 is not an exclusive ER-resident chaperone as previously reported, but instead exits the ER and localizes with Chs3-GFP at the bud neck and intracellular organelles. The fact that Chs7 remains associated with Chs3 after ER exit, and is required for its catalytic activity at the cell surface, suggests that it may represent a new functional subunit of a multi-

protein chitin synthase complex, rather than a dedicated chaperone *per se*. However, because Chs7 appears to have distinct and separable roles in Chs3 folding and function, it could also represent a folding chaperone that has taken on a secondary role as a regulatory protein. There is evidence that some chaperones have acquired additional roles in higher organisms. For example, yeast Erv14 acts as an ER escort chaperone to stabilize long transmembrane domains of selected substrates and escort them into COPII vesicles (5). However, the Erv14/cornichon homologs CNIH-2 and CNIH-3 have dual roles as evolutionarily conserved ER export chaperones, and as auxiliary subunits of the AMPA-type glutamate receptor that modulate channel gating at the postsynaptic membrane (Harmel et al., 2012; Kato et al., 2011)

We found that conserved residues in the Chs7 cytosolic domain were important for the formation of a stable Chs3-Chs7 complex but were largely dispensable for Chs3 folding. Similarly, cytosolic regions of the dedicated chaperone Shr3 are not needed for folding and ER export of the Gap1 permease (Kota and Ljungdahl, 2005). Shr3 transmembrane regions were reported to interact with the first 5 TMDs of Gap1 to prevent their aggregation and promote their association with the remaining TMDs (Kota et al., 2007). Chs3 is predicted to contain two groups of TMDs separated by a large cytosolic catalytic domain. By analogy to Shr3, the Chs7 transmembrane portion may bind and stabilize one or more TMDs of Chs3 to facilitate their interactions with the remaining TMDs. Full assembly would presumably require rearrangement and release of these TMDs, such that continued Chs3-Chs7 interaction would depend on residues in the Chs7 cytosolic domain. This model suggests that different Chs3-Chs7 interfaces mediate folding, and post-ER association, and could explain why Chs7 cytosolic domain mutations did not prevent folding but did reduce Chs3-Chs7 interactions at subsequent transport steps.

Whereas some dedicated chaperones, including Shr3, are restricted to the ER, others such as Vma21 are transported in COPII vesicles with their substrates, and may provide the COPII binding signals needed for ER exit (Ljungdahl et al., 1992; Malkus et al., 2004). Because Chs7 is largely competent for ER exit without Chs3, Chs7 may possess COPII binding signals that direct the ER exit of the Chs3-Chs7 complex. Although mutation of a putative di acidic COPII binding signal in the Chs7 tail did not block ER exit, Chs7 could have other redundant COPII binding

motifs, which could take many forms (eg. DxE, FF, LL, VV, IXM, LXXLE, Φ*X*Φ*X*Φ, RI/RL) (Otsu et al., 2013). Other proteins could also direct sorting into COPII vesicles. For example, Erv14 was recently proposed to facilitate the ER exit of Chs3 (Sacristan et al., 2013), though we and others did not find Chs3 to be retained in the ER of *erv14* strains (unpublished data) (Herzig et al., 2012). Alternatively, Chs3 could have COPII binding signals that depend on its conformational state, or are masked by Chs3 aggregation, and thus are only recognized when Chs7 is present. Further work will be needed to define the determinants responsible for the ER exit of Chs3.

Chs7 does not absolutely require Chs3 for its ER exit, yet strongly depends on its association with Chs3 for subsequent trafficking steps. Chs3 has cytosolic signals that bind exomer and AP-1 coat proteins (Rockenbauch et al., 2012; Starr et al., 2012). When Chs6, a component of the exomer coat, is mutated, Chs3 follows an AP-1-dependent recycling pathway that retains it in Golgi/endosomal compartments (Valdivia et al., 2002). Chs7 was similarly trapped in *chs6* mutants, but only when bound to Chs3; point mutations in a conserved region of the Chs7 tail that reduced its binding to Chs3 prevented its accumulation in intracellular compartments. Thus, Chs7 does not simply provide signals to assist in the trafficking and folding of Chs3, but also relies on Chs3 to provide sorting signals that direct its own itinerary.

Mutations that strongly reduced the association of Chs7 with Chs3 caused most Chs7 to be transported from the Golgi to the vacuole by way of the late endosome/MVB. Although chitin levels in were extremely low in these mutants, Chs3 was not aggregated and appeared to maintain nearly wild-type localization at endosomes and the cell surface, despite the lack of associated Chs7. This suggests that Chs7 might not be an obligatory accessory subunit, but instead may interact reversibly with Chs3 to regulate its activity. Several different models could account for the influence of Chs7 on Chs3 activity. First, Chs7 might contribute to Chs3 enzymatic activity directly. However, Chs7 is not needed for chitin synthesis by Chs1 or Chs2, two synthases with related catalytic domains, and is, therefore, unlikely to be an integral part of the catalytic mechanism. Alternatively, the continued association of Chs7 could help Chs3 maintain it's fully active conformation. A final hypothesis is that Chs7 plays a regulatory role.

Chs7 is a 7-TMD protein with a large, conserved extracellular domain that could sense and respond to extracellular signals by activating Chs3. Because dissociation of Chs7 and Chs3 did not cause gross misfolding and aggregation of Chs3, and manipulations designed to stabilize Chs3/Chs7 interactions enhanced Chs3 activity, it is tempting to speculate that the Chs3/Chs7 interaction is dynamic and regulated in response to extracellular signals.

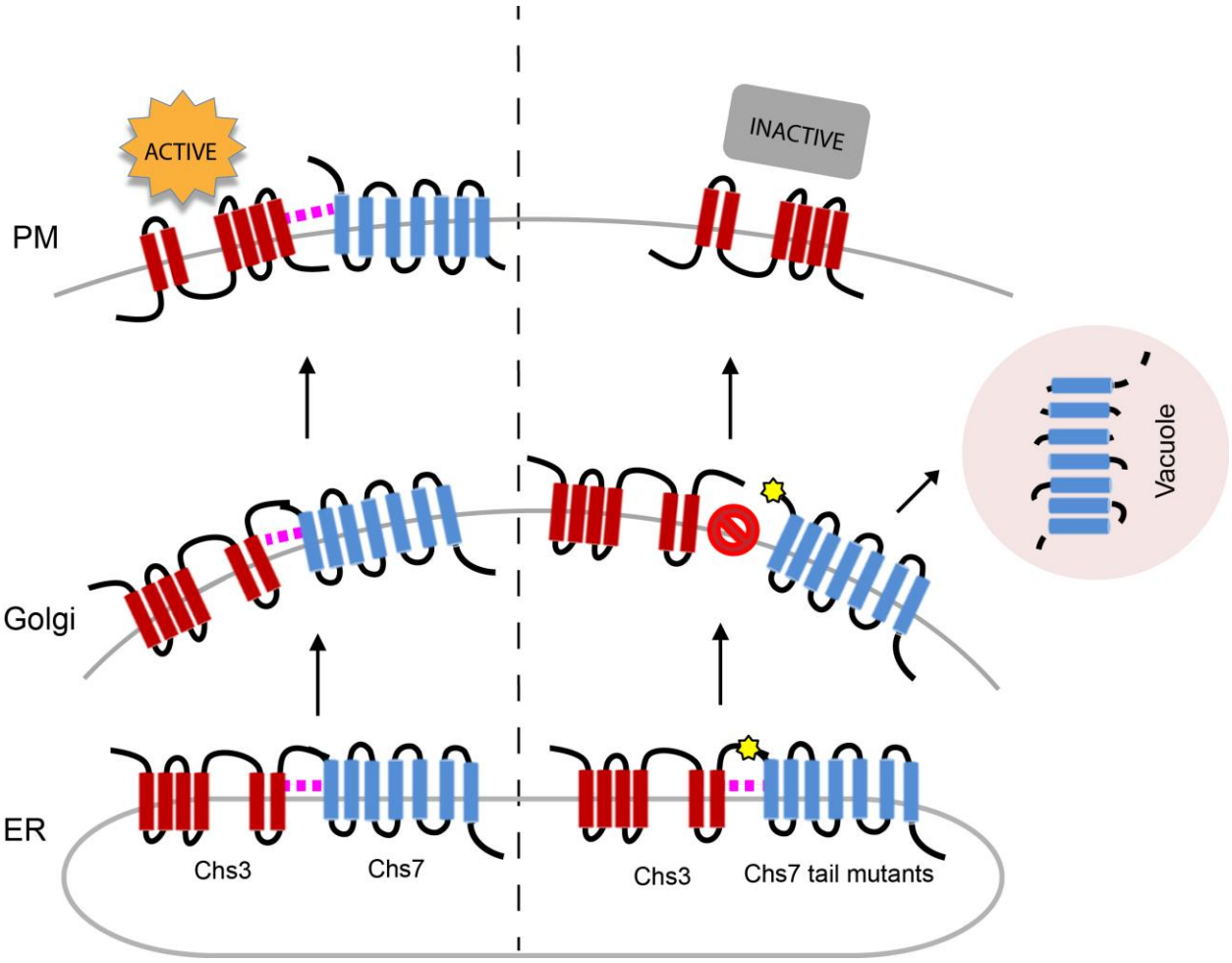

Figure 2.7 Summary
In WT cells, Chs3 and Chs7 continue to exist as a complex throughout the lifecycle of Chs3 and Chs7 is required for Chs3 activity at the plasma membrane. Chs7 tail mutants are unable to interact with Chs3 post ER and are transported to the vacuole. Chs3 is localized to the plasma membrane in these mutants but is not active.

2.5 Supplemental information

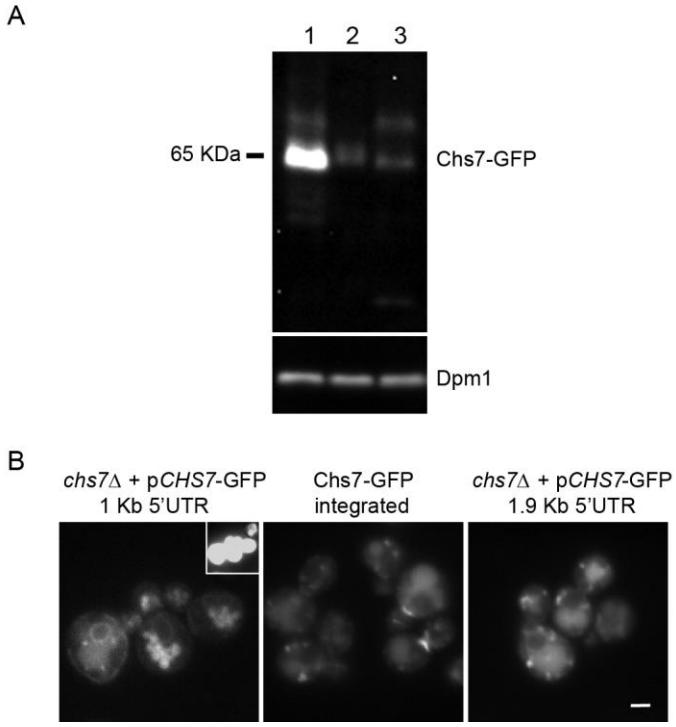

Figure 2.8 Overexpression of plasmid-expressed Chs7-GFP
(A) Lysates of strains expressing Chs7-GFP from a plasmid containing 1 Kb 5'UTR (Lane1), genomically integrated Chs7-GFP (Lane 2) or from a plasmid containing 1.9 Kb 5'UTR (Lane 3), were separated on a 10% SDS-PAGE gel. Chs7 was detected with a α-GFP mAb and Dpm1 (dolichol phosphate mannose-1) was detected using α-Dpm1 mAb as a loading control. (B) Fluorescence microscopy of Chs7-GFP of the indicated strains. The image in the first panel was adjusted for best visualization; the inset shows the image at the same relative brightness settings. Scale bar, 2μM.

This supplementary figure, associated with Figure 1, demonstrates that the plasmid used by Trilla et al. expresses high levels of Chs7, explaining why ER retention is observed with plasmid expressed but not integrated, Chs7-GFP.

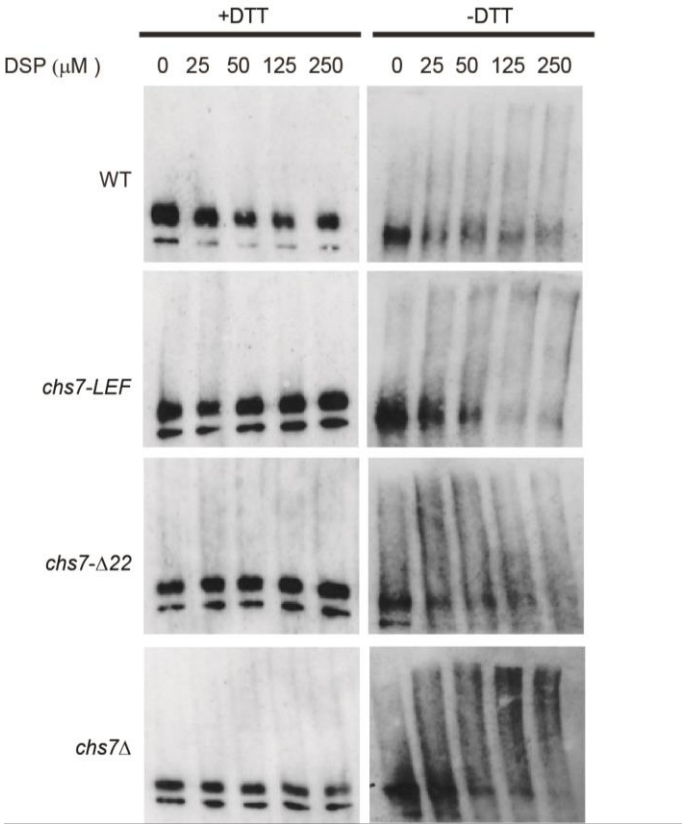

Figure 2.9 Chs3 aggregates at high concentrations of DSP in Chs7 tail mutants
Log phase cells of the indicated strains were treated with DSP and separated on a 10% SDS-PAGE gels. Chs3-HA was detected using α-HA mAb.

This supplementary figure is associated with Figure 3. It uses an alternative technique to examine Chs3 aggregation in mutant strains.

Chapter 3: Quality control of palmitoylation-deficient Chs3 at the ER and Golgi

3.1 Introduction

Molecular chaperones at the endoplasmic reticulum (ER) help fold membrane proteins and shield their transmembrane domains (TMDs) to prevent protein aggregation and degradation (Lecomte et al., 2003). At the ER protein modifications such as N-glycosylation, lipidation and ubiquitination are added co or post-translationally to the proteins. They can reversibly or irreversibly alter signaling, protein structure, function, protein-protein interactions and further trafficking (Wang et al., 2013). Among lipid modifications, S-Palmitoylation is unique, in that it is a reversible post-translational thioester linkage of a palmitate (16 carbons) group to the cysteine residues of the cytosolic domain of the protein (Conibear and Davis, 2010). Reversibility of palmitoylation provides an additional regulatory control in the trafficking, localization and function of many membrane proteins.

Palmitoylation of membrane proteins usually occurs on cysteines adjacent to the TMDs. It plays a significant role in targeting proteins to the specific membrane domains as seen for calnexin and transmembrane thioredoxin family protein (TMX). Palmitoylation of these proteins at the ER targets them specifically to the mitochondria-associated membrane (MAM) domain of the rough ER (Lynes et al., 2011). Palmitoylation is also implicated in tilting of membrane proteins in the lipid bilayer to prevent hydrophobic mismatch, regulate protein-protein interactions and protein quality control pathways (Blaskovic et al., 2013). For example, in yeast, lack of Tlg1 palmitoylation by Swf1 at the ER leads to ubiquitination by Tul1 and subsequent degradation in the vacuole (Valdez-Taubas and Pelham, 2005). In mammalian cells, palmitoylation of GluR2 on TMD2 stabilizes it in the ER and prevents its degradation in the lysosome (Yang et al., 2009).

Palmitoylation of proteins is achieved by Palmitoyl acyl transferase (PAT) enzymes. PATs are a family of multi-pass membrane proteins which encompass a broad range of cysteine-rich DHHC domain proteins. The DHHC (Asp-His-His-Cys) domain is responsible for the catalytic transfer of palmitate to cysteines. Mutations in this domain render the enzyme inactive (Mitchell et al.,

2006). While 23 distinct DHHC proteins have been identified in mammalian cells, yeast cells contain a total of seven PAT enzymes that include Pfa3, Pfa4, Pfa5, Akr1, Akr2, Swf1 and Erf2. A global analysis of palmitoylation revealed several PAT enzyme substrates in yeast, which include AAPs, G-proteins, and SNAREs (Roth et al., 2006).

We use the polytopic (multiple TMDs) yeast chitin synthases III (Chs3) as a model to study the effect of palmitoylation on protein folding and transport. Folding and ER exit of Chs3 depend on its dedicated chaperone, Chs7, and palmitoylation by Pfa4. We have previously reported that Chs3 is largely ER-retained when not palmitoylated by Pfa4, one of the seven yeast PATs (Lam et al., 2006). In *pfa4Δ* mutants, Chs3 forms molecular aggregates, a phenotype that mirrors mutants lacking the Chs3-specific ER chaperone Chs7 (Kota and Ljungdahl, 2005; Lam et al., 2006). Although we know palmitoylation is necessary for protein folding and trafficking, a precise mechanism of how it regulates these is not well understood.

In this study, we explored the role of palmitoylation in Chs3 folding and trafficking. We found that Chs3 needs to be palmitoylated for efficient interaction with its dedicated chaperone Chs7 and for ER exit via COPII vesicles. We have identified the palmitoylated cysteines on Chs3 to be C1014 and C1018. Further, using a genome-wide screen, we have identified suppressors that allow surface expression of misfolded, non-palmitoylated Chs3. Taken together, our results help us understand how palmitoylation effects protein folding and how ER surveillance mechanisms target unlipidated proteins for destruction.

3.2 Materials and methods

3.2.1 Strains and plasmids

General molecular biology methods were as described (Conibear and Stevens, 2000; Conibear and Stevens, 2002). Strains and plasmids used in this study are listed in Table S1. Integration of C-terminal tags (GFP, 3xHA, or 13xMyc) into the *CHS3* locus was done by the PCR tagging method as described previously (Longtine et al., 1998). C-terminal tagging of SEC61 with RFP was carried out according to Sheff and Thorn (Sheff and Thorn, 2004). Nat^R (Nourseothricin resistance), Kan^R (Kanamycin resistance) and HIS3 knockout strains were created by PCR-based

homologous recombination using gene specific primers (Tong et al., 2001). The pND2115 plasmid (pRS316: GAL1-CHS3-3xHA-FLAG-His) was constructed as described in Lam et al., 2006. pHV7 (YCp50::Chs3-3xHA) was a gift from Cesar Roncero. NP03 was constructed by recombinational cloning resulting in the integration of UBP3 coding sequence and its upstream and downstream sequences into the multiple cloning sites of pRS416.

Table 3.1 Strains and plasmids used in Chapter 3

Strain	Genotype	Source
BY4741	*MATa his3-1 leu2-0 met15-0 ura3-0*	Open Biosystems
BY4741	BY4741 *chs7*Δ::kan^R	Open Biosystems
BY4741	BY4741 *pfa4*Δ::kan^R	Open Biosystems
BY4741	BY4741 *bre5*Δ::kan^R	Open Biosystems
BY4741	BY4741 *ubp3*Δ::kan^R	Open Biosystems
KLY3	BY4741 *chs7*Δ::kan^R *CHS3*-GFP::*HIS3*	Lam et al., 2006
KLY5	BY4741 *pfa4*Δ::kan^R *CHS3*-GFP::*HIS3*	Lam et al., 2006
KLY9	BY4741 *CHS3*-GFP::*HIS3*	Lam et al., 2006
KLY41	BY4741 *CHS3*-3xHA:: kan^R	Lam et al., 2006
KLY46	BY4741 *chs7*Δ::HIS3 *CHS3*-3xHA:: kan^R	This study
KLY45	BY4741 *chs7*Δ::HIS3 *pfa4*Δ::nat^R *CHS3*-3xHA:: kan^R	Lam et al., 2006
TDY40	BY4741 *chs3Δ*:: nat^R *chs7*Δ::HIS3 *pfa4*Δ:: kan^R	This study
KLY24	BY4741 *chs7*Δ::HIS3 *pfa4*Δ:: kan^R	This study
NP22	BY4741 *bre5*Δ::kan^R *pfa4*Δ::nat^R *CHS3*-GFP::*HIS3*	This study
NP11	BY4741 *ubp3*Δ::hph^R *pfa4*Δ::kan^R *CHS3*-GFP::*HIS3*	This study
NP21	BY4741 *ubp3*Δ::hph^R *chs7*Δ::kan^R *CHS3*-GFP::*HIS3*	This study
KLY44	BY4741 *pfa4*Δ::nat^R *CHS3*-3xHA:: kan^R	This study
MDY701	BY4741 *pfa4*Δ::nat^R *ubp3*Δ::hph^R *CHS3*-3xHA:: kan^R	This study
KLY90	BY4741 *ubp3*Δ:: kan^R *CHS3*-3xHA::*HIS3*	This study
NP20	BY4741 *chs7*Δ::nat^R *ubp3*Δ:: kan^R *CHS3*-3xHA::*HIS3*	This study
KLY86	BY4741 *CHS3*-GFP*::HIS3 pfa4*Δ::nat^R *ubp3*Δ:: kan^R	This study
KLY47	BY4741 *CHS3*-GFP*::HIS3 SEC61*-RFP*::URA*	This study
KLY54	BY4741 *pfa4*Δ::kan^R *CHS3*-GFP*::HIS3 SEC61*-RFP*::URA*	This study

Strain	Genotype	Source
KLY96	BY4741 *pfa4*Δ::kan^R *sec21-1 CHS3*-GFP*::HIS3 SEC61*-RFP*::URA*	This study
CKY10	*ura3-52 leu2-3,112*	This study
KLY97	CKY10 *CHS3*-3xHA::kan^R	This study
KLY99	CKY10 *sec21-1 CHS3*-3xHA::kan^R	This study
KLY98	CKY10 *pfa4*Δ::nat^R *CHS3*-3xHA::kan^R	This study
KLY100	CKY10 *pfa4*Δ::nat^R *sec21-1 CHS3*-3xHA::kan^R	This study
MDY918	BY4741 *CHS3 C1014A*-GFP$^+$::*HIS3*	This study
NP105	BY4741 *CHS3 C1018A*-GFP$^+$::*HIS3*	This study
MDY896	BY4741 *CHS3 C1014A/C1018A*- GFP$^+$::*HIS3*	This study
TDY11	BY4741 *ubp3*Δ::hph^R *CHS3*-GFP*::HIS3*	This study
NP86	BY4741 *ubp3*Δ::hph^R *CHS3 C1014A/C1018A*- GFP$^+$::*HIS3*	This study
BY4742	*MATα his3-1 leu2-0 lys2-0 ura3-0*	Open Biosystems
KLY70	BY4742 *CHS3*-GFP::kan^R	This study
KLY71	BY4742 *pfa4*Δ::nat^R *CHS3*-GFP::kan^R	This study
KLY72	BY4742 *chs7*Δ::nat^R *CHS3*-GFP::kan^R	This study
KLY73	BY4742 *ubc6*Δ::*HIS3 ubc7*Δ::*LEU2 CHS3*-GFP::kan^R	This study
KLY74	BY4742 *ubc6*Δ::*HIS3 ubc7*Δ::*LEU2 pfa4*Δ::nat^R *CHS3*-GFP::kan^R	This study
KLY75	BY4742 *ubc6*Δ::*HIS3 ubc7*Δ::*LEU2 chs7*Δ::nat^R *CHS3*-GFP::kan^R	This study
MDY747	BY4741 *doa10*Δ*::hph, hrd1*Δ*:: nat^R CHS3-GFP::HIS3*	This study
MDY739	BY4741 *doa10*Δ*::hph, hrd1*Δ*:: nat^R chs7*Δ::kan^R *CHS3-GFP::HIS3*	This study
MDY740	BY4741 *doa10*Δ*::hph, hrd1*Δ*:: nat^R pfa4*Δ::kan^R *CHS3-GFP::HIS3*	This study
Plasmid	**Genotype**	**Source**
pCS40	*CHS7*-GFP$^+$ with 1.9Kb 5'UTR in pRS316	This study
pKL1	*PFA4*-3xHA in pRS415	Lam et al., 2006
pKL6	*PFA4 DHHA*-3xHA in pRS415	Lam et al., 2006
pNP3	*UBP3* in pRS416	This study

3.2.2 Co-immunoprecipitations

Co-precipitation was performed as previously described (Conibear and Stevens, 2000; Lam et al., 2006). Briefly, 20 OD_{600} of spheroplasts were resuspended in 1 mL lysis buffer containing 0.1% CHAPSO, 50 mM KPO4, pH7.5, 50 mM NaCl, and a protease inhibitor cocktail. The cleared lysate was incubated with 60 µL of a 75% slurry of IgG–Sepharose (GE Healthcare; Mississauga, ON) for 1 h at 4°C to immunoprecipitate GFP-tagged proteins. The supernatant was incubated with rabbit α-GFP (A6455; Life Technologies; Burlington, ON) antibody Sepharose beads were washed twice with lysis buffer, resuspended in sample buffer, and analyzed by SDS-PAGE. Co-immunoprecipitated proteins were detected by Western blotting with mouse α-GFP (Clone 13.1, Roche Diagnostics; Indianapolis, IN) and mouse α-HA (HA.11; Covance; Berkley, CA) antibodies followed by HRP-conjugated goat anti-mouse antibody (Jackson Immunoresearch Laboratories, West Grove PA).

3.2.3 BN-PAGE

5 OD_{600} spheroplasts were suspended in 1mL 1X Novex® NativePAGE™ Sample Buffer pH 7.2 (Life Technologies; Burlington, ON), with 1% Digitonin (Sigma-Aldrich; Oakville, ON) and Halt Protease Inhibitor Cocktail (Fisher; Ottawa, ON) . The protein concentration was equalized to 60µg of protein using Pierce® BCA Protein Assay Kit, and 0.5 µL of Coomassie G-250 sample additive (Life Technologies) was added to the cleared lysates just prior to loading. Proteins were detected by Western blotting using mouse anti-HA and mouse anti-GFP antibodies, followed by HRP-conjugated goat anti-mouse antibody. Blots were developed with ECL and imaged using Kodak Film Developer. Quantity One software was used for densitometry of scanned images and relative pixel values were plotted using MS excel 2010.

3.2.4 Fluorescence microscopy

For fluorescence microscopy of living cells expressing GFP or RFP, cells were grown to log phase at 30°C in synthetic complete minimal media and observed directly. Temperature sensitive *sec21-1* strains were grown 3 hours at 25°C and shifted to 33°C for 45 minutes. Cells were viewed using a 100x oil-immersion objective on a Zeiss Axioplan2 fluorescence microscope

(Thornwood, NY), and images were captured with a CoolSnap camera using MetaMorph software (Universal Imaging, West Chester, PA), and adjusted using Adobe Photoshop.

3.2.5 Protease accessibility

The protease accessibility assay was modified from (Chen and Davis, 2000; Schluter et al., 2008). 5 OD_{600} of log phase cells expressing Chs3-3xHA were harvested and washed in 1mL Pronase Buffer (PB) (1.4M Sorbitol, 25mM Tris-HCl pH 7.5, 10mM NaN3, 10mM NaF). Cells were resuspended in 350uL PB and incubated for 30 min at 30°C. Half was treated with a final concentration of 2mg/mL Pronase (Calbiochem-Novabiochem; La Jolla, CA) at 37°C for 1h, and the other half was mock treated with an equal volume of PB. Cells were collected at 6500rpm, resuspended in 225uL PB, and proteins were precipitated with 50uL of 100% TCA. Washed TCA pellets were resuspended in SDS-PAGE sample buffer and analyzed by Western blotting with α-HA (1:1000) or α-PGK (1:800) mAbs followed by HRP-labeled anti-mouse secondary antibody. Blots were developed with ECL and imaged on a Fluor S-Max Multi-imager. Quantity One software was used for densitometry and relative pixel values were plotted using MS excel 2010.

3.2.6 Acyl-biotin exchange

The acyl-biotin exchange assay on strains carrying the pND2115 plasmid (pRS316: GAL1-Chs3-3xHA-FLAG-His) was performed as described previously (Lam et al., 2006; Politis et al., 2005).

3.2.7 COPII budding assay

In vitro COPII budding assays were conducted as described previously (Pagant et al., 2007). Briefly, purified microsomal membranes from cells expressing Chs3-3xHA were washed with 20 mM HEPES, pH 6.8, 250 mM sorbitol, 160 mM potassium acetate, and 5 mM magnesium acetate. 125 µg of membranes were incubated with COPII proteins (10 µg/ml Sar1p, 10 µg/ml Sec23p/24p, and 20 µg/ml Sec13/31p) in 0.1 mM GTP with a 10x ATP regeneration system or in 0.1 mM GDP. Vesicles were separated from donor membranes by centrifugation at 16,000 rpm for 5 min, and the vesicle fraction was concentrated by high-speed centrifugation at 55,000 rpm for 20 min. Vesicle pellets were resuspended in SDS sample buffer and heated at 55°C for 5 min

before separation by SDS-PAGE. Chs3-3xHA was detected by Western blotting with α-HA as described above. The control protein Sec22 was detected with polyclonal antibodies (gift from R. Schekman, U.C. Berkeley, USA).

3.2.8 Click based palmitoylation assay

The click based palmitoylation assay on strains containing Chs3- 3xHA in the indicated strain backgrounds was performed as described previously (Roth et al., 2011).

3.3 Results

3.3.1 Chs3 ER exit is dependent on both Chs7 and Pfa4

Chs3 is ER retained in the absence of Chs7 and Pfa4 (Lam et al., 2006). Chs3 ER retention in *chs7Δ* and *pfa4Δ* mutants could be a consequence of inefficient entry into COPII vesicles. In order to determine this, we investigated the ER export of non-palmitoylated Chs3 in COPII vesicles by an *in vitro* COPII budding assay (Pagant et al., 2007). Microsomal membranes prepared from wild type, *pfa4Δ*, and *chs7Δ* mutants were incubated with purified COPII coat proteins (Sar1, Sec23/Sec24, Sec13/31) and either GTP (Guanosine triphosphate) (+) or GDP (Guanosine diphosphate) (-). Vesicles generated from this reaction were isolated by differential centrifugation, and the incorporation of cargo proteins into the vesicles was monitored by immunoblotting. Chs3 was able to efficiently enter COPII vesicles in WT cells only in the presence of GTP, whereas Chs3 entry into COPII vesicles was severely depleted in *chs7Δ* and *pfa4Δ* mutants in the presence of GTP and GDP. However, a small, reproducible amount of Chs3-HA budding was seen in *pfa4Δ* (Fig 3.1 A), consistent with the observation of a small amount of Chs3 at the budneck in these cells. Therefore, Chs7 and Pfa4 are both necessary for COPII export of Chs3.

To analyze if Chs7 and Pfa4 work together at the ER to aid Chs3 folding and ER exit by forming a folding complex, we compared Chs3-Pfa4, Chs3-Chs7 and Chs7-Pfa4 binary interactions by co-immunoprecipitation. We found that Chs3 and Chs7 and Chs3 and Pfa4 interact efficiently with each other in WT cells, while only a small percentage of Chs7 and Pfa4 interact with each

other and this interaction is further reduced in the absence of Chs3. These results suggest that the Chs3-Chs7-Pfa4 complex is unstable and transient (Fig3.1 B).

It is possible that Chs7 and Pfa4 interact with Chs3 sequentially, instead of forming a trimeric complex. Previously published results from our laboratory show that Chs3-Chs7 interaction does not require palmitoylation of Chs3 by Pfa4 (Lam et al., 2006). However, upon careful quantification we found that Chs3 and Chs7 do not interact efficiently in the absence of Pfa4compared to WT cells (Fig 3.1C). Thus, loss of palmitoylation greatly reduces the interaction between Chs7 and Chs3. Because Chs7 association is absolutely required for Chs3 to exit the ER, we speculate that the low levels of non-palmitoylated Chs3 that enter COPII vesicles results from the inefficient binding to Chs7.

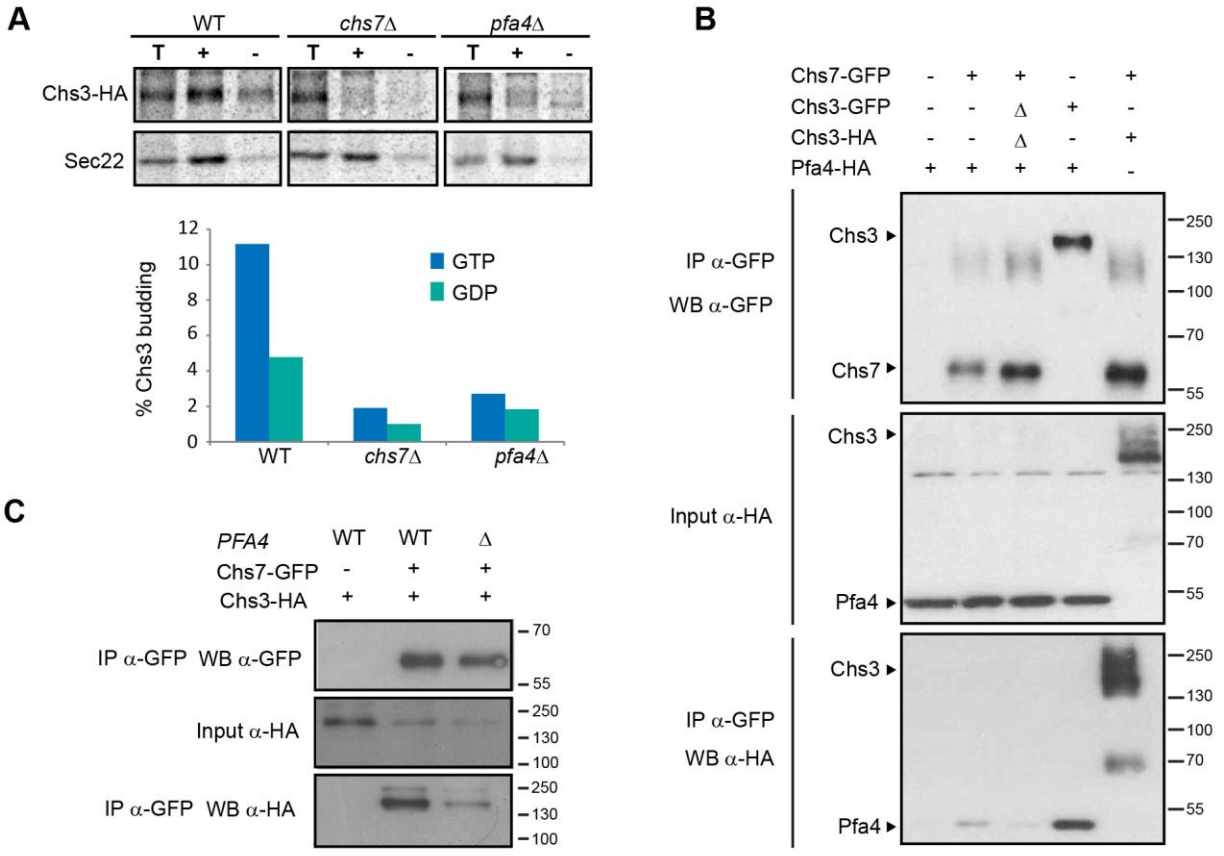

Figure 3.1 Chs7 and Pfa4 are required for ER exit of Chs3
(A) *In vitro* COPII budding reactions were performed on ER membranes isolated from WT, *chs7Δ*, *pfa4Δ* strains expressing genomically tagged Chs3-HA. Cell lysates were incubated with purified COPII proteins in the presence of GTP (+) or GDP (-), T indicates the total amount of protein. Chs3-HA was immunoprecipitated and analyzed by SDS-PAGE. Sec22 was used as a control to analyze COPII budding. (B) Cell lysates of the indicated strains expressing genomically tagged Chs3-HA and Chs7-GFP on a single copy plasmid were co-immunoprecipitated with α-GFP Ab. Lysates were separated and run on SDS-PAGE and Chs3 and Chs7 were detected using mouse α-GFP and α-HA Abs. Loading of the lysates relative to the IP was 1:17. (- indicates non-tagged version of Chs3, Δ indicates a deletion of Chs3). (C) Detergent solubilized lysates from the indicated strains expressing Chs3-HA, Chs3-GFP, Chs7-GFP and Pfa4-HA were co-immunoprecipitated with α-GFP Ab and co-precipitating proteins were detected with α-HA Ab. Loading of the lysates relative to the IP was 1:18.

3.3.2 Chs3-Pfa4 interaction is enhanced in the absence of Chs7

Similarly, we used co-immunoprecipitation to test if Chs7 contributes to the formation of the Chs3-Pfa4 complex. Surprisingly, we found that in the absence of Chs7, the interaction between Chs3 and Pfa4 was greatly enhanced. (Fig 3.2 A).

We next used BN-PAGE (Blue Native polyacrylamide gel electrophoresis) as an alternate approach to examine the interaction between Chs3 and Pfa4 at steady state in the presence and absence of Chs7 (Fig 3.2 B). We separated digitonin-solubilized lysates from cells co-expressing tagged forms of Chs3 and Pfa4 on blue native gels. SDS-PAGE (Sodium dodecyl sulfate polyacrylamide gel electrophoresis) gels were also run in parallel to monitor protein levels in all strains. To quantitate Chs3-Pfa4 complex formation, we plotted lane intensity profiles of Chs3-GFP and Pfa4-HA normalized to SDS-PAGE protein levels (Fig. 3.2 C). In WT cells, Chs3 migrated as a single peak, whereas Pfa4 was found in two forms. One form of Pfa4 comigrated with Chs3 at a higher M_r (Relative Molecular weight), suggesting it represents a Pfa4-Chs3 complex. No corresponding Chs3-GFP peak coincided with the lower M_r form of Pfa4, indicating this faster-migrating form of Pfa4 is not bound to Chs3.

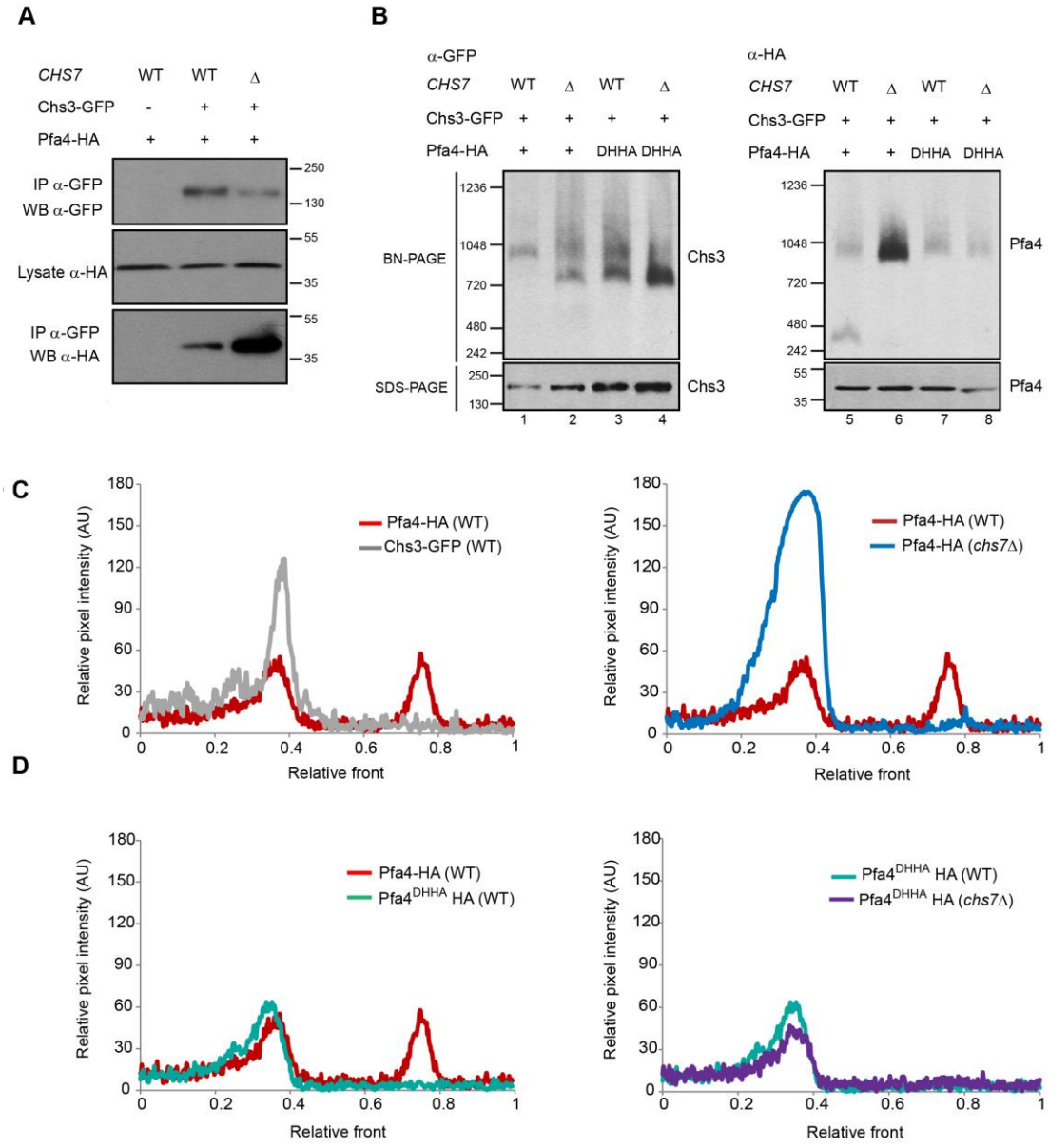

Figure 3.2 Absence of Chs7 enhances Chs3-Pfa4 interaction
(A) Lysates from cells expressing Chs3-GFP and Pfa4-HA in WT and *chs7Δ* mutants were co-immunoprecipitated with α-GFP Ab and co-precipitating proteins were detected using α-HA Ab. Loading of lysates relative to the IP was 1:8. (B) Lysates from cells co-expressing genomically tagged Chs3-GFP and Pfa4-HA or Pfa4^{DHHA}-HA on a plasmid were separated on parallel 3-12% Bis-Tris Native Page and 10% SDS-PAGE gels. Chs3 and Pfa4 were detected using α-GFP and α-HA Ab respectively. (C) Densitometry plots of Lane 1 and 5 in the left panel indicate relative migration of Chs3 and Pfa4 in WT. Right panel compares the migration of Pfa4-HA in WT vs. *chs7Δ* strains (plots of lanes 5 and 6). (D)

Densitometry plots of lanes 5 and 6 indicate relative migration of WT Pfa4 and Pfa4^{DHHA}. Right panel shows relative migration of Pfa4^{DHHA} in WT and *chs7Δ* strains. AU – Arbitrary unit.

The faster migrating species of Pfa4 was abolished in *chs7Δ* mutants, and there was a dramatic increase in the intensity of the higher M_r peak comigrating with Chs3. This is consistent with the results of the co-immunoprecipitation, and suggests that the amount of Pfa4 bound to Chs3 increases in the absence of Chs7. One hypothesis to explain this increase is that ER retention of misfolded Chs3 in *chs7Δ* mutants allows Chs3 to interact with the ER resident Pfa4 for extended periods of time. We attempted to test this by using a catalytically inactive form of Pfa4, where the cysteine of the DHHC domain is mutated to an alanine. Chs3 is not palmitoylated in a Pfa4^{DHHA} strain and is mostly ER retained. We did not detect a change in the relative amounts of Pfa4^{DHHA} and WT Pfa4 bound to Chs3 (Fig 3.2 D). However, we also no longer observed enhanced binding of Pfa4^{DHHA} to Chs3 in the absence of Chs7. Mutation of the DHHC domain has been shown to affect substrate binding. Therefore, one possible explanation for these results is that the reduced interaction between Chs3 and Pfa4^{DHHC} is compensated by the ER retention of Chs3 in these mutants. Taken together, we conclude that Chs7-mediated folding of Chs3 is not a prerequisite for interaction with Pfa4.

3.3.3 Deletion of Ubp3/Bre5 complex relieves ER retention of non-palmitoylated Chs3

It is not known how a defect in palmitoylation causes Chs3 to be retained in the ER. One hypothesis is that quality control proteins recognize the misfolded, non-palmitoylated form of Chs3 and prevent its ER exit. Inhibiting the ERAD machinery rescues the cell surface expression of misfolded Gap1 (Kota et al., 2007). However, loss of Hrd1/Doa10 or Ubc6/Ubc7 did not alter the ER localization of Chs3 (Fig 3.7; supplemental information). Blocking ERAD is therefore not sufficient to circumvent the ER retention of Chs3. Chs3 may fail to attain an export-competent conformation in these ERAD mutants, or may be retained by interactions with other components of the ER quality control machinery.

We predicted that the loss of proteins responsible for ER retention would restore the cell surface transport of Chs3 in a mutant lacking the Pfa4 palmitoyltransferase. To systematically uncover such proteins, we introduced the *pfa4*Δ mutation into the genome-wide knockout collection of

non-essential genes using synthetic genetic array techniques (Tong et al., 2001). The resulting double mutants arrays were assessed for cell surface Chs3 using a fluorescence assay based on the chitin-binding drug calcofluor white (CW) (Lam et al., 2006; Burston et al., 2008). While several mutations that impair endosomal trafficking partially restored the fluorescence of *pfa4*Δ on CW media, the ER localization of Chs3-GFP was not altered in these double mutants (data not shown), suggesting the enhanced cell surface expression of Chs3 was not due to suppression of ER retention. However, we found deletion of the cytosolic deubiquitinating enzyme Ubp3 or its obligate subunit Bre5 partially restored the fluorescence of *pfa4Δ*. In *pfa4Δ ubp3Δ* double mutants, Chs3-GFP was no longer observed at the ER but instead displayed the typical budneck localization of wild-type cells (Fig 3.3 A). Deletion of this deubiquitinating complex did not restore the wild-type localization of Chs3 in the *chs7Δ* background.

We developed a protease accessibility assay to quantify cell-surface localized Chs3. Treatment of intact cells with Pronase, a broad specificity protease, produced a Chs3-specific degradation band (Fig 3.3 B). The fraction of protease-accessible Chs3 in wild-type cells corresponded to approximately 0.13 of total Chs3 (Fig 3.3 C), corroborating previous observations that up to 70-80% of Chs3 is normally stored in internal compartments (Santos and Synder, 1997). Protease accessibility was greatly reduced in *pfa4*Δ mutants, where Chs3 is largely retained in the ER. However, the cell surface abundance of Chs3 was restored to wild-type levels in *pfa4*Δ *ubp3*Δ or *pfa4Δ bre5Δ* double mutants (Fig 3.3 B, C). A higher than WT rescue in Chs3 surface expression in a *ubp3Δ* single mutant could be because of changes to the endocytic pathway in these mutants. The rescue seen in a *pfa4*Δ*ubp3*Δ strain could be counteracted by adding *UBP3* on a single-copy plasmid (Fig 3.3 B, C). We found deletion of *BRE5* or *UBP3* failed to restore cell surface localization of Chs3 in a *chs7*Δ strain by GFP microscopy (Fig 3.3 B) and by protease accessibility (Fig 3.3 B, C). These results suggest the Bre5/Ubp3 complex has a role in the ER retention of palmitoylation-deficient Chs3. Moreover, it appears that the cellular response to misfolded Chs3 differs with the nature of the folding lesion: defects due to loss of the Chs7 chaperone are dealt with differently compared to that arising from the lack of lipid modification. It should also be noted that deleting Pfa4 did not alleviate the ER retention of Chs3 in the

absence of Chs7, suggesting that Pfa4 is not a part of quality control machinery that retains misfolded Chs3.

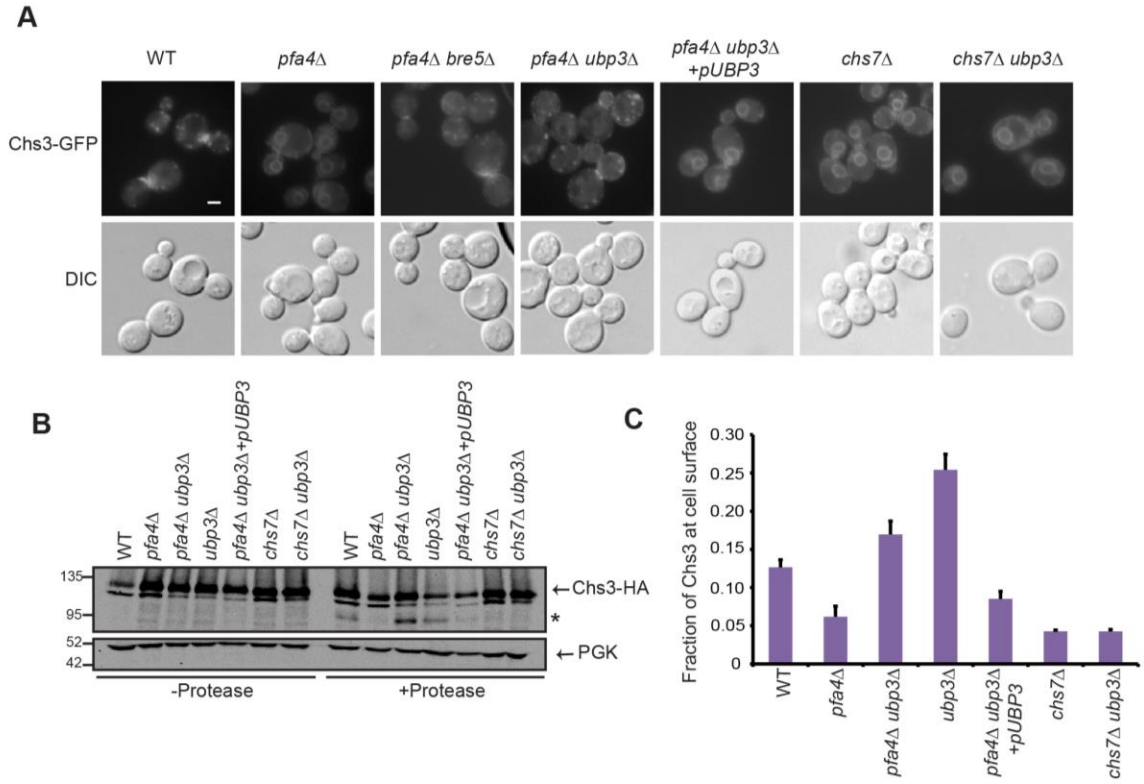

Figure 3.3 Deletion of *BRE5* or *UBP3* restores ER export of palmitoylation-deficient Chs3
(A) Log phase cultures of cells expressing Chs3-GFP in the indicated strains were observed by DIC and fluorescence microscopy. Scale bar, 2μM. (B) Protease-accessibility assay of cells expressing Chs3-3xHA in the indicated strains. Pronase treated (+ protease), and parallel samples mock-treated with buffer (- protease) were separated on 10% SDS-PAGE and detected using α-HA, and α-PGK (loading control). * Indicates Chs3 fragment generated by pronase cleavage. (C) Levels of Chs3 degradation products relative to total (shown in B) from three independent experiments were quantified by densitometry. Error bars represent SEM (Standard error of the mean).

3.3.4 Ubp3 does not alter palmitoylation or ubiquitination of Chs3

The deubiquitinating enzyme Ubp3 and its cofactor Bre5 are known to act on substrates involved in processes such as autophagy, ribophagy, mitogen-activated protein kinase (MAPK) signaling,

trafficking and transcription (Kraft et al., 2008; McCullock et al., 2006; Wang et al., 2008). Chs3 was identified as an ubiquitinated protein in a large-scale proteomics study (Peng et al., 2003). We first considered the possibility that Ubp3/Bre5 acts directly on Chs3 to modulate its ubiquitination status. Immunoprecipitation of Chs3 from cells expressing high levels of myc-tagged ubiquitin confirmed Chs3 is indeed ubiquitinated (Fig 3.4 A). However, the level of ubiquitination was unchanged in *pfa4*Δ compared to *pfa4*Δ *ubp3*Δ mutants, demonstrating Chs3 is not a substrate of Ubp3/Bre5. We hypothesized that loss of Ubp3/Bre5 might upregulate one of the remaining six DHHC palmitoyltransferases to restore Chs3 palmitoylation. To examine the palmitoylation status of Chs3, we used the acyl-biotin exchange assay (Drisdel and Green, 2004; Lam et al., 2006; Politis et al., 2005), in which potential palmitoyl-thioester linkages are cleaved by hydroxylamine, exposing cysteinyl thiols that are subsequently replaced by biotin. Chs3 was not palmitoylated in pfa4 single mutants or *pfa4Δ ubp3Δ* and *pfa4Δ bre5Δ* double mutants (Fig 3.4 B), demonstrating that loss of the Bre5/Ubp3 complex does not restore Chs3 palmitoylation. Taken together, these results suggest loss of *UBP3* does not alter the palmitoylation or ubiquitination status of Chs3.

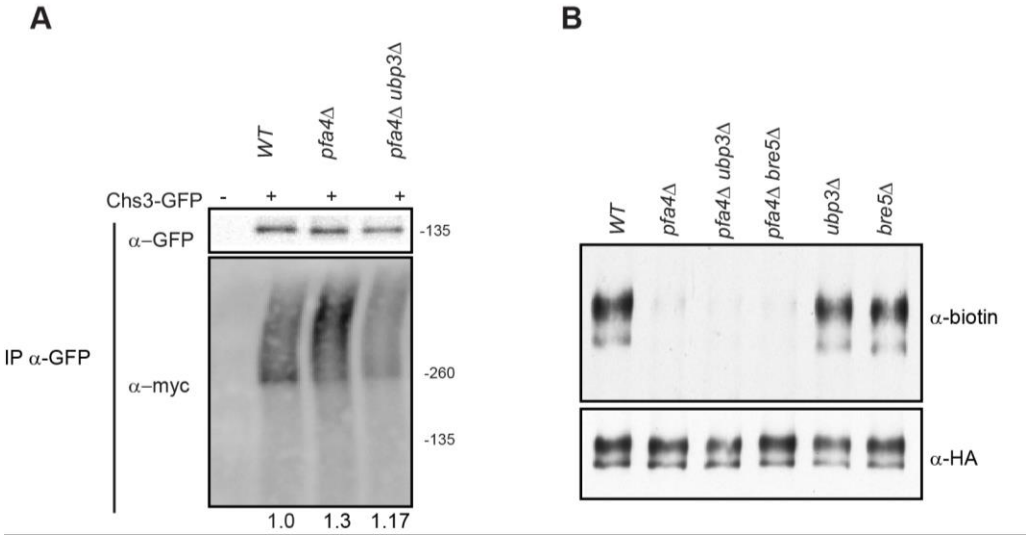

Figure 3.4 The Bre5\UBP3 complex does not alter the ubiquitination or palmitoylation of Chs3
(A) Lysates from cells expressing genomically tagged Chs3-GFP and 6-myc tagged ubiquitin from a multi-copy plasmid were immunoprecipitated with α-GFP Ab and separated on SDS-PAGE gels. Chs3 and Ubiquitin were detected with α-GFP and α-myc Ab respectively. The intensity of ubiquitination was measured using the Quantity One Image Analysis software and normalized to the intensity of the Chs3-

GFP band for each strain. The relative intensity (R.I) of ubiquitination in the different strains is indicated at the bottom. (B) Protein extracts from cells expressing Chs3-HA-FLAG on a plasmid in the indicated strains were subjected to thioester cleavage by hydroxylamine and subsequent biotin-specific replacement of cleaved palmitate. Chs3 was immunoprecipitated with α-FLAG, and immunoprecipitates were analyzed by SDS-PAGE. α-biotin and α-HA antibodies were used to detect modified and total Chs3, respectively.

3.3.5 COPI retrieval as a mechanism for Chs3 ER retention

Loss of either *BRE5* or *UBP3* destabilizes COPII and COPI, the vesicle coats that govern anterograde and retrograde transport between the ER and Golgi, respectively (Cohen et al., 2003a; Cohen et al., 2003b). Destabilization of COPII and disruption of forward transport would not be expected to restore ER export of Chs3. Instead, we considered that *ubp3Δ* suppression of *pfa4Δ* could be caused by destabilization of COPI. If palmitoylation-deficient Chs3 reaches the early Golgi, COPI retrograde transport from this compartment could retrieve Chs3 back to the ER, and failure to do so could result in transport to the plasma membrane. Indeed, we found palmitoylation-deficient Chs3 was no longer ER-retained in the temperature-sensitive COPI mutant *sec21-1* at non-permissive temperatures (Fig 3.5 A). The Sec61-RFP marker shows largely wild type ER morphology in *pfa4Δ sec21-1* mutants ruling out any indirect effects due to disruption of ER structure. As seen in the protease-accessibility assay, the level of cell surface-localized Chs3 in *pfa4Δ sec21-1* mutants was comparable to that of wild-type cells (Fig 3.5 B, C). Deleting the only nonessential COPI subunit, Sec28, gave rise to the comparable suppression. Similar effects on Chs3 localization were not observed in *chs7Δ sec21-1* double mutants (data not shown). These findings imply that *UBP3* regulates ER retention of non-palmitoylated Chs3 via disrupting COPI retrieval.

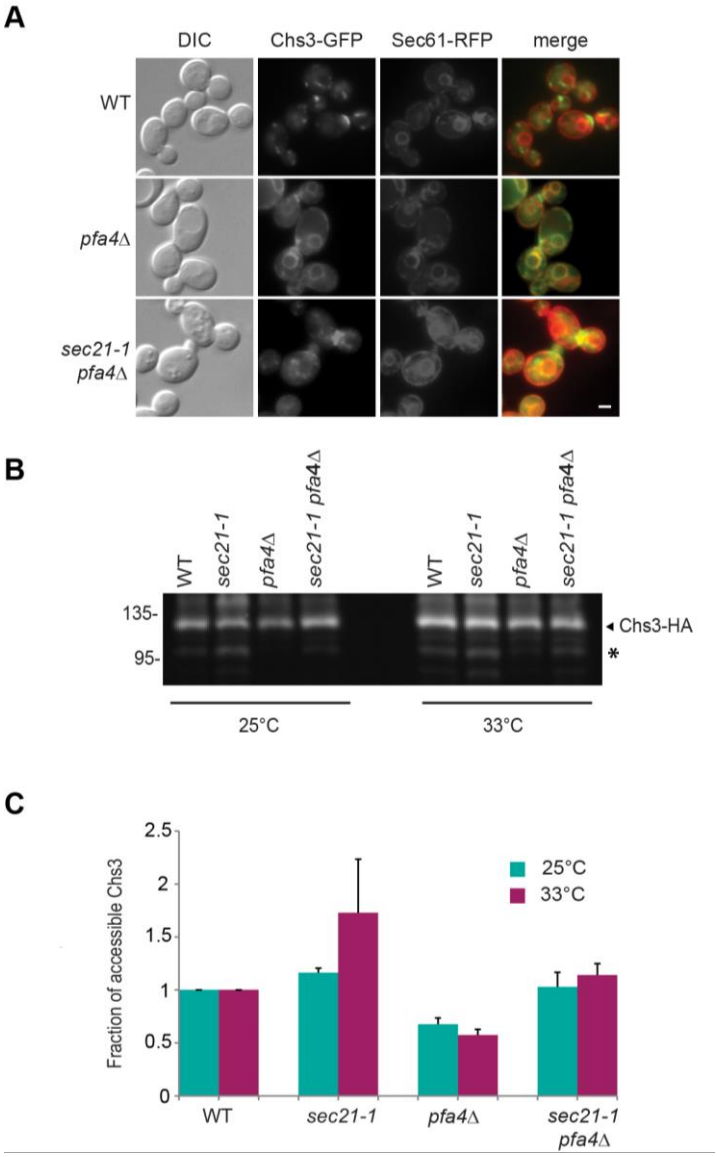

Figure 3.5 Inhibition of COPI retrograde transport restores cell-surface expression of palmitoylation-deficient Chs3

(A) Log phase cultures of cells expressing Chs3-GFP and Sec61-RFP in the indicated strains were observed by fluorescence microscopy after a 45 min shift to the restrictive temperature of 33°C. Scale bar, 2μM (B) Protease-accessibility assay of the indicated strains expressing genomically integrated Chs3-3xHA was performed as in Fig 3C, at permissive (25°C) and after a 1 hr shift to non-permissive (33°C) temperature. * Indicates Chs3 fragment generated by protease cleavage. (C) Levels of Chs3 degradation products relative to total (shown in B) from three independent experiments were quantified by densitometry. Error bars represent SEM (Standard error of the mean).

3.3.6 Chs3 is palmitoylated at cysteines 1014 and 1018

In order to uncover the mechanism of Chs3 palmitoylation by Pfa4, we wanted to first identify the palmitoylated cysteines. We made a series of point mutants by individually exchanging conserved cysteines in the putative cytosolic domains of Chs3 to alanines. We evaluated their palmitoylation status initially by ABE, followed by Click based palmitoylation assay and their localization using fluorescence microscopy. We found that mutation of Cys 1018 to Ala caused a localization defect and since palmitoylation usually occurs on proximal cystines we mutated the nearby Cys at position 1014 as well to elaborate this localization defect.

In order to test the palmitoylation status of Chs3 single and double cysteine mutants we subjected them to click based palmitoylation analysis (Roth et al., 2011). Chs3 was immunoprecipitated with α-HA, and immunoprecipitates were analyzed by SDS-PAGE. α-HA antibody was used to detect lysate and immunoprecipitated Chs3, whereas modified Chs3 labeled with Alexa Fluor 647 was detected by in gel fluorescence using a Typhoon 9200 scanner. Although Chs3 C1018A showed reduced palmitoylation compared to Chs3 C1014A, palmitoylation of Chs3 was completely absent only when both C1014 and C1018 were simultaneously changed to alanines (Fig 3.6 A). Thus, we conclude that Chs3 is palmitoylated at both these positions.

Consistent with the palmitoylation analysis, Chs3 C1014A/C1018A-GFP accumulated in the ER mimicking Chs3-GFP in a *pfa4Δ* strain (Fig 3.6 B). Chs3 C1014A-GFP showed mostly WT localization and Chs3 C1018A-GFP was mostly localized to the ER We also wanted to determine if the loss of Ubp3 would rescue the localization of Chs3 C1014A/C1018A similar to the rescue seen in the absence of Pfa4. Indeed, loss of Ubp3 rescued the ER retention of Chs3 (C1014A, 1018A) - GFP restoring cytosolic and cell surface localization (Fig 3.6 C, D). This rescue could be counteracted by adding back a single copy of *UBP3*. In conclusion, we have identified the palmitoylation sites of Chs3 to be C1014 and C1018, with C1018 being the major site of palmitoylation, and shown that mutation of these palmitoylated cysteines results in Chs3 phenotypes similar to those of a *pfa4Δ* strain. This further confirms that ER retention of Chs3 in

the absence of *PFA4* was indeed due to lack of the palmitoyl moiety on Chs3 and not due to indirect effects.

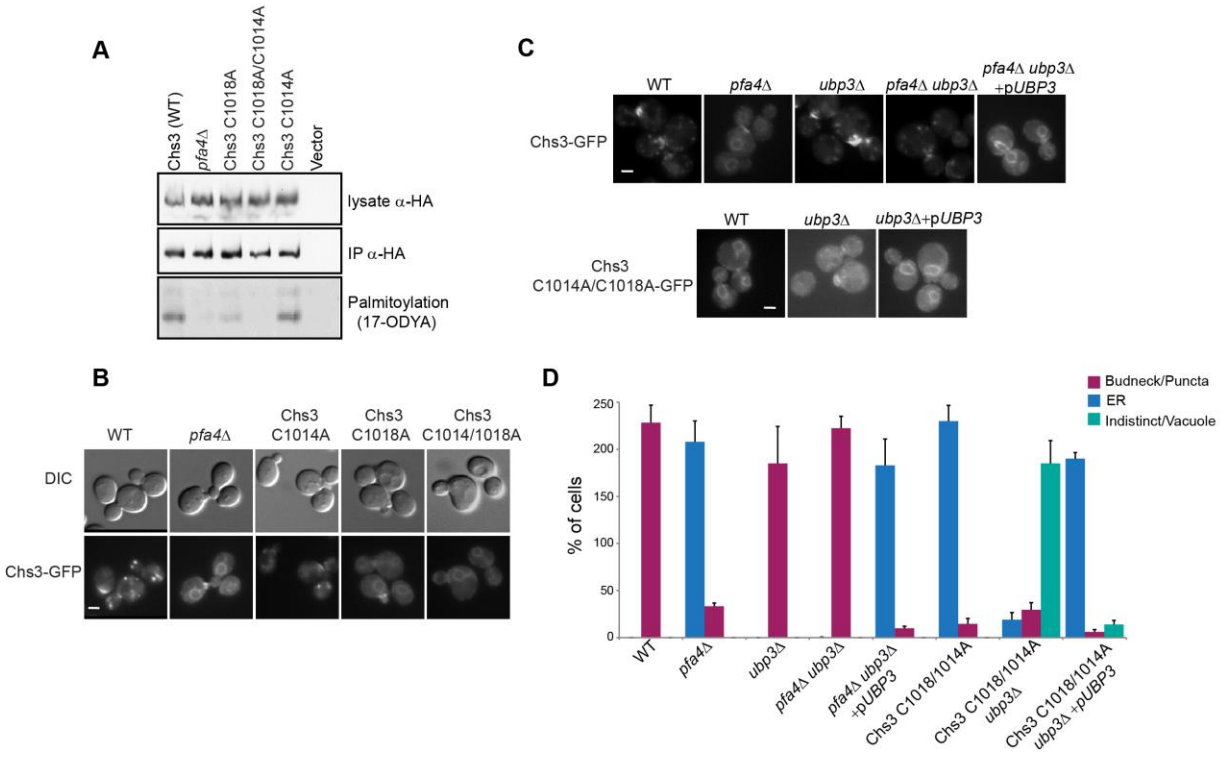

Figure 3.6 Mutation of *CHS3* at cysteines 1014 and 1018 leads to ER retention and loss of palmitoylation mimicking *pfa4Δ* mutants

(A) Cells expressing Chs3-GFP, Chs3 C1014A- GFP, Chs3 C1018A-GFP or Chs3 C1014A, C1018A-GFP were subjected to click based palmitoylation assay. Immunoprecipitated Chs3 and the lysate were analyzed by SDS-PAGE and detected using α-HA antibody. Alexa Fluor 647 labelling which indicates modified Chs3 was detected using a Typhoon 9200 scanner. (B) Log phase cultures of cells expressing Chs3-GFP, Chs3 C1014A-GFP, Chs3 C1018A-GFP and Chs3 C1014A/C1018A-GFP were observed using fluorescence microscopy (left). Scale bar, 2μM. . (C) Log phase cultures of cells expressing Chs3-GFP and Chs3 C1014A/C1018A-GFP in the indicated strain backgrounds were observed under fluorescence microscopy. Scale bar, 2μM. (D) Graphical representation of proportion of cells in each strain showing ER, budneck/ puncta and vacuolar/ indistinct localization from the above-indicated strains. An Average of 600 cells per strain were manually counted in three independent experiments. Error bars represent SEM.

3.4 Discussion

Our results highlight the role of palmitoylation in the folding and ER export of Chs3, a multi-pass membrane protein in yeast. Several proteins show a similar dependence on palmitoylation for ER exit. For example, palmitoylation of lipoprotein receptor-related protein 6 (LRP6) of the canonical Wnt signaling pathway at the ER is necessary for proper folding and ER exit. Palmitoylation induces tilting of the long TMDs of LRP6 and stabilizes it in the lipid bilayer. (Abrami et al., 2008). We identified the sites of Chs3 palmitoylation to be cysteines 1014 and 1018 adjacent to TMDs. In contrast to Chs3, the other yeast chitin synthases Chs1 and Chs2 do not require palmitoylation by Pfa4 and folding by Chs7 (Lam et al., 2006). Chs3 has several short TMDs compared to the other two chitin synthases, leading us to believe palmitoylation stabilizes these short TMDs and prevents Chs3 aggregation. However, aggregation might not be the only reason for ER retention of non-palmitoylated Chs3 since a small amount of Chs3 is able to get to the PM and function there.

Palmitoylation is also known to affect protein-protein interactions. For example, phosphorylation of LRP6 by Casein kinase I γ (CKI γ) requires palmitoylation of both proteins. When not palmitoylated, CKI γ is mislocalized and unable to phosphorylate LRP6 thus blocking Wnt signaling in cells (Davidson et al., 2005). Additionally, palmitoylation of the *β*4-subunit of the large conductance potassium channel (BK channel) is predicted to alter its orientation in the membrane and regulate its interaction with the *α*-subunit (Chen et al., 2013). Similarly, we found that palmitoylation influences interaction of Chs3 with its dedicated chaperone Chs7. In the absence of Pfa4, we detected a reduced interaction between the two proteins. Although the exact sites of Chs3-Chs7 interaction have not been mapped, it is possible this interaction is similar to other chaperone-substrate interactions and occurs via TMDs. Our results suggest that palmitoylation promotes Chs3-Chs7 binding by altering the structure of Chs3 TMDs. Chs3 might require continuous association with Chs7 for entry into COPII vesicles and for activity at the plasma membrane (PM). Thus, the ER exit defect seen in *pfa4Δ* mutants could be a consequence of both aggregation and reduced interaction with Chs7 leading to slower ER export via COPII.

Many DHHC enzymes require stable association with co-factors for activity. DHHC6 requires association with Selenoprotein K (SelK) at the ER for palmitoylation of inositol 1, 4, 5-triphosphate (IPR3) (Fredericks et al., 2014). We also explored the possibility that Chs7 could be a Pfa4 co-factor. However, we were unable to detect a strong physical interaction between Chs7 and Pfa4 indicating that Pfa4 does not depend on Chs7 for its association with Chs3. To support this idea, we also showed that there is enhanced interaction between Chs3 and Pfa4 in the absence of Chs7.

In the absence of palmitoylation, membrane proteins such as Tlg1, CD36 and AMPAR are subjected to ubiquitination and destruction by ERAD (Yang et al., 2009; Thorne et al., 2010). Our data shows non-palmitoylated Chs3 is turned over by ERAD, but it is not the only factor involved in Chs3 degradation. Our results suggest that ER retention of palmitoylation deficient Chs3 happens through two mechanisms: first, unlipidated Chs3 is statically retained by exclusion from COPII export vesicles as seen for other membrane proteins such as aggregated Gap1 (Kota et al., 2007). Second, a COPI retrieval pathway dynamically returns non-palmitoylated Chs3 to the ER. Dynamic retrieval has also been reported for membrane protein Fet3. Misfolded Fet3 escapes the ER and is actively retrieved back to the ER by Rer1 (Sato et al., 2003). We found that only non-palmitoylated Chs3 escapes the ER and is retrieved back in COPI vesicles. It is possible that the loss of palmitoylation causes a conformational change different from that of misfolding due to loss of Chs7. This change could expose a COPI site or could lead to recognition by a retrieval receptor, although we did not find a role of Rer1 in this process. Others have shown that Chs3 that cannot oligomerize is also returned to the ER from Golgi by COPI vesicles (Sacristan et al., 2013). Therefore, Chs3 ER retention mechanisms vary depending on the folding defect.

ER retention of non-palmitoylated Chs3 is regulated by the Ubp3/Bre5 deubiquitination complex. Through its role in removing ubiquitin from various substrates, the Bre5/Ubp3 complex is implicated in pathways such as ER-Golgi transport (Cohen et al., 2003a; Cohen et al., 2003b), cytoplasm-to-vacuole trafficking (Baxter, 2005), transcriptional elongation (McCullock et al., 2006), ribophagy (Kraft et al., 2008), and Pkc1 MAP kinase signaling (Wang et al., 2008).

In many cases, Ubp3 has a protective role, and its deletion results in degradation of its substrates. In others, the ubiquitinated protein is not destabilized but has altered function. We found that loss of Ubp3/Bre5 complex does not alter the ubiquitination or palmitoylation status of misfolded Chs3. Ubp3 is known to regulate anterograde transport by stabilizing COPII coats (Cohen, 2003a), but inhibition of forward transport in *ubp3* mutants is not a plausible mechanism for restoring cell surface targeting of Chs3. Loss of Ubp3 also leads to an accumulation of ubiquitinated forms of the COPI subunit Sec27 (β'-COP), resulting in increased proteasomal degradation (Cohen et al., 2003b) and inhibition of COPI-mediated retrieval to the ER. Our results support a model in which misfolded Chs3 is exported from the ER at low levels in *pfa4* mutants and subjected to later quality control by COPI at the Golgi. Loss of COPI-mediated Golgi-to-ER retrieval due to UBP3 deletion would, therefore, restore cell surface transport of Chs3.

We also observe that there is an increased expression of Chs3 at the surface in *ubp3Δ* mutants. This could be due to disruption of COPI retrieval causing more Chs3 to be trafficked to the plasma membrane. Alternatively, it is possible that Ubp3 alters endosomal trafficking and thus alters the amount of Chs3 stored in intracellular compartments, as COPI is also believed to play a role in retrieval of cargo from endosomes. Taken together our results show that quality control of non-palmitoylated Chs3 occurs in two steps. We also show for the first time that Chs3 is palmitoylated on C1014 and C1018, although the major site of palmitoylation seems to be C1018. Palmitoylation is necessary for efficient interaction of Chs3 with its chaperone Chs7, and this decrease in interaction could explain the reduced incorporation of Chs3 into COPII vesicles in *pfa4Δ* mutants. Our results highlight multiple effects of palmitoylation on the trafficking of polytopic membrane proteins similar to Chs3.

3.5 Supplemental information

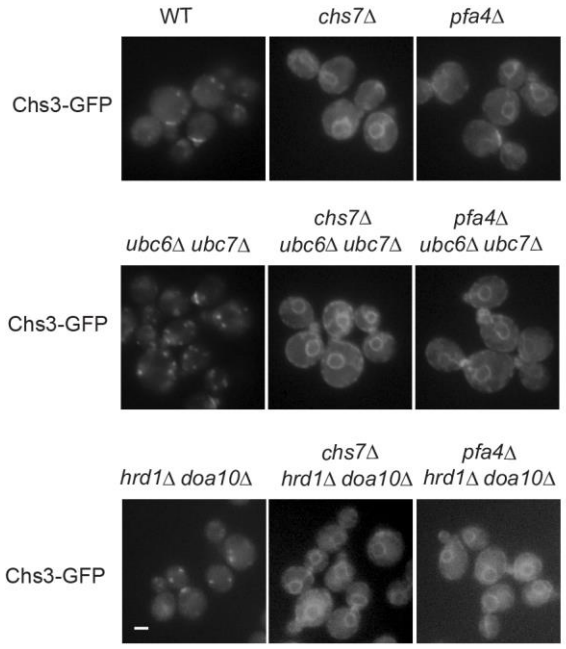

Figure 3.7 Inhibition of ERAD components does not suppress ER retention of Chs3 in *pfa4Δ* and *chs7Δ* mutants.
Log phase cultures of cells expressing Chs3-GFP in the indicated strain backgrounds were observed under fluorescence microscopy. Scale bar, 2μM.

Chapter 4: Conclusions and future directions

4.1 Overview of significant findings

The overall objective of my research was to understand how chaperones and post-translational events such as palmitoylation work together at the ER to help fold and traffic polytopic membrane proteins. We used Chs3, a model protein that requires both a dedicated chaperone Chs7 and palmitoylation by Pfa4, a palmitoyl acyltransferase, for proper folding and ER export in yeast. Previously published results from our lab indicated that in the absence of Chs7 and Pfa4, Chs3 is aggregated and unable to exit the ER in COPII vesicles. We tried to analyze if chaperone binding and palmitoylation at the ER occur in an obligatory sequence and if either displayed an absolute requirement for the other in order to bind and modify Chs3.

Chapter II of this thesis focused on the roles of Chs7 in Chs3 trafficking. Contrary to published results, we discovered that Chs7 was able to leave the ER and localize with Chs3 at the budneck and in intracellular puncta. We also showed a requirement for the presence of Chs7 for Chs3 activity at the plasma membrane, and demonstrated a requirement for the Chs7 C-terminal tail for post-ER binding to Chs3. Taken together, these results suggested that Chs7 has secondary roles in Chs3 trafficking and function.

Chapter III described in depth the role of palmitoylation in Chs3 trafficking and identified regulators that specifically alter trafficking and degradation of non-palmitoylated Chs3. We found that Chs3 palmitoylation status affected its interaction with its chaperone Chs7. This observation suggests that Chs3 palmitoylation may induce a conformational change that enhances binding to Chs7. A suppression screen was done to identify regulators that specifically affected palmitoylation-deficient Chs3. The Ubp3/Bre5 deubiquitination complex was one of the top 50 hits from this screen. Absence of Ubp3 rescued the surface expression of Chs3. Our results showed that lack of Ubp3/Bre5 did not alter the ubiquitination or palmitoylation status of Chs3 but instead altered COPI-mediated retrograde transport from the Golgi to the ER. We were also able to identify the palmitoylated cysteines on Chs3 to be C1014 and C1018. Both these cysteine residues are close to a transmembrane domain – membrane interface of Chs3 suggesting

a possible role for membrane anchoring and stabilization of Chs3 in the thin lipid bilayer of the ER.

In summary, we were able to dissect palmitoylation and folding events at the ER involved in the folding and trafficking of Chs3 and show that the Ubp3/Bre5 complex plays a critical role in the degradation of non-palmitoylated Chs3. Our work suggests that palmitoylation may generally happen first, followed by chaperone binding and folding. This discovery gives way to many questions about the exact molecular mechanism of Chs3 folding at the ER. The reason for this preferential interaction of Chs7 and Pfa4 in a sequence with Chs3 is yet to be fully understood. The next section will discuss some of the key points and future directions for the project.

4.2 Post-ER functions of Chs7 in Chs3 trafficking

4.2.1 Does Chs7 provide an ER exit signal for Chs3?

Localization of Chs7 at the budneck and at intracellular puncta similar to that of Chs3 suggests that Chs7 and Chs3 are probably trafficked together to the cell surface. Of course one caveat here is that we were unable to directly compare the co-localization of Chs7 and Chs3 due to the low intensity of the RFP signal. We considered two possibilities to explain the fact that Chs3 is unable to exit the ER in the absence of Chs7. Chs3 misfolding and aggregation could exclude it from entering COPII vesicles. Alternatively, Chs7 could provide the ER exit signal for Chs3. We based this idea on the fact that several chaperone proteins act as escorts as well. Examples such as Vma21 and NinaA have been discussed in Chapter I in detail. Through site-directed mutagenesis, we were able to generate point mutations in the conserved cytosolic C-terminal tail and a few short loops on the cytosolic side of Chs7. We discovered a few established COPII signals such as DxE and others in the C-terminal tail, however, none of these seemed to disrupt Chs3 localization and although some Chs7 was ER retained, the majority of Chs7 localized to the vacuole. It is possible that there are other COPII exit signals in the Chs7 structure, and these remain to be discovered. Therefore, we cannot entirely rule out the possibility that Chs7 is indeed an escort type chaperone for Chs3 or that there are redundant COPII exit signals in the protein. A more intense approach is needed to comb through and mutate other conserved regions of both Chs7 and Chs3 in parallel to look for COPII signals.

4.2.2 Why is Chs7 required for cell surface activity of Chs3?

A major conclusion from chapter II was that Chs3 requires the presence of Chs7 throughout its life cycle for efficient function. A similar dependence of an auxiliary subunit/chaperone can be seen for AMPAR. Cornichon proteins are suspected to be required for export, trafficking and surface expression of AMPAR. Cornichon proteins have been shown to regulate AMPAR gating at the synapse by stabilizing the open state of the channel (Schwenk et al., 2009). As discussed earlier we see that Chs3 was able to reach the surface in Chs7 tail mutants, however since their trafficking diverges beyond the Golgi Chs3 was unable to function at the surface. The reason for this dependence on Chs7 for Chs3 catalytic activity at the surface is not entirely understood. One attractive hypothesis is that Chs7 stabilizes the three-dimensional structure of Chs3 at the plasma membrane. Chs3 possesses a unique N-terminal structure, not found in Chs1 and Chs2. Hence, it is not surprising to find that Chs1 and Chs2 trafficking is independent of Chs7 and Pfa4. We could use a cellular fractionation approach and compare Chs3 folding status in the plasma membrane fraction via BN-PAGE in WT and Chs7 tail mutants. Another possibility is that Chs7 could also enhance Chs3 interaction with Chs4 and Bni4, both of which are required for localization and activity of Chs3 at the budneck. To test this, we could compare the strength of Chs3-Chs4 interaction in WT and Chs7 tail mutants. A decreased interaction could indicate a requirement for Chs7. Overexpression of Chs7 could also indicate if the strength of the Chs3-Chs4 interaction changes based on the levels of Chs7. As a next step, it would be interesting to see if Chs7 and Chs4 directly interact with each other. At the surface, Chs7 could also prevent Chs3 endocytosis and stabilize it longer at the surface.

4.3 Roles of Chs7 and Pfa4 in Chs3 folding and export at the ER

4.3.1 Chs7 and Pfa4 interact sequentially with Chs3.

Our results from Chapter III indicate that Chs7 binds well to palmitoylated Chs3. This suggests that palmitoylation of Chs3 could occur first followed by binding to Chs7 and folding. After translocation, Chs3 is palmitoylated by Pfa4, and this could cause a conformational change in Chs3, which is then recognized by Chs7. Palmitoylation could also target Chs3 to a specific region of the ER membrane enriched in Chs7 and thus bring Chs3 and Chs7 in proximity to each

other for binding. This is seen for proteins such as CD8, which depend on palmitoylation for lipid raft targeting. We also show that Chs7 and Pfa4 don't interact very well with each other. The strength of their interaction is severely reduced compared to the binary interactions of Chs7-Chs3 and Pfa4-Chs3 and Chs3 seems to bridge this interaction. This also supports the sequential interaction hypothesis by eliminating the possibility of a trimeric interaction between the three proteins. We cannot however at this point rule out the idea that Pfa4 also directly binds and modifies Chs7. The palmitoylation status of Chs7 has never been reported in the literature. Therefore, comparing the palmitoylation and folding status of Chs7 in the presence and absence of Pfa4 could explain the direct interaction between Chs7 and Pfa4.

Interestingly, we observe a steep increase in interaction between Chs3 and Pfa4 in the absence of Chs7. Based on these observations we propose a "kick-off" model. In this model, following the initial interactions between Chs3 and Pfa4, Chs7 binds the Chs3-Pfa4 complex and kicks Pfa4 off. Therefore, in the absence of Chs7, Chs3 and Pfa4 continue to bind each other and never dissociate. One way to test this model would be overexpress Chs7 and observe if this causes a reduction in Chs3-Pfa4 interaction or Chs3 palmitoylation.

4.3.2 Do Chs7 and Pfa4 bind similar regions of Chs3?

It is possible that Chs7 and Pfa4 sequentially bind Chs3 because they compete for binding to the same regions of Chs3. As stated in Chapter II, we already know that some chaperone proteins interact and fold only a particular subset of transmembrane domains of a client protein, so it is possible Chs7 also interacts with only a subset of Chs3 TMDs. In order to test this, we have generated various Chs3 truncation mutants which include: the N-terminal region of Chs3 (first 622aa), the C-terminal region of Chs3 (last 595aa), and a mutant where the first 126aa have been deleted. One caveat to this approach is that the individually expressed Chs3 C and N-terminal mutants are less stable than the FL protein. Hence, it is hard to compare the true binding potential of these mutants. However, there is comparable expression between the N and C-terminal mutants, and preliminary data suggests that Chs7 and Pfa4 bind to the C-terminal region of Chs3. This could explain why Chs7 and Pfa4 cannot bind Chs3 together and exist as a stable trimer as they are mutually exclusive.

4.3.3 Absence of the oligomerization domain does not alter the interaction between Chs3 and either Chs7 or Pfa4

A recently published study suggests that the first 126 aa of Chs3 represent an oligomerization domain. When this conserved region is deleted, Chs3 is no longer able to exist as a dimer (Sacristan et al., 2013). Co-immunoprecipitation experiments from our lab suggest that the first 126 AA are not required for binding to Chs7 or Pfa4, and that both these proteins bind Chs3$^{\Delta 126}$ just as well as full-length Chs3 protein. However, a strong binding does not indicate proper folding or palmitoylation. Hence, it would be important to check the folding and palmitoylation status of Chs3$^{\Delta 126}$. Experiments are underway to check if Chs3$^{\Delta 126}$ is misfolded in the absence of Chs7 and Pfa4.

4.4 Role of Ubp3/Bre5 in the retention of non-palmitoylated Chs3

As discussed in Chapter III, we identified the Ubp3/Bre5 deubiquitination complex as the factor responsible for ER retention and degradation of palmitoylation deficient Chs3. Non-palmitoylated Chs3 is dynamically retained in the ER via COPI mediated retrieval from the Golgi. Our results suggest that Ubp3/Bre5 does not alter the palmitoylation or the ubiquitination status of Chs3. However, it could alter the folding of Chs3 although our preliminary findings suggest otherwise. We were able to show indirectly that deletion of Ubp3/Bre5 probably disrupts the COPI retrograde transport to restore the cell surface localization of Chs3 as Chs3 localization is similar to WT in both COPI mutants and *ubp3Δ/bre5Δ*. It is prudent to test the levels of COPI protein in the absence of Ubp3/Bre5 to confirm that it alters the degradation of COPI proteins. A careful quantification of the amount the Chs3 able to interact with COPI components in WT vs. *ubp3Δ/bre5Δ* could provide further evidence that COPI mediated retrieval is indeed responsible for Chs3 retention. It would also be interesting to see if palmitoylation deficient Chs3 can interact well with Chs7 in *ubp3Δ/bre5Δ* strains. It is possible Ubp3/Bre5 alters this interaction promoting proper trafficking of Chs3. It is also necessary to verify if the palmitoylation deficient Chs3 can function normally at the surface. If it can function normally, then the functional significance of Chs3 palmitoylation could be restricted to the ER, and palmitoylation might not be necessary beyond promoting its interaction with Chs7 at the ER.

4.5 Conclusions

The purpose of this project was to understand how dedicated chaperones and other post-translational modifications work together to achieve folding and assembly of polytopic membrane proteins. Based on the results presented here it is possible that palmitoylation influences the interaction of substrate proteins with their chaperones thereby affecting their trafficking and function. This study also reveals a novel secondary role for a dedicated chaperone protein. Of course, more work is required to analyze if other client specific chaperones also take on additional roles in the post-ER trafficking of their substrates. Understanding these principles will help us design better therapeutic options such as chemical chaperones that need to operate not just at the ER but throughout the trafficking pathway of the protein to support both its structure and function.

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