THE ROLE OF PALMITOYLATION IN ENDOPLASMIC RETICULUM TRANSPORT AND QUALITY CONTROL OF THE YEAST POLYTOPIC PROTEIN CHS3

by

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ABSTRACT

All secretory proteins must pass the strict quality control (QC) imposed by the endoplasmic reticulum (ER), where they are first synthesized. Various chaperones, degradation machinery, and vesicular transport factors act together to ensure only properly folded proteins can leave this compartment for subsequent sorting. Failure in QC contributes to misfolding, intracellular retention, and frequently degradation, all of which are known to cause disease. QC is particularly crucial for polytopic proteins, which often represent plasma membrane transporters and channels important for cell function. Recent work suggests ER surveillance systems for polytopic proteins are specialized towards substrates and specific misfolding defects. The underlying mechanisms, especially the roles of post-translational modifications, are poorly understood, thus necessitating examination of various model proteins.

Here, the yeast chitin synthase Chs3 was used as a paradigm for polytopic protein trafficking. By high-throughput analysis of the yeast deletion collection, a novel Chs3 ER transport factor was identified. This protein, Pfa4, contains a conserved DHHC-domain, signifying its putative function as a protein acyltransferase. These enzymes of protein palmitoylation were only recently discovered, and few substrates are known. The work described here showed that Chs3 was palmitoylated by Pfa4, and this modification was required for ER export. Both palmitoylation and association with the chaperone Chs7 were necessary for preventing Chs3 aggregation at the ER, indicating that palmitoylation is required for Chs3 to attain an export-competent conformation. Retention of misfolded Chs3 appeared independent of known ER-associated degradation machinery. Instead, a high-throughput search identified the Ubp3 deubiquitination enzyme as a retention factor; deletion of *UBP3* restored ER export of unpalmitoylated Chs3 through palmitoylation-independent means. Ubp3-mediated deubiquitination may be regulating the levels of proteins involved in both Chs3 folding and Golgi-to-ER retrieval of misfolded Chs3.

The role of palmitoylation in folding at the ER is not well known, and many substrate-specific retention pathways for polytopic proteins have not been identified. These findings suggest palmitoylation can contribute to ERQC of polytopic proteins, and point to potentially novel QC factors that are regulated by deubiquitination. A better understanding of these fundamental molecular mechanisms could contribute to discovery of therapeutic targets for ER misfolding diseases.

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LIST OF ABBREVIATIONS

AAP	amino acid permease
ABC	ATP-binding cassette
AP	adaptor protein
APT	acyl-protein thioesterase
ATP	adenosine triphosphate
CF	cystic fibrosis
CFTR	cystic fibrosis transmembrane conductance regulator
CNX	calnexin
COP	coatomer protein
CPY	carboxypeptidase Y
CRT	calreticulin
CW	Calcofluor White
DHHC	aspartate-histidine-histidine-cysteine
DIC	differential interference contrast
DSP	Dithiobis[succinimidyl] propionate
DUB	deubiquitination enzyme
ER	endoplasmic reticulum
ERAD	endoplasmic reticulum associated degradation
ERGIC	ER-to-Golgi intermediate compartment
ERQC	ER quality control
GAP	GTPase activating protein
GDP	guanosine diphosphate
GEF	guanine nucleotide exchange factor
GFP	green fluorescent protein
GPCR	G-protein coupled receptor
GPI	glycosylphosphatidylinositol
GTP	guanosine triphosphate
HA	hemagglutinin
HSP	heat shock protein
kD	kilo Dalton
LDLR	low density lipoprotein receptor
MAPK	mitogen-activated protein kinase
NDI	nephrogenic diabetes insipidus
OD_{600}	optical density at 600nm
ORF	open reading frame
PAT	protein acyl-transferase
PDI	protein disulfide isomerase
PM	plasma membrane
QC	quality control
RFP	red fluorescent protein
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SGA	Synthetic Genetic Array
SNAREs	soluble N-ethylmaleimide-sensitive factor attachment protein receptors
TAP	tandem affinity purification
TGN	trans-Golgi network
TMD	transmembrane domain
UBP	ubiquitin-specific protease
UPR	unfolded protein response
V_2R	V_2 vasopressin receptor
YPD	yeast extract peptone dextrose

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> "I have no data yet. It is a capital mistake to theorize before one has data. Insensibly one begins to twist facts to suit theories, instead of theories to suit facts."
>
> *Sherlock Holmes, A Scandal in Bohemia* — Arthur Conan Doyle

To my mother,

For understanding the meaning behind all this,
even though the pages beyond are incomprehensible.

And

To Vicki,

A most cherished friend lost before her time,
for teaching me the most important lesson in life.

CO-AUTHORSHIP STATEMENT

CHAPTER 2

I wrote the manuscript and carried out all the work described in this chapter, except for the acyl-biotin exchange assays (Figure 2.4.A), which were performed by Amy Roth and Nick Davis (Wayne State University).

CHAPTER 3

I wrote the manuscript and carried out all the work described in this chapter, except for the following experiments, which were performed by others as listed:

- Calcofluor array testing (Figure 3.1.A)
  – Mike Davey (Conibear Lab)
- Chs3 pulse chase and *in vitro* COPII studies (Figures 3.2.D, Fig 3.5)
  – Silvere Pagant and Liz Miller (Columbia University)
- Acyl-biotin exchange assays (Figure 3.3)
  – Amy Roth and Nick Davis (Wayne State University)

APPENDIX B

I did all experiments in this section except the following:

- Acyl-biotin exchange assays for Chs3 cysteine mutant palmitoylation
  – Amy Roth and Nick Davis (Wayne State University).
- Plasmid construction for a subset of Chs3 cysteine mutants
  – Mike Davey and Xiao Chen (Conibear Lab)

CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

1.1. FOREWORD: MEMBRANE PROTEIN TRAFFICKING

Proteins that enter the secretory pathway represent about one quarter of the cell's proteome (van Anken and Braakman, 2005). All secretory proteins are first synthesized at the endoplasmic reticulum (ER) before transport to the Golgi, where they are sorted to other destinations such as lysosomes, the plasma membrane, or the extracellular environment (Figure 1.1). From the moment a protein is synthesized on ribosomes and co-translationally inserted across the ER membrane, it undergoes a strict regimen of folding and assembly before being exported to more distal compartments. The ER therefore serves not only as a protein factory, but also as a gatekeeper for entry into the secretory pathway. How this organelle executes and achieves these vital functions have been under intense study since its discovery with the electron microscope (Porter et al., 1945). This large, continuous, membrane-bound system is resident to a diverse army of accessory factors monitoring three major functions: protein production, folding, and transport. The ER surveillance system must provide for a vast number and variety of proteins; however, many mechanisms regarding folding and transport are still unclear.

Approximately 20-30% of the proteome consists of integral membrane proteins (Liu and Rost, 2001; Wallin and von Heijne, 1998). Membrane spanning proteins with a single transmembrane domain can be further classified as Type I (extracellular N-terminus and cytoplasmic C-terminus), and Type II (cytoplasmic N-terminus and extracellular C-terminus). Multi-pass proteins with more than one transmembrane domain are called polytopic proteins (van Anken and Braakman, 2005). One other class of membrane-associated proteins are those covalently modified by lipid anchors that insert into the bilayer; these proteins do not necessarily have membrane-spanning segments (van Anken and Braakman, 2005). Many polytopic proteins are plasma membrane receptors and transporters that play important roles in cell communication, mobility, and homeostasis. Proper cell surface expression of these membrane proteins is not only scrutinized at the level of the ER, but also at the trans-Golgi network (TGN) and endosomes, where many stimulus-driven trafficking events are controlled (De Matteis and Luini, 2008). In this way, secretory and endocytic compartments act in concert to tightly regulate cell surface targeting of membrane proteins.

It is therefore not surprising that the failure to present membrane proteins at the cell surface is the basis of various human diseases (Aridor and Hannan, 2000). Misfolding and retention of

polytopic proteins at the ER are causative factors in many inherited ER storage disorders, such as cystic fibrosis and nephrogenic diabetes insipidus (Aridor and Hannan, 2000). These and other pathologies can also be caused by impairment of ER quality control and transport functions. Intracellular accumulation of aberrant proteins can be a further source of cytotoxicity, and therefore must be properly managed. The ER can achieve this through a combination of preventing deployment, refolding, and disposing of aberrant proteins. Currently, some of the major unanswered questions include how proteins are recognized as being misfolded, and how the cell adapts to these aberrant forms. Do degradation or refolding pathways dominate? What are the determinants of retention and forward transport? The influence of various post-translational modifications such as glycosylation and ubiquitination are also significant, but not all of their effects have been well characterized. A system comprised of chaperones, vesicle transport factors, and degradative machinery exists to direct ER quality control processes, but many components of this system have not been identified and the coordination of their actions is not understood.

In an effort to understand the regulated cell surface trafficking of polytopic proteins, the work here describes the discovery and characterization of novel aspects of quality control and transport at the ER. This opening chapter will review what is currently known and what further questions remain unanswered about the vital processes of vesicle trafficking, protein folding, and post-translational modifications at the ER.

1.2. TRANSPORT AT THE ENDOPLASMIC RETICULUM

1.2.1. OVERVIEW OF VESICULAR TRANSPORT

Protein transport from the ER to downstream organelles of the secretory pathway, as well as the intracellular transport between these various compartments, is mediated by vesicular carriers (Schekman and Orci, 1996) (Figure 1.1). The first step of vesicle transport involves concentration of both soluble and membrane-associated cargo into sites of export at the donor compartment, often through the action of cargo-binding transport adaptors. Next, vesicle formation and budding are mediated by a set of cytosolic coat proteins that are recruited to export sites. A budded vesicle docks and fuses with the acceptor compartment with the aid of tethering complexes and SNAREs (soluble N-ethylmaleimide-sensitive factor attachment protein receptors). The precision in vesicle transport is ensured by: the specific recognition and capture of cargo, the exclusion of non-cargo or resident proteins, and the action of compartment-specific coats, tethers, and SNAREs. These mechanisms of vesicle transport are well conserved in eukaryotes (Schekman and Orci, 1996).

Most transport carriers are bound by one of three major vesicle coats that have been well characterized: clathrin, and the coatomer proteins COPI and COPII (Figure 1.1). Clathrin, the first coat to be identified, is a triskelion-shaped protein complex involved in sorting at the cell surface and Golgi to endocytic and lysosomal compartments. It exerts its specificity with compartment-specific adaptor protein (AP) complexes that recognize different signal motifs. Some characterized motifs are sequences intrinsic to cargo proteins; others involve the post-translational addition of ubiquitin (Bonifacino and Traub, 2003; Hinners and Tooze, 2003).

1.2.2. TRANSPORT AT THE ER

Vesicle traffic between the ER and Golgi is directed by the two non-clathrin coats, COPI and COPII. Anterograde cargo transported from the ER to the Golgi are packaged into COPII coated vesicles, while Golgi-to-ER retrograde cargo are transported in COPI vesicles. Like clathrin,

remarkable conservation of COPII and COPI is observed from yeast to humans. Their vital role in intracellular transport is reflected in the fact that, with the exception of the COPI structural component Sec28, all components of COPII and COPI are essential for survival in yeast (Lee et al., 2004). Therefore, the discovery and subsequent analysis of these mutants have traditionally involved temperature-sensitive alleles (Teasdale and Jackson, 1996). The next sections will focus on the basic structure and formation of these two coats.

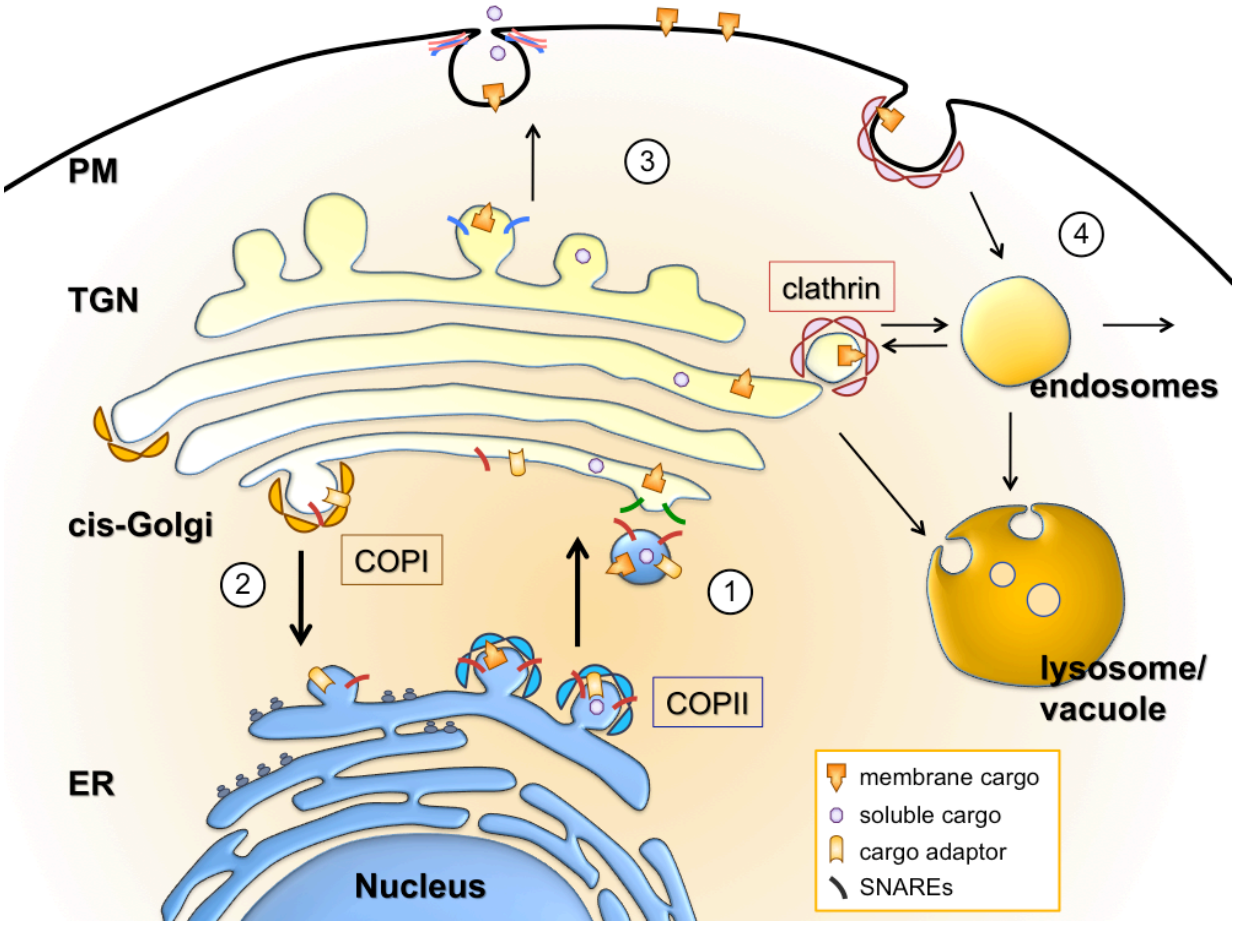

Figure 1.1. Secretory and Endocytic Transport pathways.
(1) After their synthesis and assembly at the endoplasmic reticulum (ER), secretory proteins are incorporated into COPII coated vesicles for export to the Golgi. Vesicle fusion with target membranes is mediated by vesicle and compartment-specific SNAREs (soluble N-ethylmaleimide-sensitive factor attachment protein receptors). (2) ER resident proteins such as some cargo adaptors are transported from the cis-Golgi back to the ER in COPI-coated vesicles. (3) The trans-Golgi network (TGN) is responsible for all major protein sorting to the plasma membrane (PM), and other intracellular compartments such as endosomes and lysosomes (yeast vacuole). Some of these trafficking events are known to take place in clathrin-coated vesicles. (4) Proteins that are endocytosed from the plasma membrane or extracellular environment, a process that can also involve clathrin-coated vesicles, are further sorted in endosomes.

1.2.2.1. COPII export

The COPII coat is made up of the Sar1 GTPase, an inner layer consisting of the Sar1-GTPase activating protein (GAP) Sec23 and Sec24 (Sec23/24), and an outer layer of Sec13 and Sec31 (Sec13/31) (Stagg et al., 2008). Formation of COPII vesicles requires recruitment and activation of Sar1 to the ER membrane by Sec12, the Sar1-guanine nucleotide exchange factor (GEF). This is followed by the sequential recruitment of the rest of the coat: Sar1 first recruits the inner cargo-binding Sec23/24 heterodimer, forming the prebudding complex, which concentrates COPII cargo. The prebudding complex then recruits the outer structural layer that is made up of the Sec13/31 heterotetramer (Lee et al., 2004)(Figure 1.2).

Cargo to be exported from the ER exhibit specific exit signals that are recognized by several components of the COPII coat. For example, the A site of Sec24 binds a YNNSNPF motif on the Sed5 SNARE, and the B site recognizes diacidic and LXXLE export motifs (Lee et al., 2004). Sar1 is also known to have a role in cargo recognition of a C-terminal dibasic signal (specifically, a di-arginine RXR sequence) found on mammalian glycosyltransferases (Giraudo and Maccioni, 2003). Mutation of critical export signals has been shown to result in the loss of interaction with adaptors, thus blocking forward transport.

While transmembrane proteins can interact directly with Sec23/24 through signals on their cytosolic domains, soluble proteins and glycosylphosphatidylinositol (GPI) anchored membrane proteins are captured by cargo-specific adaptors that associate with Sec23/24 (Figure 1.2). For example, the transport of soluble yeast pheromone α-factor into COPII vesicles requires Erv29 (ER vesicle protein 29kDa) (Belden and Barlowe, 2001a), a membrane receptor that also functions in packaging of other soluble proteins such as vacuolar hydrolases, carboxypeptidase Y, and proteinase A (Lee et al., 2004). In mammalian cells, the lectin ERGIC-53, which binds Sec23/24 through a C-terminal diphenylalanine motif, has a similar function for secretion of soluble glycoproteins (Nufer et al., 2003).

Some transmembrane proteins also require binding to adaptor proteins for export. Recent work suggest that the conserved Erv family of proteins may serve as COPII adaptors for ER export of different subsets of transmembrane cargo by continuously cycling between the ER and Golgi (Powers and Barlowe, 2002; Nakanishi et al., 2007). The p24 family of integral membrane

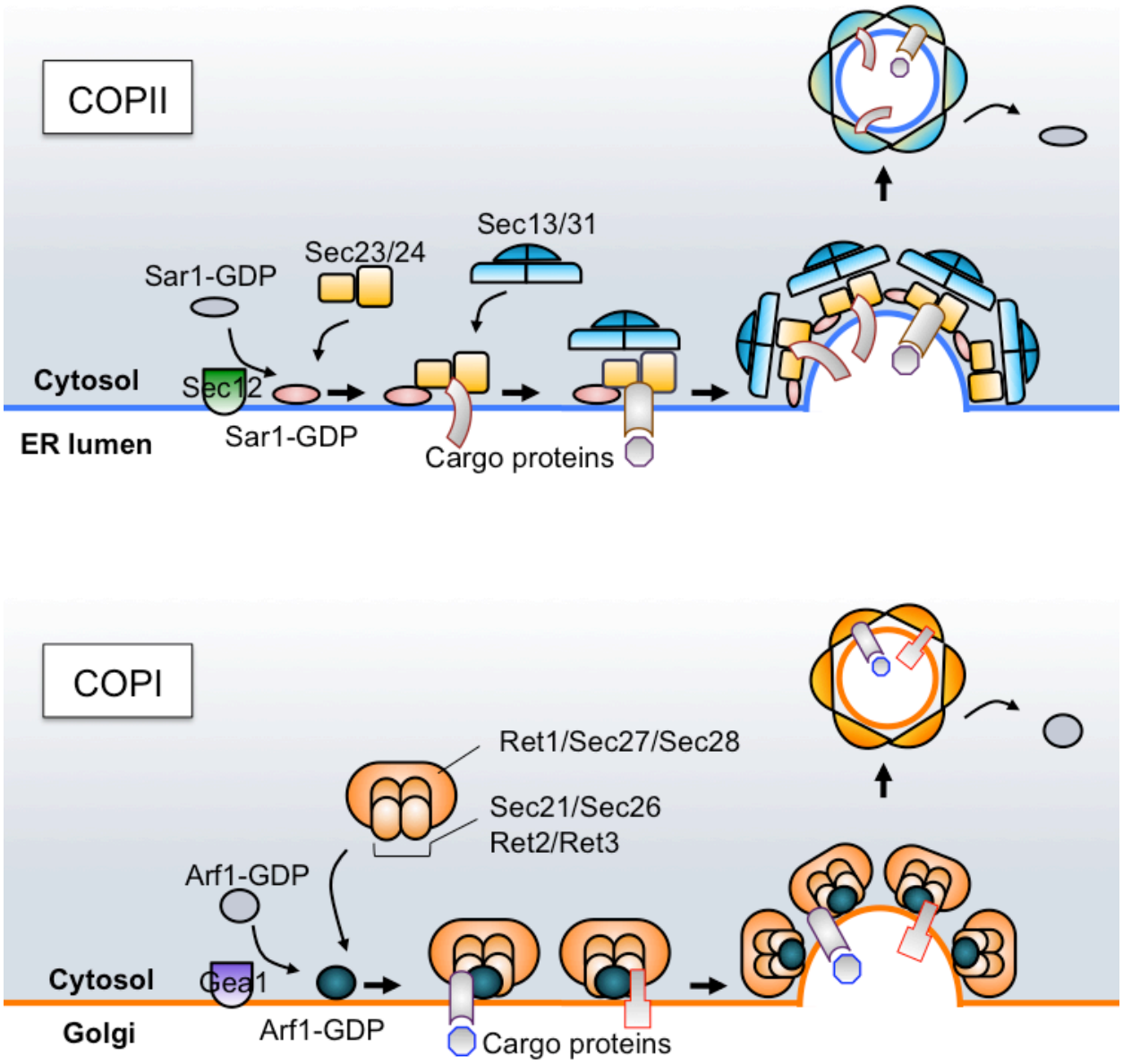

Figure 1.2. COPII and COPI coat formation.
Top: Formation of the COPII coat requires membrane recruitment and activation of the Sar1-GTPase by the ER-localized guanine GEF, Sec12. The COPII coat is comprised of the inner Sec23/24 and the outer Sec13/31 layers. Sec23/24 is responsible for binding to specific cytosolic export motifs on cargo proteins. Soluble proteins (octagons) can be incorporated into coated vesicles through membrane-associated adaptors that also bind the coat proteins. Sar1-GTP is deactivated after vesicle budding. Bottom: The COPI GTPase, Arf1, is recruited to Golgi membranes by the Arf1-GEF, Gea1. The COPI coat is a pre-assembled complex consisting of the COPI-b heterotrimer (Ret1/Sec27/Sec28) and the COPI-f heteroteramer (Sec21/Sec26/Ret2/Ret3). Various COPI subunits are responsible for cargo recognition and binding (see text). (Adapted from Kirchhausen, 2000, with permission.)

proteins has also been suggested to mediate cargo-coat interactions through binding both COPII and COPI. For example, one p24 member, Emp24, is suggested to function as a receptor for Gas1, a GPI-anchored protein (Belden and Barlowe, 2001b; Lee et al., 2004). Additional transport adaptors, as well as recognition motifs, are likely to exist, as some proteins do not have obvious export signals.

1.2.2.2. COPI retrograde transport

The COPI coat is structurally distinct from COPII, but its initial assembly is analogous, requiring membrane recruitment of the Arf1 GTPase by the Golgi-localized Arf1-GEF, Gea1. Activated Arf1 then recruits the preassembled heptameric COPI complex, which consists of the α/β'/ε (yeast Ret1/Sec27/Sec28) outer subcomplex and the β/γ/δ/ζ (yeast Sec26/Sec21/Ret2/Ret3) inner subcomplex, which are also known respectively as COPI-b and COPI-f (Gabriely et al., 2007) (Figure 1.2). COPI-b shares homology with clathrin, while components of the heterotetrameric COPI-f complex are structurally analogous to, and show some sequence homology with, subunits of the clathrin adaptor protein AP complexes (Lee et al., 2004; DeRegis et al., 2008).

Although COPI also has a post-ER role in retrograde transport through the Golgi stacks (a process important for maintaining Golgi integrity), its major function is the retrieval of proteins to the ER. This is important for proteins like transport escorts that continually cycle between the ER and the Golgi, as well as ER-resident proteins that may have escaped to the Golgi. Many such proteins contain specific retrieval signals to ensure ER localization is maintained. Soluble proteins displaying KDEL motifs (HDEL in yeast) are retrieved from the Golgi by the KDEL receptor, Erd2 (ER retention defective 2) (Lewis and Pelham, 1992). Retrieval of membrane proteins has been shown to be mediated by dibasic (dilysine or di-arginine) signals that are recognized directly by COPI (Teasdale and Jackson, 1996). COPI subunits with known cargo binding functions include Sec27 and Ret1, which bind dilysine signals through their WD40 domain, and Sec26 and Ret2, which bind arginine-based motifs (Duden et al., 1994; Eugster et al., 2004). COPI adaptors such as Rer1 can also retrieve transmembrane proteins, most likely by recognizing specific transmembrane determinants (Sato et al., 2003; Kaether et al., 2007; Spasic et al., 2007). Similar to other vesicle trafficking pathways, additional COPI transport signals and substrate specific adaptors are likely to exist.

1.2.3. DOWNSTREAM ORGANELLES

Despite much study on how coat proteins confer cargo specificity, many mechanisms such as cargo recognition signals and binding sites, as well as accessory factors that mediate these interactions, are still unclear. Furthermore, new coat complexes continue to be identified at downstream compartments, including FAPP (Godi et al., 2004) and exomer (Wang et al., 2006), which specialize in Golgi to cell surface transport. There is keen interest in post-translational signals such as phosphorylation and ubiquitination, which are key to protein sorting especially at post-ER compartments like the TGN and endosomes. Emerging data also highlight the importance of other modifications in vesicle transport, such as the reversible attachment of lipid moieties. Characterization of these modifications and how they are recognized by trafficking machinery such as coat proteins will lead to a better overall picture of how the specificity of intracellular transport is ensured.

1.3. PROTEIN FOLDING

To be incorporated into COPII vesicles at the ER and transported to their sites of function, proteins must first be properly folded and assembled after synthesis. Folding intermediates and misfolded proteins often do not expose the proper sequences for capture by COPII or COPII adaptors. They can also either alternatively or concurrently display signals that are recognized by retention machinery. The importance of protein folding can be understood in the context of the outcomes stemming from misfolding. Misfolded proteins represent a danger to the cell in that, not only are they retained and absent from their normal sites of function, but they can also become toxic aggregates (Gregersen, 2006). Because protein folding occurs almost exclusively at the ER, an industrious system exists here to circumvent this potentially deadly situation and prevent the export of inadequately folded species. This process is termed "quality control" (Hammond and Helenius, 1995; Kopito, 1997), which involves 1) facilitation of protein folding, 2) recognition between native and non-native states, 3) selective export of native proteins, 4) retention of both pre-folded and aberrant forms, and 5) refolding or removal of defective conformers (Figure 1.3).

Newly synthesized proteins are prone to misfolding and are quickly disposed of by ER degradative processes (Schubert et al., 2000). This narrow window of achieving a stable state makes folding highly susceptible to environmental or genetic assaults (Liberek et al., 2008). Transmembrane proteins must also complete proper insertion through the Sec61 translocon complex as they are co-translationally inserted into the ER; during this process, general topology and some secondary structure are determined. However, topology can be further altered in the crucial, post-insertion folding stage that establishes tertiary and quarternery structures (Bowie, 2005). Membrane proteins are also subject to an added complexity of having domains that are exposed to multiple cellular environments: the cytosol, the hydrophobic lipid bilayer, and the ER lumen which is the topological equivalent of extracellular space. Thus, monitors for their folding must be present to recognize folding defects that can arise in these different settings. Currently, our understanding of membrane protein insertion and folding is still rudimentary.

The next sections are dedicated to three distinct but related processes that regulate ER quality control: 1) the action of folding factors or chaperones, 2) ER associated degradation, and 3) vesicular transport. Although some key players have been identified in each of these processes,

many substrate specific factors remain unknown. Another question is how these processes are coordinated to ensure the adequacy of secretory traffic. Examination of the underlying molecular mechanisms is crucial to avoiding the deleterious consequences brought about by protein misfolding.

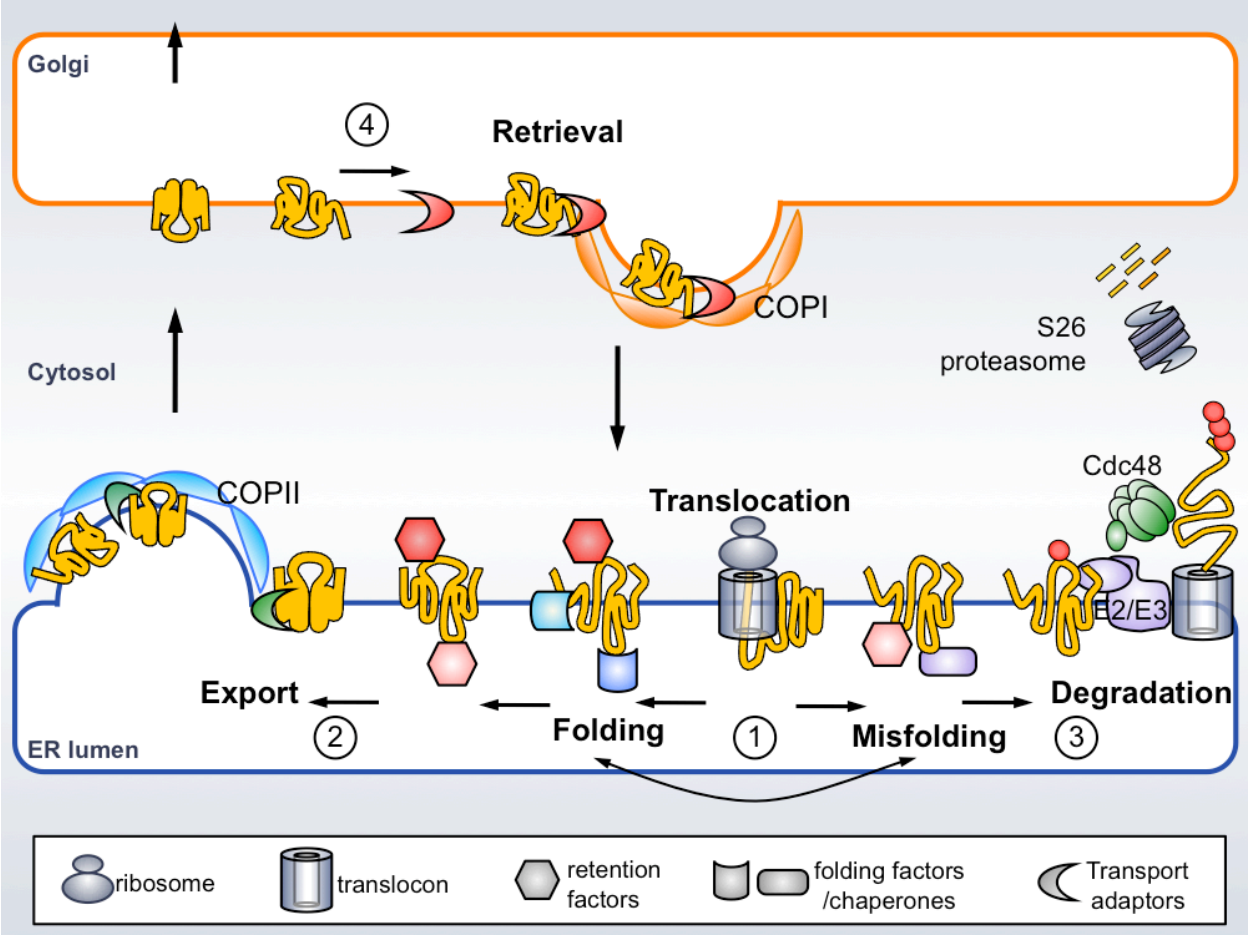

Figure 1.3. ER quality control
(1) Proteins entering the secretory pathway are synthesized from ER localized ribosomes and co-translationally inserted into the ER membrane through the Sec61 translocon. The subsequent folding process is aided by folding factors, as well as retention factors which retain folding intermediates. (2) Export in COPII coated vesicles occurs when proper export signals are exposed and recognized by COPII or COPII transport adaptors, and also when retention factors dissociate. (3) Terminal misfolding often leads to ubiquitination (red circle) by E2/E3 ubiquitin conjugating enzymes, and retrotranslocation, which is mediated by the Cdc48 complex and the translocon. The proteasome is responsible for final degradation of polyubiquitinated substrates in the cytosol. (4) At the cis-Golgi, properly folded proteins continue to distal compartments, while misfolded forms that have escaped ER quality control can be recognized by COPI or specific retrieval adaptors and returned to the ER in COPI vesicles for further rounds of folding, or degradation.

1.3.1. CHAPERONES AND FOLDING FACTORS

The traditional view of protein folding asserted that the primary amino acid sequence contains all required information for a protein to self assemble *in vitro*. However, it is now accepted that the precision and rates of folding demanded under physiological conditions in the crowded milieu of the ER lumen require other machinery such as folding enzymes and molecular chaperones (Ellis, 1993; Hebert and Molinari, 2007). Indeed, although chaperones do not reside only in the ER, their concentration is highest at this compartment. In addition to misfolded proteins themselves being causative disease factors, loss of function of accessory folding proteins has also been implicated in misfolding disorders (Kim and Arvan, 1998).

1.3.1.1. Definition of a molecular chaperone

Classically, molecular chaperones are defined as proteins that assist in folding and assembly by binding to their substrates, but are not part of the final functional structure (Ellis, 1993). They are typically distinguished from 1) true folding enzymes such as protein disulfide isomerase (PDI) and peptidyl prolyl isomerases (PPI), which catalyze rate limiting steps in folding, and 2) molecular escorts, which accompany substrates out of the ER (Kim and Arvan, 1998; Hebert and Molinari, 2007; Anelli and Sitia, 2008). This definition is not strictly adhered to in the literature, however, possibly because many chaperones are known to demonstrate overlapping functions. For example, PDI may serve chaperone-like functions independent of its role in disulfide bond formation (Wang and Tsou, 1993), and some chaperones like yeast Shr3 are proposed to have both folding and export functions (Gilstring et al., 1999). In general, however, chaperones bind reversibly to their substrates that have not completely folded, aid in the folding process through retention or preventing aggregation, but do not typically catalyze the attachment of covalent post-translational modifications (Ellis, 1993).

Chaperones are further classified based on the specificity of their action: “Generalist” or “public” chaperones function in primary quality control of nearly all newly synthesized proteins, and therefore have many substrates. “Specialist” or “private” chaperones act in secondary quality control, demonstrating high levels of substrate-specificity, and sometimes tissue-specificity in the case of mammalian cells (Ellgaard et al., 1999; Anelli and Sitia, 2008;).

1.3.1.2. General chaperones

Known as the "master regulator" of the ER, BiP (immunoglobulin binding protein, also glucose regulated protein Grp78, yeast Kar2) is one of the most abundant general ER chaperones, and the first that a nascent non-glycosylated polypeptide encounters in the ER lumen (Hebert and Molinari, 2007). It belongs to the family of 70kD heat shock proteins (Hsp70s), a large group of chaperones involved in refolding and proteolysis, conserved from prokaryotes to higher eukaryotes (Kim and Arvan, 1998). BiP influences various steps of protein quality control by binding different co-factors within the ER lumen. Its functions include translocation, folding and oligomerization, and dislocation of misfolded proteins that are targeted for ER associated degradation (Section 1.3.2) (Hebert and Molinari, 2007). BiP also binds hydrophobic patches that are exposed in the ER lumen, shielding these regions from forming inappropriate interactions and thus preventing aggregation of both soluble and membrane proteins (Fujita et al., 2006; Pety de Thozee and Ghislain, 2006). The expression of BiP is tightly controlled, and is upregulated under conditions of cellular stress such as the accumulation of misfolded proteins (Hebert and Molinari, 2007).

Aside from BiP, other members of the Hsp70 and Hsp90 families located in the cytosol bind misfolded or partially-folded membrane proteins, preventing their aggregation and facilitating ER associated degradation (Fewell et al., 2001; Picard, 2002; Morano, 2007). Like BiP, they have general functions as chaperones, although some members also appear to demonstrate considerable substrate specificity. For example, yeast Hsp90s are required for degradation of the misfolded plasma membrane ATP-binding cassette (ABC) transporter Yor1 (Pagant et al., 2007), but not the misfolded plasma membrane ATPase, Pma1-D378S (Han et al., 2007).

Another major class of classical ER chaperones includes the lectins calnexin (CNX) and calreticulin (CRT), which are required for folding and quality control of glycoproteins within the ER lumen (Helenius and Aebi, 2004). Although this process is not the focus of the work described here and may not apply to all proteins, it is nevertheless a well-studied system that highlights how post-translational modifications operate in ER quality control, and may thus provide paradigms for other systems.

1.3.1.2.1. N-Glycosylation and the calnexin cycle

Because glycoproteins represent a substantial portion of the proteins synthesized at the ER, their processing in this compartment has been thoroughly examined (Helenius and Aebi, 2004). The mechanisms of N-glycosylation, as well as pathways presiding over quality control of glycoproteins, are also conserved from yeast to humans (Zapun et al., 1999).

Newly synthesized glycoproteins at the ER are first modified with a preassembled oligosaccharide ($Glc_3Man_9GlcNAc_2$) that is attached to asparagine (Asn, N) residues at the sequence Asn-X-Ser/Thr. In the ER lumen, this core oligosaccharide is trimmed of two terminal glucoses by glucosidases I and II. This trimming results in an unfolded, monoglucosylated ($GlcMan_9GlcNAc_2$) protein that interacts with the lectin chaperones CNX and CRT, which have roles in folding and preventing aggregation. Release from CNX/CRT occurs when the final terminal glucose is removed by glucosidase II (producing $Man_9GlcNAc_2$). If properly folded and assembled, the glycoprotein is brought to sites of ER export, which is mediated by lectin adaptors such as ERGIC-53. Misfolded or partially folded forms are recognized by UDP-glucose glycoprotein glucosyltransferase (UGGT), which adds a single glucose back to the N-glycan. This once again produces a monoglycosylated protein which binds CNX/CRT, and the cycle repeats until the native state is achieved. A terminally misfolded protein could also break this cycle: after removal of the final glucose by glucosidase II, aberrant forms undergo further cleavage of mannose sugars by ER α-mannosidase I. The resulting $Man_8GlcNAc_2$ signals recognition by other lectins (eg. EDEMs, OS9) that target misfolded forms for degradation. Mannose trimming therefore has the important task of distinguishing between unfolded and terminally misfolded proteins (Helenius and Aebi, 2001; Anelli and Sitia, 2008). Mannosidases are known to be “timers” of folding, while UGGT controls re-entry into the CNX cycle for further folding. The binding to CNX/CRT ultimately determines the fate of the glycoprotein (degradation or export), but also prevents premature degradation of conformers that have not reached their final native state.

Quality control of glycoproteins is conserved between humans and the fission yeast *Schizosaccharomyces pombe*, but is slightly different in the budding yeast *Saccharomyces cerevisisae*. Although a similar situation exists in which the number and linkage of mannose residues affects proteasomal degradation, *S. cerevisiae* is considered to have a linear pathway

due to the absence of UGGT. A UGGT homolog, Kre5, is present in *S. cerevisiae*, but may not have a conserved function in quality control (Levinson et al., 2002). Interestingly, while CNX deletion in *S. pombe* and CNX or CRT null mice display lethal phenotypes, CNX and/or CRT in some mammalian cell lines is not required for viability due to tissue specificity. The *S. cerevisiae* CNX/CRT homolog Cne1 is also non-essential (Williams, 2006); however, it similarly functions as a glycoprotein chaperone (Xu et al., 2004), and retains misfolded glycoproteins such as the α pheromone receptor Ste2-3 at the ER (Parlati et al., 1995). Deletion of Cne1 may have a less global effect in *S. cerevisiae* due to the presence of other ER chaperones such as Pbn1 that also have vital roles in quality control (Subramanian et al., 2006).

The functions of CNX/CRT are not limited to glycoproteins, as they act on a variety of non-glycosylated proteins as well (Williams, 2006). For example, CNX was also shown to monitor folding of the polytopic and non-glycosylated proteolipid protein (PLP) by binding to misfolded PLP through its transmembrane domain (Swanton et al., 2003). The precise molecular mechanism of how CNX/CRT binding promotes folding is still unclear. Moreover, recognition signals and regulation mechanisms have not been elucidated for non-glycosylated proteins. It has also been suggested that BiP and CNX may form temporary scaffolds with other resident chaperones within the ER lumen to physically immobilize misfolded proteins (Nehls et al., 2000). However, more work is required to support this hypothesis.

That $BiP^{-/-}$, $CNX^{-/-}$, and CRT $^{-/-}$ mice suffer from embryonic or perinatal lethality is evidence of the importance and breadth of function of these general chaperones (Morano, 2007; Anelli and Sitia, 2008). In yeast, the essential protein Rot1 was also shown to be a general chaperone that may function in concert with BiP, acting on soluble, single-membrane-spanning, and polytopic substrates (Takeuchi et al., 2008). One additional specialized ER mechanism of primary quality control is the retention of proteins that have not properly formed disulfide bonds, a process monitored by the thiol oxidoreductase PDI (Sevier and Kaiser, 2002). Although important in ensuring proper protein conformation, this enzymatic folding process known as oxidative folding will not be covered in detail here.

1.3.1.3. Substrate-specific chaperones

Specialist chaperones that act in secondary quality control are highly heterogeneous, with roles from multimeric assembly to assisting in translocation or membrane insertion. They often act through binding to specific sequence features of their substrates (Ellgaard et al., 1999). For instance, the Wnt coreceptor subset of low-density lipoprotein receptors (LDLRs) requires the dedicated Mesd (mesoderm development) chaperone to avoid aggregation and to properly fold. These events are required for ER export and subsequent cell surface expression, which is crucial for Wnt signaling (Li et al., 2006). Hsp47 is an ER-lumenal chaperone specific to folding and assembly of pro-collagen molecules, and is implicated in fibrotic diseases (Taguchi and Razzaque, 2007).

Other substrate-specific chaperones function in both folding and cargo packaging. For example, folding and aggregation avoidance of LDLRs require the receptor-associated protein (RAP) chaperone, which also acts as an escort to the Golgi (van Anken and Braakman, 2005). In yeast, the Shr3 chaperone facilitates folding and export of a family of related polytopic amino acid permeases, although Shr3 itself does not exit the ER (Gilstring et al., 1999). Similar roles have been proposed for two other functionally similar yeast proteins Chs7 and Pho86, which act as specific ER chaperones for the polytopic chitin synthase Chs3 and phosphate transporter Pho84, respectively (Trilla et al., 1999; Lau et al., 2000). It is unclear at present how these mediate both folding and export processes at the molecular level, and whether they leave the ER as transport escorts.

The highly divergent and specialized actions of ER chaperones are not surprising, given that each protein has, theoretically, multiple ways to reach the native state. In addition, chaperone requirements are highly dependent on substrate-specific features such as size, hydrophobicity, disulfide bonds, oligomerization, and glycosylation, as well as cellular stress conditions. Currently, little is known about the interplay between these pathways, the specific signals chaperones recognize, and the precise molecular functions of many substrate-specific chaperones. It is clear, however, that proteins cannot reach a stable folded state *in vivo* by relying solely on their amino acid sequences. Identification and characterization of molecular chaperones is therefore essential in understanding the requirements of protein folding demanded by the ER.

1.3.2. ER ASSOCIATED DEGRADATION

Should proteins fail to be properly folded or assembled despite the various ER checkpoints, their continued presence would represent a cellular threat. These terminally misfolded proteins are eradicated in a process known as ER associated degradation (ERAD). Unlike other compartments such as mitochondria, however, the ER lumen is not resident to its own proteolytic enzymes (Raasi and Wolf, 2007), as this would likely cause massive and spurious degradation of newly synthesized, unfolded peptides, as well as folding intermediates. Consequently, ERAD exploits the cytosolic proteasomal system for protein disposal. The basic process of ERAD involves specific recognition and ubiquitination of misfolded substrates, their retro-translocation into the cytosol, and degradation by the cytosolic 26S proteasome (Figure 1.3). It has been hypothesized that up to 30% of newly synthesized proteins do not fold properly at the ER and are prematurely degraded by the proteasome (Schubert et al., 2000).

1.3.2.1. Ubiquitin-mediated proteasome degradation

All ERAD substrates characterized to date are ubiquitinated. Like glycosylation, mechanisms of ubiquitination and the proteins regulating this process are conserved from yeast to higher eukaryotes (Hebert and Molinari, 2007). The process of ubiquitination during ERAD is initiated with the activation of ubiquitin by an E1 ubiquitin activating enzyme. Ubiquitin is then transferred to E2 ubiquitin conjugating enzymes (Ubc6 or Ubc7), which are associated with the ER membrane. Finally, an E3 ubiquitin ligase (Hrd1 or Doa10) binds the ubiquitinated substrate and transfers ubiquitin from the E2 enzymes to lysine residues. The ubiquitin moiety itself also becomes modified at lysine 48, resulting in polyubiquitination. This signal is recognized by various receptors, and functions in both proteasomal-targeting and ER-to-cytosol dislocation, the latter of which requires the Cdc48-Ufd1-Npl4-Ubx2 complex (Elsasser and Finley, 2005; Sayeed and Ng, 2005; Nakatsukasa and Brodsky, 2008).

1.3.2.1.1. Substrate recognition

The recognition of terminally misfolded substrates for ERAD is a chaperone-mediated process. The general chaperones BiP and CNX/CRT and the protein disulfide isomerase PDI have been

implicated in recognition of lumenally misfolded proteins (Williams, 2006), often acting in conjuction with other cofactors. For example, the "gate-keeping" Yos9 lectin, which forms a complex with BiP, is also required for substrate selection in yeast; similar roles have been proposed for its two mammalian homologs Os-9 and XTP3B (Nakatsukasa and Brodsky, 2008). Yeast PDI and the Htm1 lectin are known to facilitate ERAD by capturing misfolded luminal domains through recognition of unbound cysteine residues and non-native N-glycans, respectively (Pety de Thozee and Ghislain, 2006). Although recognition of the N-glycan on misfolded glycoproteins is required for their disposal by ERAD, the existence of a critical or common recognition motif has not been elucidated for misfolded non-glycosylated proteins. It is likely that there are different pathways for recognition of non-glycosylated proteins (Nakatsukasa and Brodsky, 2008). For integral membrane proteins, cytosolic chaperones such as the Hsp70/Hsp40 system appear to be required. These substrates are ubiquitinated by the E3 Doa10 and the E2s Ubc6 and Ubc7 (Nishikawa et al., 2005).

Further substrate recognition is conferred by the E3 ligases, Hrd1 and Doa10, which belong to separate membrane protein complexes similar in basic composition (Figure 1.4). Both associate with the Cdc48 complex and share some parallel functions, but differ in substrates modified. The Hrd1 complex ubiquitinates lumenal and membrane proteins, whereas the Doa10 complex has a broader range of substrates, including polytopic, cytosolic, and nuclear proteins. Both complexes function with the Ubc7 E2; however, unlike Doa10, the Hrd1 complex does not typically require the Ubc6 E2 (Kostova et al., 2007). E3 enzymes have some overlapping specificity, but their utilization may also depend on the ERAD pathways activated. In particular, membrane proteins appear to enter different ERAD pathways depending on the location of their misfolding lesions (Vashist and Ng, 2004). Cytosolic lesions are recognized by the ERAD-C pathway, which degrades substrates through Doa10. Lumenal lesions are recognized by the ERAD-L pathway, which, similar to the ERAD of soluble proteins, employs the Hrd1 complex (Figure 1.4). Substrates displaying both cytosolic and lumenal lesions are subjected to ERAD-C. In this way, ERAD-C and ERAD-L are proposed to act sequentially, with ERAD-C being the first of two crucial checkpoints that must be passed prior to export (Vashist and Ng, 2004).

Due to the different locations of potential misfolding lesions, the mechanisms for recognition of misfolded membrane proteins are more complex than for soluble proteins. Details on how ERAD-C and ERAD-L differ mechanistically are still unknown. Recently, the existence of an

ERAD-M pathway for transmembrane lesions has been proposed, and appears to rely on the ERAD-L Hrd1 ligase but not the ERAD-L accessory proteins Der1 or Usa1 (Carvalho et al., 2006). Few substrates of ERAD-M are known, however.

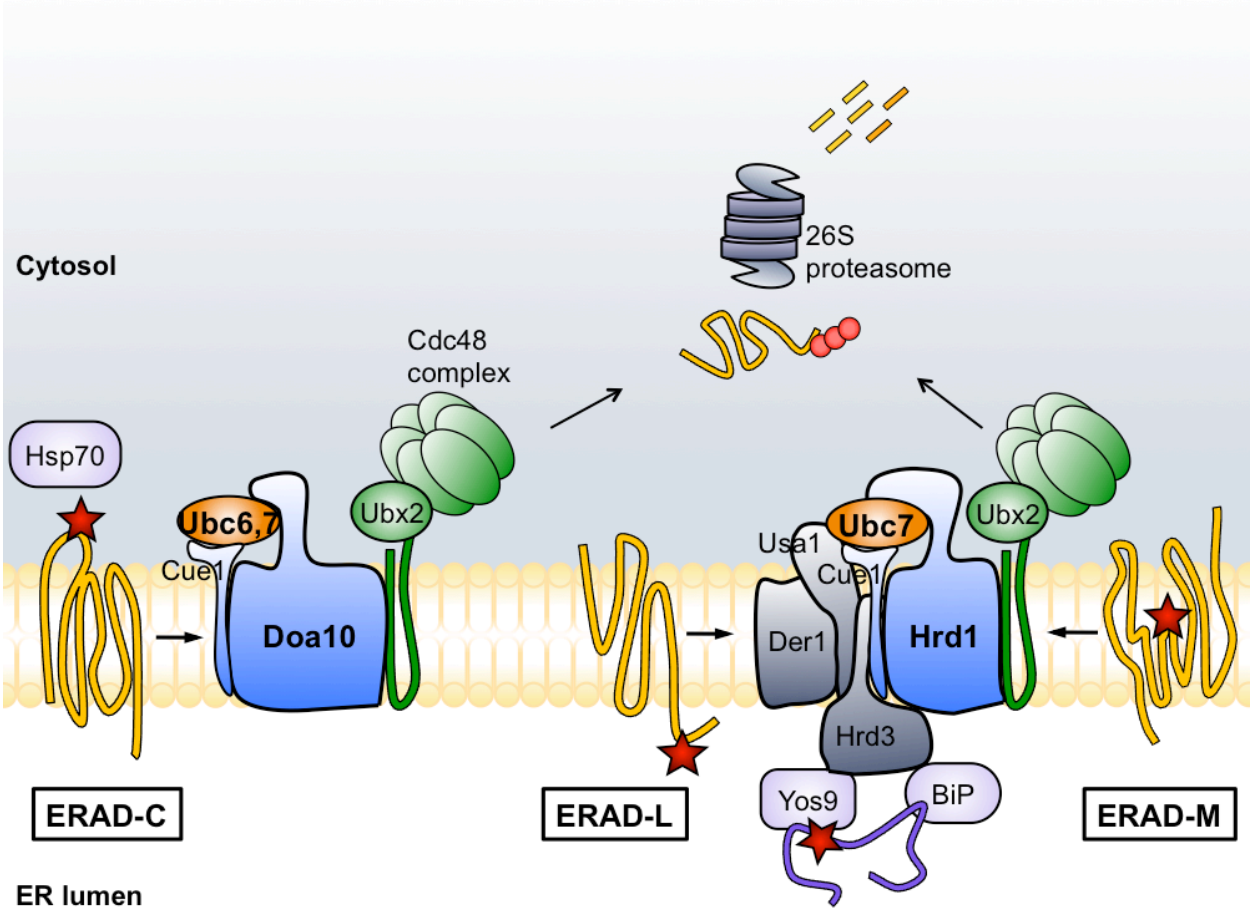

Figure 1.4. ERAD pathways for transmembrane proteins.
Transmembrane proteins displaying misfolding lesions (red stars) in the cytosol, ER lumen, and transmembrane domain are recognized by ERAD-C, ERAD-L, and ERAD-M, respectively. ERAD-C is monitored by the Doa10 complex, comprised of the Doa10 E3 ubiquitin ligase and Ubc6 and Ubc7 E2 ubiquitin conjugating enzymes. Substrates of ERAD-L and ERAD-M are ubiquitinated by the Hrd1 complex, made up of the Hrd1 ubiquitin ligase, Der1, Der3, and Usa1. Misfolded soluble substrates within the ER lumen (purple) are also recognized by the Hrd1 complex, which is less reliant on Ubc6. Cytosolic (eg. Hsp70) and lumenal chaperones (eg. BiP, Yos9) contribute to substrate recognition. Both Hrd1 and Doa10 complexes interact with the Cdc48-Ufd1-Npl4-Ubx2 complex, which mediates retrotranslocation of ubiquitinated (red circles) substrates. After retrotranslocation into the cytosol the misfolded protein is degraded by the 26S proteasome. (Adapted from Carvalho et al., 2006, and Ismail and Ng, 2006, with permissions.)

1.3.2.2. Deubiquitination in ER quality control

A crucial downstream process of proteasomal degradation is the removal and recycling of ubiquitin from substrate proteins, a process catalyzed by deubiquitination enzymes (DUBs) (Millard and Wood, 2006). Accordingly, initial studies on DUBs were focused on the few enzymes responsible for maintaining ubiquitin homeostasis. It has only become clear in recent years that many more potential DUBs are present in the mammalian genome, and their substrates vary in localization and function. The human genome has 84 DUBs, divided into five families, a major one being the 55 ubiquitin-specific proteases (USPs, or yeast UBPs). The yeast genome contains 21 DUBs, 16 of which are UBPs (Clague and Urbe, 2006). Currently, the substrates modified by many of these DUBs are unknown. Generally, the removal of ubiquitin from substrate proteins prevents their proteasomal and lysosomal degradation (Amerik and Hochstrasser, 2004). However, many membrane protein trafficking events, particularly plasma membrane internalization and endocytic sorting of signaling proteins and cell surface receptors, are regulated by ubiquitination (Hicke and Dunn, 2003), and consequently by deubiquitination (d'Azzo et al., 2005) (Figure 1.5). Deubiquitination can further affect protein sorting indirectly through regulating levels of vesicle transport machinery.

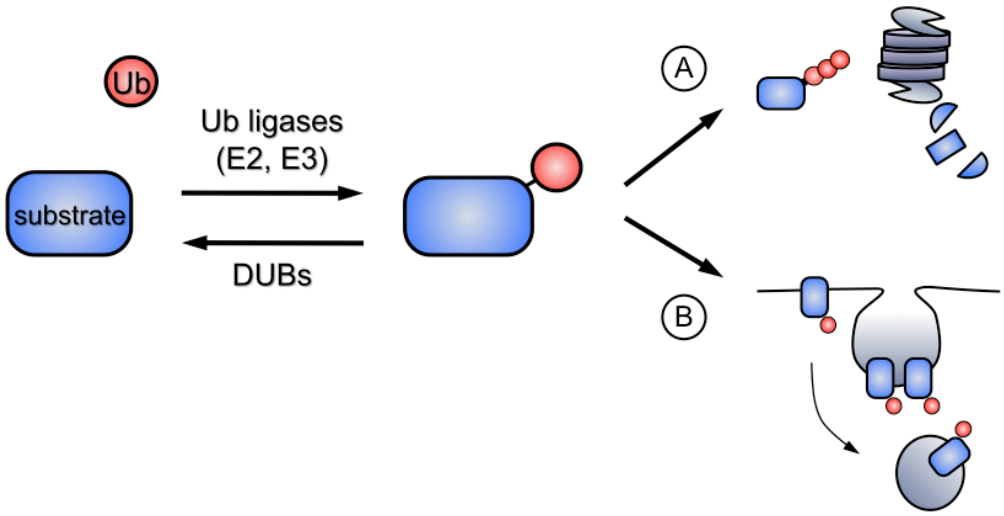

Figure 1.5. Mechanisms and consequences of ubiquitination.
Ubiquitinated substrates are modified through E2 ubiquitin conjugating enzymes and E3 ubiquitin ligases, and deubiquitinated by deubiquitination enzymes (DUBs). Often, ubiquitination can be a signal for proteasomal degradation (A), or can mediate intracellular trafficking events such as endocytosis (B). Ub (red circles), ubiquitin.

Ubiquitination/deubiquitination cycles can determine the degradative fate of misfolded substrates at the ER, in an analogy similar to glycosylation cycles in the ER lumen. For instance, the DUB Usp4 binds to the cytosolic C-terminus of the A_{2A} adenosine receptor ($A_{2A}R$), a G protein-coupled receptor (GPCR) with seven transmembrane domains (Milojevic et al., 2006). In the absence of Usp4, $A_{2A}R$ is ubiquitinated; this prevents its folding and leads to its degradation by the proteasome. Binding by Usp4 leads to $A_{2A}R$ deubiquitination and ER export, allowing proper cell surface expression (Milojevic et al., 2006). Usp4 shows high specificity for $A_{2A}R$, and it appears that $A_{2A}R$ undergoes some basal ubiquitination and retention under normal conditions, suggesting ubiquitination is important in regulating the maturation and transport of $A_{2A}R$. Thus, like the ligases that catalyze the addition of ubiquitin, DUBs have the potential to modulate the ubiquitination status of a protein, and therefore have garnered much attention in recent years.

1.3.3. OTHER QC PATHWAYS INDUCED BY MISFOLDING: UPR AND AUTOPHAGY

Persistent misfolding or the accumulation of aberrant species induces two other ER-related pathways important for quality control and degradation. One is the unfolded protein response (UPR), which involves the upregulation of molecular chaperones such as BiP and other folding factors. Not all misfolded proteins, however, activate UPR (Schroder, 2008). Another pathway is autophagy, a conserved ERAD-independent pathway for disposal of aberrant proteins. During this process, bulk degradation of damaged organelles occurs. Autophagy is usually induced during ER stress, and is also a target of UPR (Yorimitsu and Klionsky, 2007). Both UPR and autophagy are important for maintaining homeostasis and cell survival under conditions of stress brought about by the presence of misfolded proteins. They are, however, beyond the scope of this work.

1.3.4. VESICULAR TRANSPORT IN QUALITY CONTROL

Both folding intermediates and misfolded conformers targeted for ERAD must not be erroneously deployed beyond the ER. Aside from chaperones that physically retain their substrates, ER retention can also be achieved through the exclusion from COPII vesicles, or the retrieval of escaped proteins from the early Golgi. These selective vesicular transport pathways are mediated through interactions with transport adaptors and coats. In this way, vesicle trafficking systems can act as additional ER "barriers" for misfolded proteins.

1.3.4.1. Retention by COPII exclusion

Failure to be incorporated into ER export vesicles can happen through several mechanisms that are by no means exclusive. The masking of proper export signals, either due to the misfolding event itself or the association with retention factors, could prevent recognition by transport receptors or vesicle coats. Interestingly, the presence of an ER export signal does not necessarily guarantee COPII packaging. For example, an ER exit signal on some misfolded soluble proteins leads to export (Kincaid and Cooper, 2007), but on not the misfolded polytopic protein Yor1 (Pagant et al., 2007). Chaperones can further prevent export by physically segregating misfolded proteins from ER exit sites, or by binding to retention signals displayed by folding intermediates. Aggregation and inappropriate transmembrane domain length could also prevent entry into transport vesicles (Hendershot, 2000; Tu et al., 2006), and recent work suggests that transmembrane domain length and hydrophobicity could affect partitioning into sites of ER export independent of receptor-mediated mechanisms (Ronchi et al., 2008). Together, these observations indicate that the mechanisms of ER retention are complex, and not solely a consequence of not having proper export signals.

1.3.4.2. Retention by COPI retrieval

A more dynamic situation of retention can involve initial ER export followed by COPI retrieval at the early Golgi. Although the precise role of COPI in quality control is not clear, post-ER quality control pathways nevertheless have significant roles. Under stress conditions, for instance, the quality control intrinsic to the ER can become saturated, leading to the escape of

misfolded proteins. In addition, some lesions may not be recognized at the ER (Pety de Thozee and Ghislain, 2006). Several proteins are known to enter a recycling or retrieval pathway instead of being statically retained, and also require export prior to ERAD. For example, mutations that impair ER-to-Golgi transport delay ERAD of the soluble misfolded protein carboxypeptidase Y (CPY*) in yeast, although it was suggested that only a portion of CPY* recycles, and that disorganized ER structure or the mislocalization of the yeast BiP (Kar2) could also impair degradation (Taxis et al., 2002). The existence of recycling pathways is further supported by a reliance on Golgi retrieval factors. The Golgi-localized Erv29 retrieval adaptor is required for efficient ERAD of CPY* and misfolded proteinase A (PrA*) (Caldwell, 2001). Similarly, a misfolded form of hemagglutinin neuraminidase is packaged into COPII vesicles, and its retrieval and subsequent ERAD degradation depends on the Golgi-localized Bst1 adaptor, which may also retrieve CPY* (Vashist et al., 2001).

Like ERAD processes, the trafficking requirements that govern quality control of soluble and transmembrane proteins also differ. The role of Bst1 as an adaptor for retrieval of soluble proteins is not observed for the misfolded transmembrane protein, Ste6-166 (Vashist et al., 2001). At first, this observation suggested that retrieval pathways do not apply to transmembrane proteins, but it was later found that the sites of lesion (cytosolic or lumenal) might determine static or dynamic retention (Vashist and Ng, 2004). The Bst1 adaptor appears to be specific towards soluble substrates and transmembrane substrates that display lumenal lesions and signal ERAD-L (Vashist and Ng, 2004). Another Golgi-localized retrieval adaptor, Rer1, was also discovered to recognize misfolded transmembrane proteins by binding to specific features within transmembrane domains (Sato et al., 2004). Thus it would appear that ERAD-M substrates could also undergo retrieval.

Currently, both static retention and retrieval are believed to act in concert to achieve ER localization. There is however no clear consensus on the types of substrates or lesions that determine either retrieval or static retention. It was recently found that ERAD may also play a role in retention in addition to degradation by competing with the export machinery for binding to misfolded proteins (Kincaid and Cooper, 2007; Kota et al., 2007; Pagant et al., 2007). The precise interactions between degradation and trafficking pathways are not known, and require further study.

1.4. EMERGING ROLES FOR OTHER POST-TRANSLATIONAL MODIFICATIONS: LIPIDATION

As mentioned in previous sections, glycosylation and ubiquitination are two common post-translational modifications affecting folding and quality control at the ER, and therefore have been intensely studied. Other post-translational modifications (eg. terminal glycosylation, phosphorylation, sulfation), which are attached during transport through the Golgi, can also participate in protein function and targeting. However, they do not generally influence folding and conformation or ER export (Kim and Arvan, 1998). Another type of modification that can occur at the ER is lipidation, which is known to affect membrane attachment and hence protein sorting mainly at post-Golgi compartments. However, a few types of lipid modifications have been proposed to have roles at the ER.

1.4.1. TYPES OF LIPIDATION

A well-known ER lipidation event is the irreversible covalent attachment of glycosylphosphatidylinositol (GPI) to the C-terminus of some proteins (Figure 1.6), which, like glycosylation, occurs within the lumen. GPI anchored proteins must be deacylated before ER exit, and export of some GPI proteins require adaptor binding to the GPI anchor (Orlean and Menon, 2007). A properly deacylated GPI anchor may also be required for quality control of the misfolded Gas1* in yeast (Fujita et al., 2006); mutations in the GPI modification could bring about ER associated degradation through recruitment of chaperones such as BiP (Oda et al., 1996; Fujita et al., 2006). The role of the GPI anchor as a COPII exit signal is still debated, and ER export of GPI anchored proteins may differ from other secretory proteins as they are thought to be packaged into separate export carriers (Mayor and Riezman, 2004).

Other lipidation events are catalyzed in the cytosol: this includes N-myristoylation (attachment of 14-carbon myristate to N-terminal glycine through amide linkage), S-palmitoylation (attachment of 16-carbon palmitate to cysteines through thioester linkage), and prenylation (attachment of an isoprenoid – either 15-carbon farnesyl or 20-carbon geranylgeranyl – to a C-terminal cysteine through thioether linkage) (Figure 1.6). These lipidation events are recognized

for their roles in regulating membrane targeting at downstream compartments and the plasma membrane, but little is known about their roles in ER export (Resh, 2006a). N-myristoylation may negatively affect the ER membrane targeting and early insertion of NADH-cytochrome b5 reductase (Colombo et al., 2005). Lack of isoprenylation of the $\beta_1\gamma_1$ G protein subunits causes ER mislocalization and decreased targeting to the cell surface, although these subunits appear to be properly folded (Takida and Wedegaertner, 2003). Like GPI anchors, myristoylation and prenylation are irreversible modifications; however, palmitoylation is reversible, suggesting the potential for dynamic regulation of transport processes (Nadolski and Linder, 2007).

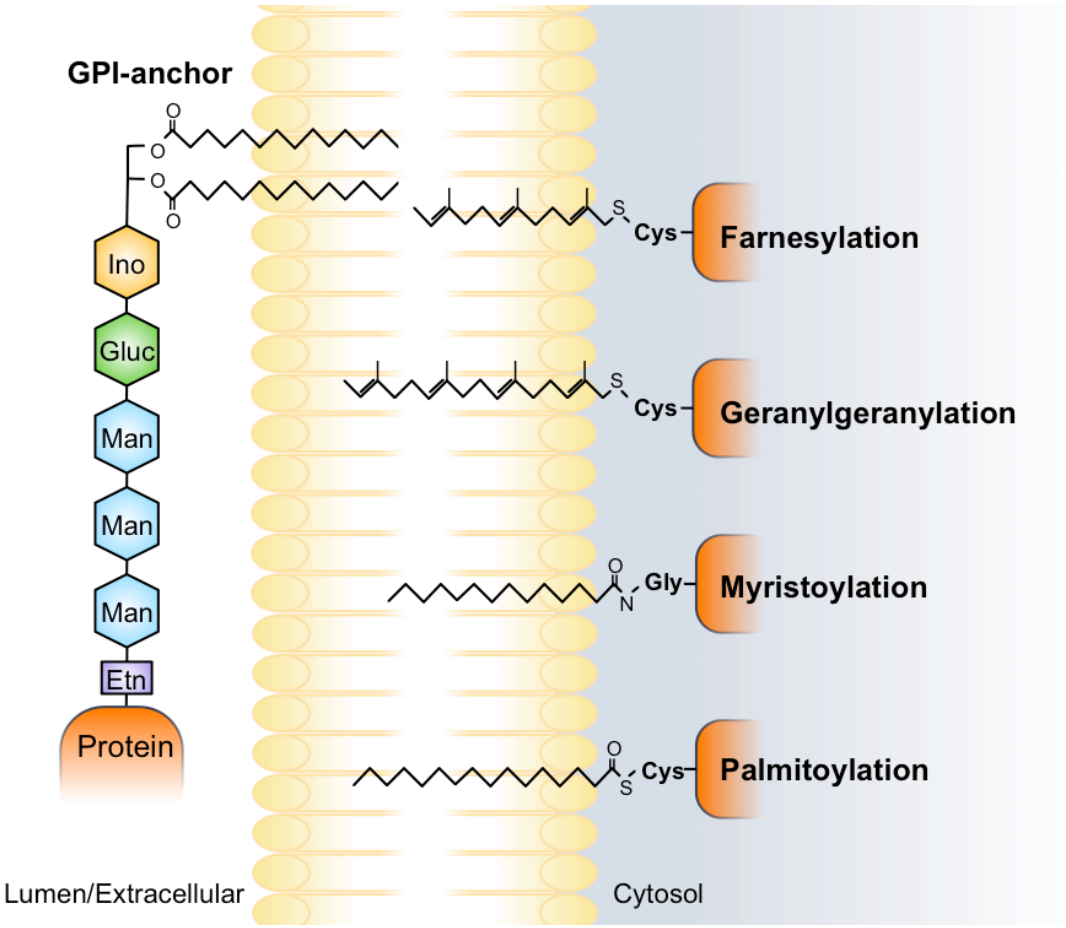

Figure 1.6. Common types of lipid modifications on proteins.
Structures of common lipid modifications that can be covalently attached to proteins (orange box). Farnesylation, the attachment of C15-farnesyl, and geranylgeranylation, the attachment of C20-geranylgeranyl, are both referred to as prenylation. They occur on C-terminal cysteines via thioether linkages. Myristoylation, attachment of C14-myristate, occurs at N-terminal glycines through amide linkages. All three lipidation events, like the lumenal/extracellular glycosylphosphatidylinositol (GPI) anchor, are irreversible modifications. Palmitoylation, the thioester linkage of C16-palmitate to cysteine residues, is reversible. Ino – inositol; Gluc – glucose; Man – mannose; Etn – ethanolaminephosphate. (Adapted from Mayor and Riezman, 2004, and Linder and Deschenes, 2007, with permissions.)

1.4.2. PALMITOYLATION

1.4.2.1. Functions

Palmitoylation has diverse cellular functions, and is known to affect transport of both soluble and membrane proteins at the plasma membrane, Golgi, and endosomes (Resh, 2006b). Especially in neurons, palmitoylation has a crucial role in regulating the trafficking of numerous postsynaptic neurotransmitter receptors to the synapse (Huang and El-Husseini, 2005). For example, $GABA_A$ receptor palmitoylation may affect its regulated transport from the late Golgi to the plasma membrane (Keller et al., 2004). Palmitoylation exerts its effects on protein sorting through several known mechanisms, the most recognized being its function as a membrane anchor. The saturated palmitoyl chain increases the hydrophobicity of proteins and thus their affinity for lipid bilayers, allowing for stable membrane association of not only soluble proteins, but also polytopic ones. For substrates such as Ras and G protein subunits, this is often through augmenting the actions of pre-existing lipid groups such as prenyl or N-myristoyl (Smotrys and Linder, 2004).

Palmitoylation is also known to affect protein activity independently of trafficking events. For example, it can influence protein structure through altering the packing of protein helices (Smotrys and Linder, 2004). There are several known examples in which protein-protein interactions, and consequently protein function, are affected through palmitoylation (Resh, 2006b). For instance, palmitoylation of some G proteins modulates protein interaction and receptor signaling (Chen and Manning, 2001), while palmitoylation of tetraspannins contributes to secondary interactions of tetraspannin complexes, which is also required for cell signaling (Hemler, 2005).

1.4.2.1.1. Sorting into lipid microdomains

Palmitoylation may also sort proteins into lipid and cholesterol enriched subdomains called lipid rafts, although this is still a matter of debate (Katzman and Longnecker, 2004; Abrami et al., 2006). Rafts are hypothesized to be platforms for signaling and transport through selective incorporation of lipids and proteins, and are often experimentally extracted as detergent resistant membranes (Ikonen, 2001). Palmitate is thought to confer an increased compatibility with the

highly ordered, tightly packed lipid environment of rafts, thus affecting the clustering and function of numerous signaling proteins such as G protein subunits and Ras (Smotrys and Linder, 2004).

There is increasing evidence that lipid rafts have trafficking functions not only at the plasma membrane, but also in the secretory and endocytic pathways. For example, sorting of several yeast polytopic membrane proteins at the Golgi and endosomes requires raft association (Bagnat et al., 2001; Umebayashi and Nakano, 2003). Proper transport of GPI anchored proteins to the cell surface also depends on raft incorporation in mammals and in yeast, which occur at the Golgi and the ER, respectively (Helms and Zurzolo, 2004). Although rafts are not believed to be present in the mammalian ER, a recent report showed that ER detergent resistant domains may have a protective function in folding of the prion protein PrP (Campana et al., 2006). Currently, both the *in vivo* existence of rafts and a positive role for palmitoylation in raft targeting are topics of much controversy in the field of membrane trafficking.

1.4.2.1.2. Roles at the ER

At the start of this thesis work in 2003, there had been no reports linking palmitoylation to ER export or quality control of membrane proteins (for example, see Resh, 2006). Palmitoylation of the soluble apolipoprotein B (apoB), a glycosylated protein, appears to mediate its interaction with the chaperone CNX. The modification may operate as an export signal by promoting dissociation from CNX and preventing apoB degradation, although it appears that non-palmitoylated apoB is properly folded (Vilas and Berthiaume, 2004). It was also found that proper cell surface targeting of some polytopic proteins, such as the GPCR delta opioid receptor (Petaja-Repo et al., 2006), and nicotinic alpha7 receptors (Drisdel et al., 2004), require palmitoylation events that occur at the ER. However, the modification does not appear to directly facilitate ER export (Drisdel et al., 2004; Petaja-Repo et al., 2006). Very recently, palmitoylation has been proposed to play a crucial role in transmembrane domain arrangement of the Wnt signaling protein LRP6 at the ER, preventing its aggregation and thus promoting export (Abrami et al., 2008).

1.4.2.2. Palmitoylation motifs

Consensus sequences are well defined for myristoylation (N-terminal glycine) and prenylation (C-terminal CaaX, CC or CXC motifs). However, aside from the requirement for the modified cysteine, no universal palmitoylation motif is known or is thought to exist. Proteins can be palmitoylated at single or multiple cysteines, and the modification is often, but not necessarily, found near transmembrane domains, at cysteine strings, or adjacent to other lipid modifications (Magee and Seabra, 2005; Nadolski and Linder, 2007). Recently, the Davis group reported that a family of yeast amino acid permeases could be palmitoylated at a shared C-terminal Phe-Trp-Cys (FWC) motif (Roth et al., 2006). The many potential locations on which a protein can be palmitoylated may be a reason why numerous cellular substrates and processes can be regulated by this lipid modification.

1.4.2.3. The DHHC family of protein acyltransferases

Although palmitoylation has been characterized for over 30 years, the existence of protein acyltransferases (PATs, also called palmitoyltransferases) remained elusive, since proteins can undergo spontaneous palmitoylation *in vitro* (Duncan and Gilman, 1996). However, it was observed that the kinetics of spontaneous palmitoylation were much too slow to account for observed *in vivo* rates, which prompted a search for PATs (Resh, 1999). The first PATs were only identified in yeast in 2002, with the discovery of Akr1, a PAT for casein kinases, and the Erf2/4 complex, a Ras PAT (Lobo et al., 2002; Roth et al., 2002). Both are involved in regulating transport of their substrates to the plasma membrane, and contain a highly conserved Asp-His-His-Cys (DHHC) cysteine-rich domain. Subsequent characterization of yeast and mammalian DHHC proteins suggested that this is a signature motif for a family of PATs with diverse subcellular localization and substrates (Linder and Deschenes, 2004). Several mammalian DHHC PATs characterized to date affect trafficking at the synapse. For example, the Akr1 homolog HIP14, a huntingtin interacting protein, is a PAT for various neuronal proteins, including SNAP-25, PSD-95, and huntingtin (Huang et al., 2004), while GODZ is the PAT for the γ subunit of $GABA_A$ receptors (Keller et al., 2004).

Many of the 23 mammalian DHHC PATs and their *in vivo* substrates remain uncharacterized and are currently under intense study. The recognition sites for PATs, which are currently unclear, are likely to differ greatly due to a lack of consensus sequences for palmitoylation. Three yeast and twelve mammalian DHHC PATs are ER-localized (Ohno et al., 2006), suggesting that palmitoylation could potentially regulate ER trafficking events. Discovery of PAT substrates and functions are key goals in understanding the many biological processes that are affected by this lipid modification.

1.5. MISFOLDING AND ER RETENTION IN HUMAN DISEASE

It is apparent that many safeguards are imposed by the ER to ensure the adequacy of secretory traffic. Protein misfolding at the ER, ultimately culminating in intracellular retention and degradation of many cell surface proteins, can be the underlying basis of many genetic diseases (Aridor and Hannan, 2002; Hebert and Molinari, 2007; Anelli and Sitia, 2008). In general, these diseases can be grouped into two categories that are not always mutually exclusive: 1) inherent mutations in a protein that causes misfolding and 2) defective processing proteins (Brooks, 1997).

1.5.1. DISEASES CAUSED BY MISFOLDED PROTEINS

Mutations leading to misfolding can cause disease through loss-of-function or toxic gain-of-function effects. The former is typically observed when mutant proteins are retained and/or degraded, and consequently absent from their sites of function (eg. plasma membrane). Well-known examples of ER-retained membrane proteins include the cystic fibrosis transmembrane conductance regulator (CFTR) in cystic fibrosis (CF), LDL receptor in familial hypercholesterolemia, and V_2 vasopressin receptor (V_2R) in recessive nephrogenic diabetes insipidus (NDI) (Aridor and Hannan, 2002). CF is caused by the absence of CFTR, a chloride channel, on the cell surface of lung epithelia. The misfolded ΔF508-CFTR mutant, which represents 70% of CF cases, is completely ER-retained despite being largely functional. Interestingly, even wild-type CFTR folds inefficiently and a large majority is degraded via ERAD (Brodsky, 2007), further signifying the vulnerability of this protein to events that can adversely influence folding. For recessive NDI, over 90% of cases involve mutations in the V_2R G-protein coupled receptor, many of which result in misfolding and ER retention. Absence of V_2R at the plasma membrane leads to a failure in activating signaling pathways that are required to concentrate urine in response to the vasopressin hormone (Morello and Bichet, 2001). V_2R is one of many GPCRs whose cell surface expression is increased by palmitoylation (Sadeghi et al., 1997).

In the second class of mutations, dominant effects are observed when misfolded proteins accumulate inside or outside cells, leading to ER stress and eventually cell death. CF represents

an interesting example here: although generally considered a loss-of-function disease, under some stress conditions the aggregation of CFTR variant proteins can increase, leading to formation of toxic aggresomes, and a gain-of-function effect (Gregersen, 2006).

1.5.2. DISEASES CAUSED BY DEFECTIVE PROCESSING

Defects in various processing proteins such as chaperones, degradation machinery, and transport factors can also be underlying mechanisms of disease. General chaperones such as CNX, CRT and BiP have been implicated in a variety of disease phenotypes due to their wide-range of substrates (Anelli and Sitia, 2008). Notably, CNX binds mutant ΔF508-CFTR, which may lead to its ER retention (Brooks, 1997). Defective BiP function, leading to misfolding of α-1-procollagen, is implicated in osteogenesis imperfecta (Aridor and Hannan, 2000). Substrate-specific chaperones have also been implicated in disease. For example, high levels of the collagen-specific chaperone HSP47 causes excessive assembly and processing of procollagen. This leads to an accumulation of collagens and can contribute to the formation of fibrotic lesions (Taguchi and Razzaque, 2007).

Mutations in the ERAD pathway are known to impair degradation of misfolded toxic species. For example, mutations in the E3 ligase Hrd1 have been linked to Parkinson's disease, rheumatoid arthritis, and diabetes, due to defective recognition and targeting of misfolded glycoproteins for ERAD (Anelli and Sitia, 2008). Currently, however, there are no known genetic disorders in which ubiquitin or the proteasome has been mutated (Jiang and Beaudet, 2004).

Finally, faulty transport components can also result in ER retention of otherwise wild-type proteins. One example is the ERGIC-53 cargo receptor, a lectin that cycles between the ER and Golgi by interacting with COPII and COPI. Missense mutations in ERGIC-53 lead to a block in the secretion of factor V and VIII, and the development of combined factors V and VIII deficiency, an inherited bleeding disorder (Baines and Zhang, 2007).

Due to the many factors that govern folding and transport of any particular protein, it is conceivable that a disease phenotype could be caused by any one, or a combination, of the above mentioned mechanisms. Therefore, understanding how proteins pass the strict ER folding surveillance system, avoid degradation, and achieve transport competency is central to the knowledge of ER storage disease development. Insight into these mechanisms can be gained through studying different model proteins in organisms with conserved pathways and machineries of transport and quality control.

1.6. MODELS FOR STUDYING ER TRANSPORT AND QUALITY CONTROL

Perhaps no other single cellular eukaryote has been as extensively exploited for studying essential biological pathways as the budding yeast *Saccharomyces cerevisiae.* In addition to being easily accessible and having a low generation time, yeast also share many basic cellular mechanisms and proteins with mammalian cells. As a result, many yeast proteins have been used as paradigms to understand vesicle trafficking and quality control processes in higher cells.

1.6.1. YEAST FUNCTIONAL GENOMICS TO MODEL TRAFFICKING PROCESSES

This work takes a yeast functional genomics approach to study intracellular transport and quality control of polytopic proteins, which is advantageous for several reasons. First, the yeast genome has been completely sequenced, and a collection of ~4800 yeast mutant strains, comprised of systematic knock-out disruptions of all non-essential genes, is available in haploid (MATa and MATα), homozygous diploid and heterozygous diploid strains (Giaever et al., 2002). Although a portion (<30%) of yeast genes are poorly characterized or have unknown functions, the deletion collection has allowed for exhaustive "reverse genetics" investigations, leading to the discovery of novel components in genetic pathways and protein complexes (Scherens and Goffeau, 2004). Moreover, collections other than the knock-out set have also been completed in recent years, allowing for studies of overexpression, localization, protein-protein interactions, and essential gene function (Boone et al., 2007). Additionally, both vesicle transport machinery (Schekman and Orci, 1996) and ER folding and degradation pathways (Hebert and Molinari, 2007) are highly conserved from yeast to mammals, with many proteins involved in these processes being first discovered in yeast. The mechanisms learned from yeast can therefore be subsequently applied to mammalian cells. Finally, *S. cerevisiae* is easy to manipulate for cell biological and biochemical studies, providing an overall robust system for functional genomics.

1.6.2. MODEL MEMBRANE PROTEINS

Several yeast cell surface transporters have been used as models to study trafficking and quality control of polytopic proteins. For example, misfolded versions of the polytopic ABC transporters Ste6 and Yor1, known ERAD substrates, are often used as models for ΔF508-CFTR (Brodsky, 2007; Pagant et al., 2007). Several polytopic amino acid permeases, such as the general amino acid permease Gap1 and the tryptophan permease Tat2, have been invaluable for understanding how modifications such as phosphorylation and ubiquitination affect ligand-dependent transport (De Craene et al., 2001; Horak, 2003). As previously mentioned, Gap1 also requires the specific action of an ER chaperone Shr3 for folding and export, and is subjected to ERAD when misfolded (Gilstring et al., 1999). A misfolded mutant of the plasma membrane ATPase, Pma1, is often used as a marker for ER subdomains. Its characterization has led to insights into folding requirements, chaperone functions in ERAD, and the discovery of novel transport machinery, for example (Ferreira et al., 2001; Han et al., 2007). Together, these different yeast model proteins have provided much information on the basic mechanisms of polytopic protein folding, quality control, and transport at the ER as well as in post-Golgi compartments.

The development of additional models is therefore a valuable pursuit, especially if these model proteins are also polytopic but not necessarily transporters. This is because similar trafficking or quality control pathways observed in a protein with a divergent function may reveal basic, conserved mechanisms. At the same time, there is also potential to uncover alternative and novel pathways. The work described in this thesis uses another protein in yeast, the chitin synthase Chs3, to study these processes. In contrast to many proteins whose cell surface levels are regulated through increased protein production followed by rapid endocytosis and degradation, a distinguishing feature of Chs3 sorting is that it is kept in stable intracellular storage at the TGN and endosomes until an extracellular stimulus is received. In this way, Chs3 shares many similar trafficking patterns with the mammalian glucose transporter GLUT4, which is stored in a stable compartment until stimulation from insulin (Bryant et al., 2002), and with the vasopressin-regulated aquaporin-2 water channel (Knoers and Deen, 2001). The lack of cell surface presentation of these proteins leads to Type II diabetes and a rare recessive form of NDI, respectively (Knoers and Deen, 2001; Bryant et al., 2002). Thus, Chs3 can be used as a model for understanding the stimulus-driven translocation of membrane proteins from intracellular storage (Ziman et al., 1998), which was the original impetus for selecting this protein.

1.6.3. CHITIN SYNTHASE 3: FUNCTION AND REGULATION

Chs3 is a ~130kD protein with six to eight predicted transmembrane domains, and is responsible for synthesizing ~90% of cellular chitin, a component of the yeast cell wall that is deposited in the lateral walls and concentrated at the budneck and septum (Chuang and Schekman, 1996; Lesage and Bussey, 2006). Chs1 and Chs2 synthesize the remaining chitin, mainly during cytokinesis for septum repair (Lesage and Bussey, 2006). Until an external stimulus such as cell stress induces its transport to the cell surface, Chs3 is kept in stable intracellular storage: at steady state, approximately 50-70% of Chs3 is located in the trans-Golgi network or early endosomes (Santos and Snyder, 1997). It has been proposed that these internal reservoirs are possibly specialized endosomal compartments called "chitosomes" (Chuang and Schekman, 1996; Ziman et al., 1996).

In response to perturbations to the cell wall, the redistribution of Chs3 is induced from these internal compartments, leading to a 20 fold increase of chitin deposited at the cell surface (Garcia-Rodriguez et al., 2000; Valdivieso et al., 2000). Inactivation of Chs3 is through endocytosis; however, the protein does not become degraded but instead recycles in chitosomes (Valdivia et al., 2002). Although its abundance at the plasma membrane varies during the cell cycle and under different conditions, Chs3 levels are constant throughout the cell cycle (Ziman et al., 1996). Additionally, an increase in Chs3 protein levels does not result in a concomitant rise in activity levels of the synthase, nor does higher activity necessitate an increased amount of protein (Choi et al., 1994; Cos et al., 1998). These observations further implicate roles for post-translational modifications and protein interactions in spatial regulation. Indeed, a proteomics approach revealed that Chs3 is both ubiquitinated and phosphorylated (Peng et al., 2003), but the *in vivo* consequences of these modifications are not known.

1.6.4. FACTORS AFFECTING CHS3 TRANSPORT

Chs3 trafficking between the late Golgi/endosomes and the cell surface is regulated tightly by the cell's response to extracellular stress. The protein kinase C (PKC) cell integrity pathway (Figure 1.7), one of five yeast mitogen activated protein (MAP) kinase cascades, is involved in this response (Valdivia and Schekman, 2003). Several trafficking proteins are also required for

proper transport or activation of Chs3 at specific cellular locations, although not all of their molecular functions have been elucidated (Figure 1.7). Chs7 mediates the ER exit of Chs3, likely by acting as a folding chaperone (Trilla et al., 1999); Chs5 and Chs6 regulate export from the TGN (Santos and Snyder, 1997; Ziman et al., 1998), possibly as a specialized coat (Wang et al., 2006). Chs3 acquires proper plasma membrane targeting and activation through Chs4 and Bni4 (DeMarini et al., 1997; Ono et al., 2000), and End4 is required for endocytosis into endosomes (Ziman et al., 1998). The recycling between TGN and early endosomes requires the clathrin adaptor complex AP-1 (Valdivia et al., 2002). The protein kinase Pkc1 appears to be responsible for phosphorylation of Chs3, although it is not clear whether phosphorylation regulates redistribution of Chs3 to the plasma membrane (Valdivia and Schekman, 2003). It is not known how the signaling and vesicle transport machinery converge molecularly, and whether other factors are required for regulating Chs3 transport. Moreover, aside from Chs7, no other ER export and quality control factors, or the signals for ER exit, are known.

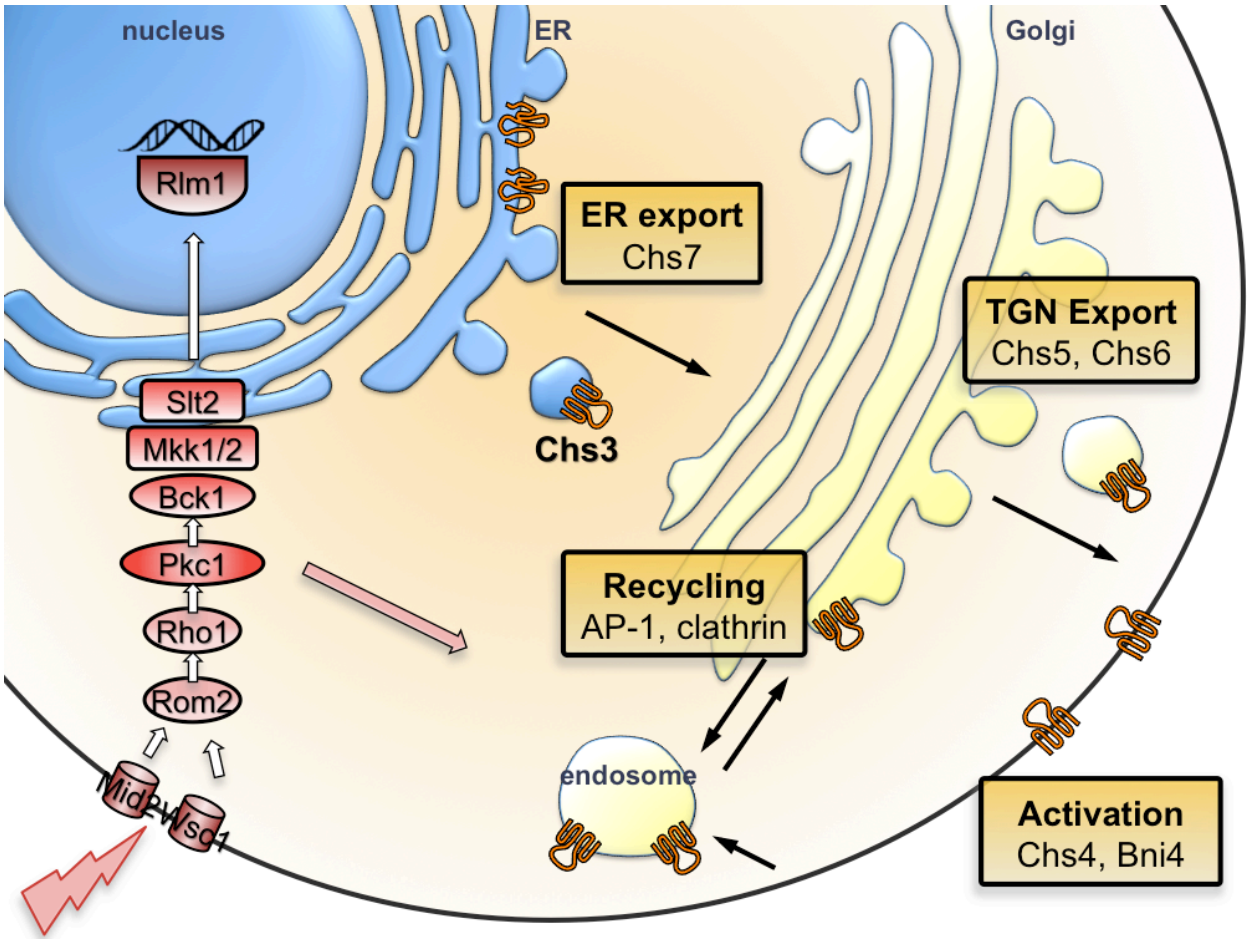

Figure 1.7. Chs3 transport factors
Several compartment-specific proteins are known to affect Chs3 trafficking. The export of Chs3 from the ER requires Chs7, a chaperone important for folding. At the trans-Golgi network (TGN), Chs5 and Chs6 form a specialized vesicle coat for export to the cell surface. Chs4 and Bni4 are involved in plasma membrane activation and recruitment of Chs3 to the budneck. The clathrin adaptor protein complex AP-1 is required for Chs3 recycling between endosomal compartments and the TGN, where most of Chs3 is stored at steady state. Chs3 cell surface levels are also affected by the cell integrity signalling pathway (red). Cell surface stress sensors such as Mid2 and Wsc1 activate the protein kinase C (PKC) mitogen actived protein (MAP) kinase cell integrity pathway through Rom2, a guanine exchange factor (GEF) for the Rho1 GTPase. Rho1 triggers the MAP kinase cascade composing of the protein kinase Pkc1, the MAP kinase kinase kinase (MEKK) Bck1, the MAP kinase kinases (MEK) Mkk1/2, and the MAP kinase Slt2. Activated Slt2 phosphorylates the Rlm1 transcription factor, which increases expression of cell-wall specific genes, including Chs3. Pkc1 and Rho1 also increase cell surface translocation of Chs3 from Golgi/endosome compartments through unknown mechanisms.

1.7. THESIS SUMMARY

1.7.1. OBJECTIVES AND HYPOTHESIS

Our current knowledge of how misfolded membrane proteins are retained or degraded through the combined actions of chaperones, retention factors, degradation machinery and vesicle transport proteins is still rudimentary. At present, one major unsolved question in ER quality control is how proteins achieve a folded, export competent state through the aid of substrate-specific factors and post-translational modifications such as ubiquitination and lipid modifications. In addition, signals required for vesicle transport to and from the ER remain obscure for many membrane proteins. More generally, many factors affecting downstream processes in regulated transport to the cell surface have not been elucidated. Before effective, targeted molecular therapies for treating retention or misfolding diseases can be developed, it is crucial that we gain a more fundamental knowledge of these processes.

The spatial regulation of the yeast chitin synthase Chs3, used in this study as a model for polytopic protein trafficking, has not been well characterized. At the start of this work, the only known ER trafficking factor was the chaperone Chs7, whose precise function was still unclear. Many late secretory and endocytic trafficking factors were also unknown. Moreover, aside from large scale studies showing Chs3 is modified by ubiquitination and phosphorylation, little was known about how its trafficking is post-translationally regulated. Finally, despite being important determinants for membrane association and protein sorting, the role of the lipid modifications specifically in ER transport of polytopic proteins has not been thoroughly investigated. The main goal of this work was therefore to expand the knowledge on the trafficking of this model protein. This was achieved by using genomic methods to uncover novel transport factors, followed by their characterization at the cell and molecular level. This work demonstrates that palmitoylation is important for quality control and ER export of Chs3 by facilitating its folding, and that palmitoylation-deficient Chs3 is ER retained through previously uncharacterized pathways that can be affected by the loss of a deubiquitination enzyme.

1.7.2. SYNOPSIS

My first objective was to identify novel proteins required for the cell surface transport of Chs3. To this end, a genomic screen was developed for Chs3 trafficking factors. This led to the discovery that Chs3 is palmitoylated by a DHHC PAT, Pfa4, and this modification was required for ER export (Chapter 2). Subsequently, I continued the theme of this work to understand how palmitoylation-deficient Chs3 is ER-retained, again starting with a genomics approach. The screen for ER-retention factors of unlipidated Chs3 revealed that the deubiquitination enzyme Ubp3 may be regulating retention through both static and retrieval mechanisms (Chapter 3). These findings raise questions about alternate pathways and factors in ER quality control. They may also provide insights into molecular mechanisms of diseases caused by protein misfolding and retention (Chapter 4).

1.7.3. SIGNIFICANCE

Defects in ER transport and quality control can have numerous influences on human disease. It is clear that different routes and machinery exist to avoid aberrant folding and ensure proper cell surface expression, and that there is considerable modulation through post-translational modifications. The work in this thesis points to a novel role for palmitoylation in folding and transport of polytopic proteins, and possible mechanisms for overcoming the lack of this modification. For pathologies involving protein misfolding and intracellular retention, model systems can contribute to a better understanding of the basic mechanisms and development of disease, and in the long term provide avenues for pharmacological interventions.

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CHAPTER 2. PALMITOYLATION BY THE DHHC PROTEIN PFA4 REGULATES THE ER EXIT OF CHS3 [1]

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2.1. INTRODUCTION

The ER is an important quality control site within the cell, where proteins destined for secretion or for sorting to post-Golgi organelles are monitored for proper folding and oligomeric assembly. Proteins that fail to fold or assemble are typically retained in the ER and, in some cases, retro-translocated to the cytoplasm for proteosomal degradation (Meusser et al., 2005). Polytopic membrane proteins receive particular scrutiny in this regard. Indeed, many diseases are attributable to the failed ER export of mutant transmembrane proteins (Schulein, 2004).

Yeast genetic analyses have identified a number of ER resident proteins that mediate the ER export of specific polytopic membrane proteins. For instance, Shr3 is required for the ER export of the yeast amino acid permeases. In *shr3*Δ mutants, these permeases are retained in the ER, whereas the transit of other polytopic proteins is unimpaired (Gilstring et al., 1999). Similarly, Gsf3, Pho86, and Chs7, which are unrelated to Shr3 at the sequence level, are specifically required for the ER export of the hexose transporter Hxt1, the phosphate transporter Pho84, and the chitin synthase Chs3, respectively (Kota and Ljungdahl, 2005). These export factors have been suggested either to direct the segregation of their target proteins into budding COPII vesicles for anterograde transport or, alternatively, to act as dedicated chaperones, regulating proper protein folding prior to transport.

The yeast chitin synthase Chs3, a polytopic protein with 6 to 8 predicted transmembrane domains, provides a genetic model for understanding mechanisms of transport through the secretory pathway. Chs3-mediated chitin deposition at the plasma membrane is highly regulated at the level of intracellular trafficking. Chs3 is maintained in an intracellular pool at steady state that may correspond to the TGN or endosomes (Ziman et al., 1996), and transported to the plasma membrane upon activation of the *BCK1-SLT2* cell integrity signaling pathway (Valdivia and Schekman, 2003). Mutants that impair cell wall chitin deposition have been found to block the plasma membrane delivery of Chs3 at different intracellular transport steps. Whereas Chs7 mediates the ER export of Chs3, Chs5 and Chs6 direct its transport from the TGN to the plasma membrane, and Chs4 is required both for Chs3 activity at the cell surface and for its localization at the bud neck (reviewed in Roncero, 2002). Here, we describe a genomic analysis of factors that regulate the transport of Chs3 to the cell surface, and identify an unexpected role for protein palmitoylation in the ER export of Chs3.

Palmitoylation, the thioester linkage of palmitate to selected cysteine residues, is one of several lipid modifications used for tethering proteins to membranes (Bijlmakers and Marsh, 2003). For transmembrane proteins, which are already embedded in the bilayer, the functional consequences of palmitoylation are not clear, though a role in directing segregation to membrane microdomains (lipid rafts) is often invoked. Enzymes for protein palmitoylation, protein acyl transferases (PATs), were identified only recently by work in yeast (Lobo et al., 2002; Roth et al., 2002). The first two PATs to be characterized, Akr1 and Erf2, were found to contain a conserved zinc finger-like Asp-His-His-Cys (DHHC) cysteine-rich domain, suggesting this motif defines a larger PAT family. Yeast has a total of seven DHHC proteins, while 23 are identifiable from the human genome. More recent reports have linked additional DHHC proteins to the palmitoylation of various substrates in both yeast and mammalian cells (reviewed in Mitchell et al., 2006). In this study, we find the uncharacterized yeast DHHC protein Pfa4 to be required for ER export by acting as the dedicated PAT for Chs3 palmitoylation.

2.2. RESULTS AND DISCUSSION

2.2.1. CHS3 CELL SURFACE ACTIVITY REQUIRES THE DHHC PAT PFA4

To identify additional genes required for Chs3 trafficking, we developed a fluorescence assay suitable for the large-scale screening of yeast deletion arrays, based on the binding of the fluorescent antifungal drug Calcofluor white (CW) to cell wall chitin (Fig. 2.1A) (Roncero and Duran, 1985). Mutants that are defective for the transport of Chs3 to the cell surface produce little chitin, and thus exhibit low levels of fluorescence. We screened three independent gene deletion collections in duplicate, and calculated the median fluorescence intensity for each strain. As expected, the mutants with the lowest fluorescence values included *chs3*Δ cells, which lack chitin synthase III, and strains deleted for the known Chs3 transport factors *CHS4-7* (Fig. 2.1B). In addition, the screen identified *slt2*Δ and *bck1*Δ, components of the cell integrity pathway that stimulates the cell surface transport of Chs3, indicating that colony fluorescence values correlate well with independent measures of cell wall chitin (Lesage et al., 2005). Unexpectedly, cells deleted for the uncharacterized ORF YOL003c (*PFA4)* consistently displayed fluorescence values comparable to *chs3-7* mutants (Fig. 2.1B, C). Pfa4 is predicted to be a 45 kDa protein containing the signature DHHC cysteine-rich domain that has been linked to protein palmitoylation (Bijlmakers and Marsh, 2003). Like *chs* mutants, *pfa4*Δ cells not only bind less CW, but are also strikingly resistant to CW toxicity (Fig. 2.1C).

2.2.2. PFA4 IS REQUIRED FOR THE ER EXIT OF CHS3

To determine if the low levels of cell wall chitin in *pfa4*Δ mutants result from alterations in the intracellular transport of Chs3, we examined the subcellular localization of Chs3-GFP in *pfa4*Δ strains and in mutants with known Chs3 trafficking defects. In wild-type cells, Chs3-GFP is present at the bud neck, bud tip, and intracellular compartments, whereas it is completely restricted to this latter compartment in *chs6*Δ mutants, consistent with a mislocalization to the

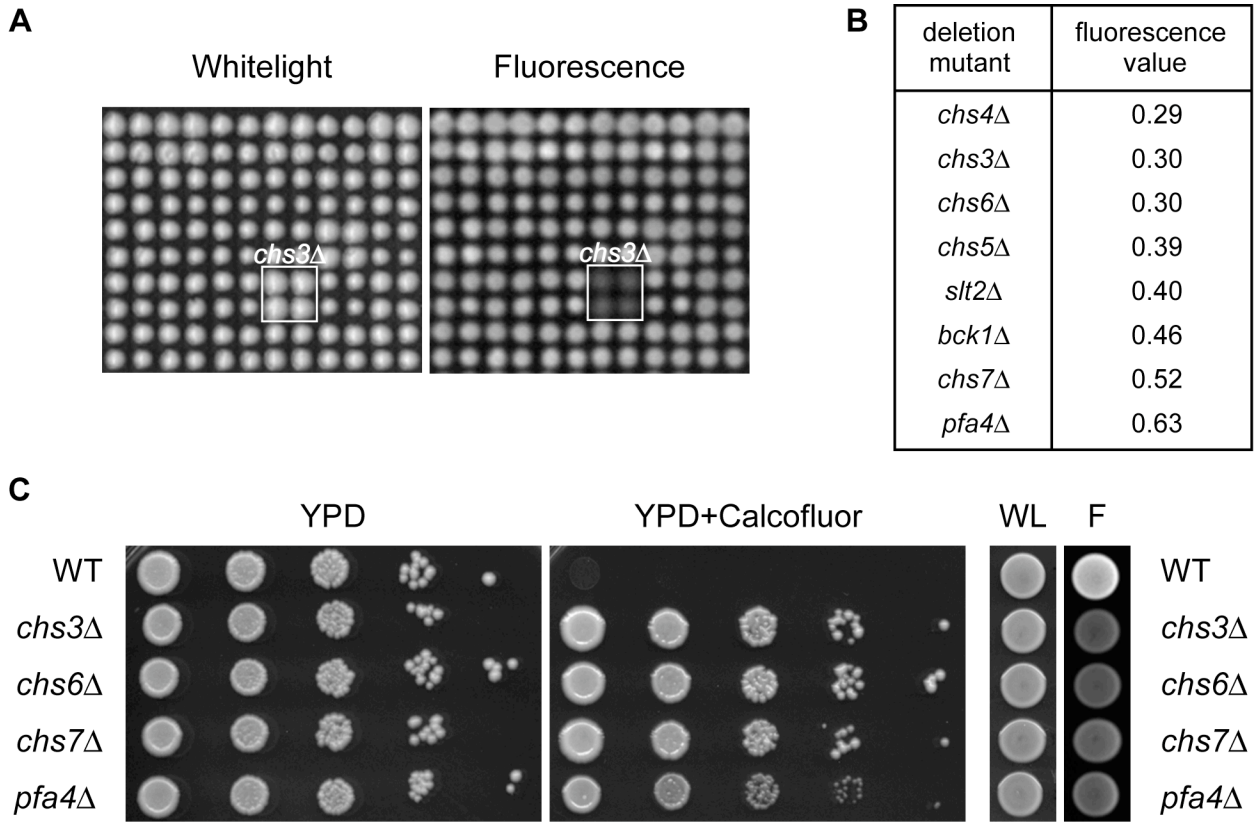

Figure 2.1. Pfa4 mutants show reduced fluorescence on CW media.
(A) Representative white light and fluorescence images showing part of a MATα yeast deletion array grown on 50 µg/mL CW. (B) Ranked list of mutants with the lowest median fluorescence values. (C) Growth and fluorescence phenotypes of wild-type (BY4741) and congenic *chs3*Δ, *chs6*Δ, *chs7*Δ, and *pfa4*Δ cells on 75 µg/mL CW. WL: white light, F: fluorescence.

TGN or endosomes (Fig. 2.2A) (Ziman et al., 1998). In contrast, Chs3-GFP localized to intracellular rings in *pfa4*Δ mutants, similar to those seen in *chs7*Δ mutants, where ER exit of Chs3 is blocked (Trilla et al., 1999). Colocalization with the ER marker Sec61 confirmed that Chs3-GFP resided primarily in the ER of *pfa4*Δ cells (Fig. 2.2B); however, unlike *chs7Δ* cells, a small proportion of cells also showed some Chs3-GFP at the bud neck or bud tip. The ER-localized pool of Chs3 is not unstable or targeted for degradation, as Chs3 is present at wild-type levels in *pfa4*Δ mutants (Fig. 2.2C). These results indicate that loss of cell surface Chs3 activity in *pfa4*Δ mutants results from a defect in transport at the ER. Moreover, this transport defect is specific to Chs3, as localization of other yeast chitin synthases Chs1 and Chs2 are unaltered in *pfa4*Δ cells (Fig. 2.2D).

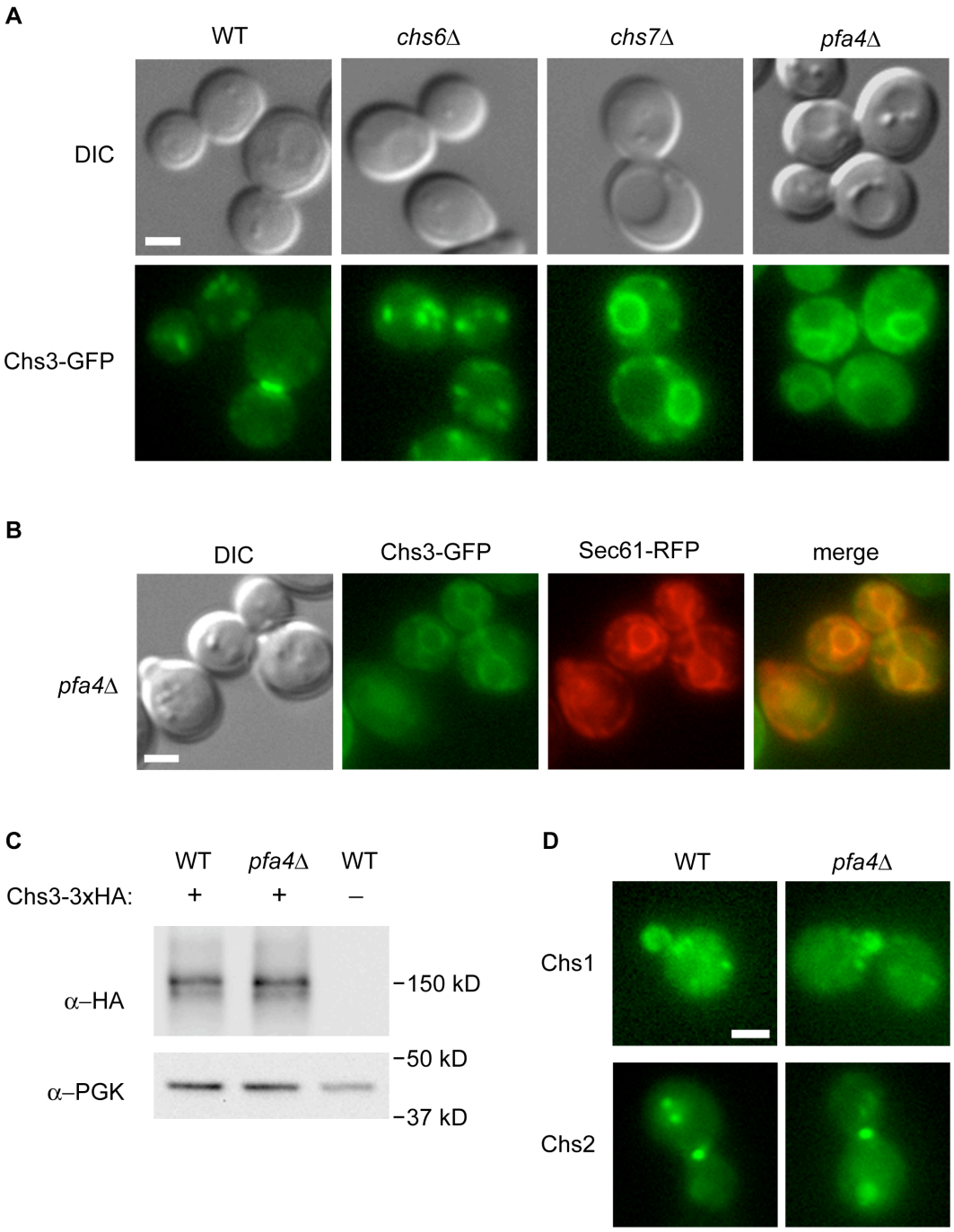

Figure 2.2. Chs3-GFP mislocalizes to the ER in *pfa4*Δ cells.
(A) Wild-type (KLY9), *chs6*Δ (KLY1), *chs7*Δ (KLY3), and *pfa4*Δ (KLY5) cells expressing *CHS3*-GFP were observed by DIC and fluorescence microscopy. (B) Chs3-GFP colocalizes with the ER-marker Sec61-RFP in *pfa4*Δ (KLY55) cells. (C) Chs3-3xHA levels in wild-type (KLY41) and *pfa4*Δ (KLY43) cells, as shown by Western blotting with α-HA mAb. α-Pgk1 was used as a loading control. (D) Localization of Chs1-GFP and Chs2-GFP in wild-type and *pfa4*Δ cells. Bars: 2μm.

2.2.3. CHS3 IS PALMITOYLATED IN A PFA4-DEPENDENT MANNER

Because Pfa4 is a predicted PAT, we considered the possibility that ER export of Chs3 requires a Pfa4-mediated palmitoylation event. For the few DHHC PATs examined to date, mutation of the cysteine within the core DHHC tetrapeptide element has been found to abolish PAT activity both *in vivo* and *in vitro* (Lobo et al., 2002; Roth et al., 2002; Smotrys et al., 2005; Valdez-Taubas and Pelham, 2005) (Fig. 2.3A). Therefore, cysteine108 within the Pfa4 DHHC sequence was mutated to alanine. The Pfa4^{DHHA} mutant protein accumulated to wild-type levels (Fig. 2.3B), indicating that the substitution does not destabilize Pfa4. Nevertheless, plasmid-borne *pfa4*^{DHHA} failed to complement Calcofluor resistance and Chs3-GFP mislocalization in *pfa4*Δ mutants (Fig. 2.3C and D), suggesting these phenotypes are due to a lack of Pfa4 enzymatic activity. Deletion of other DHHC proteins did not alter CW fluorescence (Fig. 2.3E).

To determine if Chs3 is the direct target of Pfa4, we tested Chs3 for palmitoylation using a modified acyl-biotin exchange assay (Drisdel and Green, 2004; Politis et al., 2005). This three-step protocol involves 1) blockade of free thiols with N-ethylmaleimide, 2) hydroxylamine cleavage of palmitoylation thioester linkages, and 3) thiol-specific biotinylation of the newly exposed cysteinyl thiols. To control for acyl-biotin exchange specificity, samples omitting the key hydroxylamine cleavage step were processed in parallel. We found that Chs3 is palmitoylated, and this modification is Pfa4-dependent, being abolished in *pfa4*Δ and *pfa4*^{DHHA} mutants (Fig. 2.4A). In contrast, palmitoylation of the other chitin synthases, Chs1 and Chs2, or the ER export factor Chs7, could not be detected (not shown). Other PATs have been shown to copurify with their substrates (Keller et al., 2004). Pfa4-Chs3 complexes could be detected by co-immunoprecipitation (Fig. 2.4B), suggesting that Pfa4 interacts directly with Chs3 to mediate its palmitoylation. The observation that Chs3 is ER-localized and palmitoylated in *chs7Δ* cells (Fig. 2.4C) is consistent with Chs3 palmitoylation being an early, ER-localized event.

To date, four of the seven yeast DHHC proteins have been shown to mediate protein palmitoylation in various cellular locations (Lobo et al., 2002; Roth et al., 2002; Hou et al., 2005; Smotrys et al., 2005; Valdez-Taubas and Pelham, 2005). Our finding of Pfa4-dependent palmitoylation for Chs3 adds a fifth DHHC protein to this list. Two other yeast PATs, Erf2/4 and Swf1, are also known to function at the ER but recognize distinct substrates. The emerging

picture is thus of a family of PATs that are distinguished from one another both by intracellular localization and by substrate specificity.

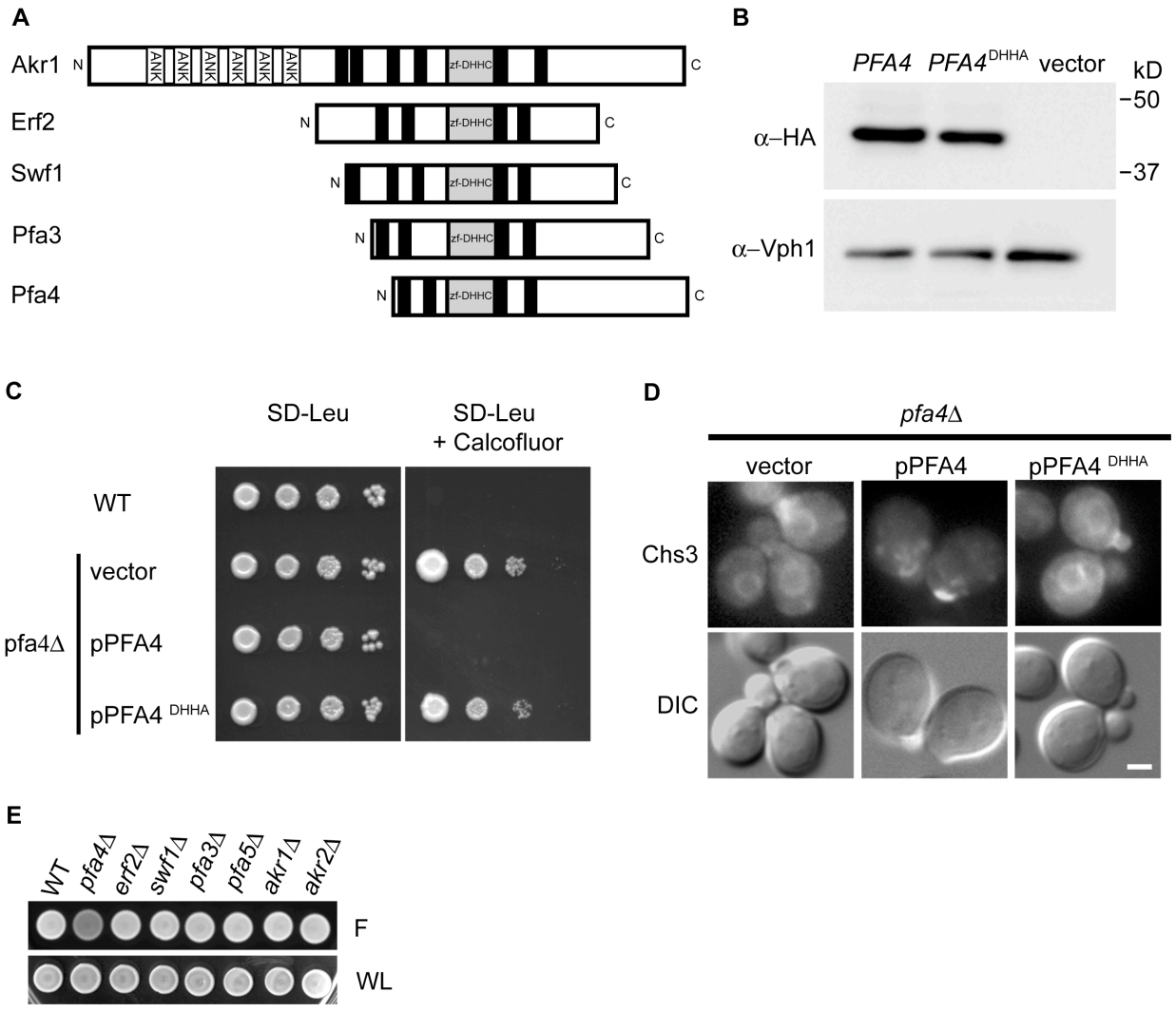

Figure 2.3. The DHHC domain of Pfa4 is required for Chs3 localization.
(A) Topology of the four characterized yeast DHHC proteins (Akr1, Erf2, Swf1, Pfa3) and Pfa4. Zf-DHHC: zinc-finger-like DHHC cysteine rich domain, black boxes: hypothetical transmembrane domains, ANK: ankyrin repeats. (B) Western blots of lysates prepared from *pfa4*Δ cells carrying plasmids expressing Pfa4-3xHA, Pfa4^{DHHA}-3xHA or vector alone indicate the DHHA mutant is stably expressed in vivo. α-Vph1 mAb was used as a loading control. (C) Serial dilutions of strains from (B) on 100 µg/mL CW. (D) *pfa4*Δ strains expressing Chs3-GFP (KLY5) were transformed with empty vector, p*PFA4*, or p*PFA4*^{DHHA}, and observed by microscopy. Bar: 2µm. (E) Fluorescence phenotype of mutants lacking other DHHC proteins on 75 µg/mL CW. WL: white light, F: fluorescence.

2.2.4. A REQUIREMENT FOR PALMITOYLATION AND CHAPERONE ASSOCIATION IN CHS3 ER EXPORT

As both Chs7 and Pfa4 are required for Chs3 ER export, we considered the possibility that these two proteins act together. Hetero-oligomeric PATs have been identified that require binding partners for activity and stability (Lobo et al., 2002). Although Chs3 palmitoylation was reduced in *chs7*Δ cells, it clearly was not abolished (Fig. 2.4C). Furthermore, Chs3 copurifies with its PAT even in the absence of Chs7 (Fig. 2.4D), while Pfa4-Chs7 interactions could not be detected under similar conditions (not shown). Therefore, Chs7 does not appear to be required for substrate recognition by Pfa4 and is unlikely to be a subunit of a dimeric PAT.

We tested an alternative model: that Chs7 preferentially interacts with lipid-modified Chs3 to promote its interaction with the COPII vesicle budding machinery (Gilstring et al., 1999). Although a Chs3-Chs7 physical interaction has not been yet reported, other polytopic yeast proteins, including Gap1 and the vacuolar H-ATPase, have been shown to interact with dedicated accessory factors at the ER during biosynthesis (Gilstring et al., 1999; Malkus et al., 2004). By co-immunoprecipitation, we demonstrated that Chs3 and Chs7 do interact (Fig. 2.5A, lane 3). In *pfa4*Δ cells, the Chs3-Chs7 interaction was subtly reduced, but not eliminated (Fig. 2.5A, lane 2), indicating that Chs3 palmitoylation is not required for recognition by Chs7.

2.2.5. CHS3 AGGREGATES IN THE ABSENCE OF PFA4

Recent work has suggested a chaperone function for Chs7 and the ER accessory proteins Shr3, Gsf3, and Pho86 (Kota and Ljungdahl, 2005). When their cognate chaperones were absent, the substrate polytopic proteins were found to accumulate in the ER in high molecular weight aggregates, which were visualized by crosslinking with Dithiobis[succinimidyl propionate] (DSP). Using the DSP cross-linking protocol of Kota and Ljungdahl, we examined Chs3 aggregation in *pfa4*Δ cells (Fig. 2.5B). Chs3-HA was readily crosslinked into high molecular weight forms in *chs7*Δ*, pfa4*Δ, and *chs7*Δ *pfa4*Δ mutants (Fig. 2.5B and C), indicating that Chs7 chaperone function and Pfa4-mediated palmitoylation are both required to circumvent Chs3 aggregation.

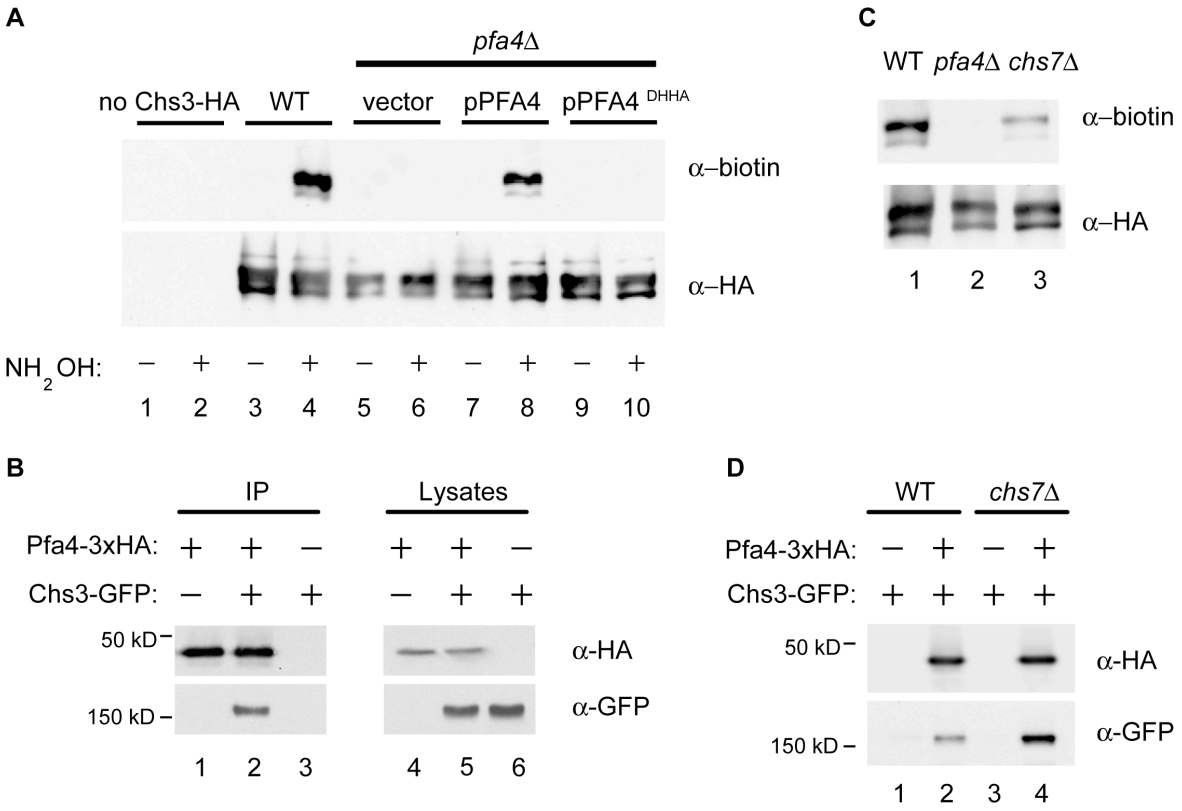

Figure 2.4. Pfa4 is required for palmitoylation of Chs3.
(A) Lysates from cells expressing C-terminally 3xHA/FLAG epitope-tagged Chs3 from the *GAL1* promoter were subjected to the acyl-biotin exchange reactions. Protein extracts were treated with (+) or without (-) hydroxylamine (NH_2OH). α-FLAG immunoprecipitates were blotted with α-biotin and α-HA to detect modified and total Chs3, respectively. (B) Cells expressing Chs3-GFP and Pfa4-3xHA were immunoprecipitated with α-HA antiserum and analyzed by Western blotting with α-HA and α-GFP mAbs. (C) Chs3 palmitoylation was assessed in wild-type, *pfa4*Δ, and *chs7*Δ cells by acyl-exchange as for (A). (D) Coimmunoprecipitation of Chs3-GFP and Pfa4-3xHA in wildtype and *chs7Δ* cells as performed in (B).

Despite the similarity of the *chs7*Δ and *pfa4*Δ phenotypes, our data do not support models that Chs7 and Pfa4 act together as part of a complex or linear pathway. Instead, our results are best accommodated by models where Chs7 and Pfa4 act in parallel, mediating separate events that are both required for ER export. Nonetheless, defects in one pathway do appear to impact the other: Chs3 palmitoylation is reproducibly reduced in *chs7*Δ cells, and the Chs3-Chs7 interaction is decreased in *pfa4*Δ cells.

As both Chs7 and Pfa4 are required to circumvent Chs3 accumulation in high molecular weight aggregates, both appear to participate in the prerequisite folding of Chs3 that precedes ER export. Hydrophobic mismatch, resulting from an incompatibility between long transmembrane domains of polytopic proteins and the thinner ER bilayer, could explain why a membrane protein such as Chs3 requires both palmitoylation and chaperone association for export. ER chaperones have been hypothesized to shield hydrophobic regions of transmembrane domains to prevent protein aggregation (Levine et al., 2000). Palmitoylation may also promote hydrophobic matching by targeting proteins to cholesterol-rich membrane microdomains, which provide a local region of higher bilayer thickness. It is interesting that Chs1 and Chs2, which are also polytopic proteins, require neither Chs7 nor Pfa4 for ER export. This suggests a unique requirement for chaperone association and palmitoylation for only a subset of membrane proteins.

Several recent results suggest that the palmitoylation requirement for ER export may not be unique to Chs3. Our concurrent proteomic analysis of yeast protein palmitoylation indicates that several amino acid permeases are palmitoylated in a Pfa4-dependent manner (Roth et al., 2006). Intriguingly, these polytopic proteins also require dedicated accessory proteins for their ER export (Kota and Ljungdahl, 2005). It will be interesting to see if, as for Chs3, palmitoylation plays a role in permease trafficking. The link between palmitoylation and ER exit may hold true for at least some polytopic proteins in higher cells, as it was recently reported that functional cell surface expression of the nicotinic acetylcholine receptor requires an ER palmitoylation event (Drisdel et al., 2004). Thus, palmitoylation may participate more generally in ER quality control mechanisms, particularly for newly synthesized polytopic integral membrane proteins.

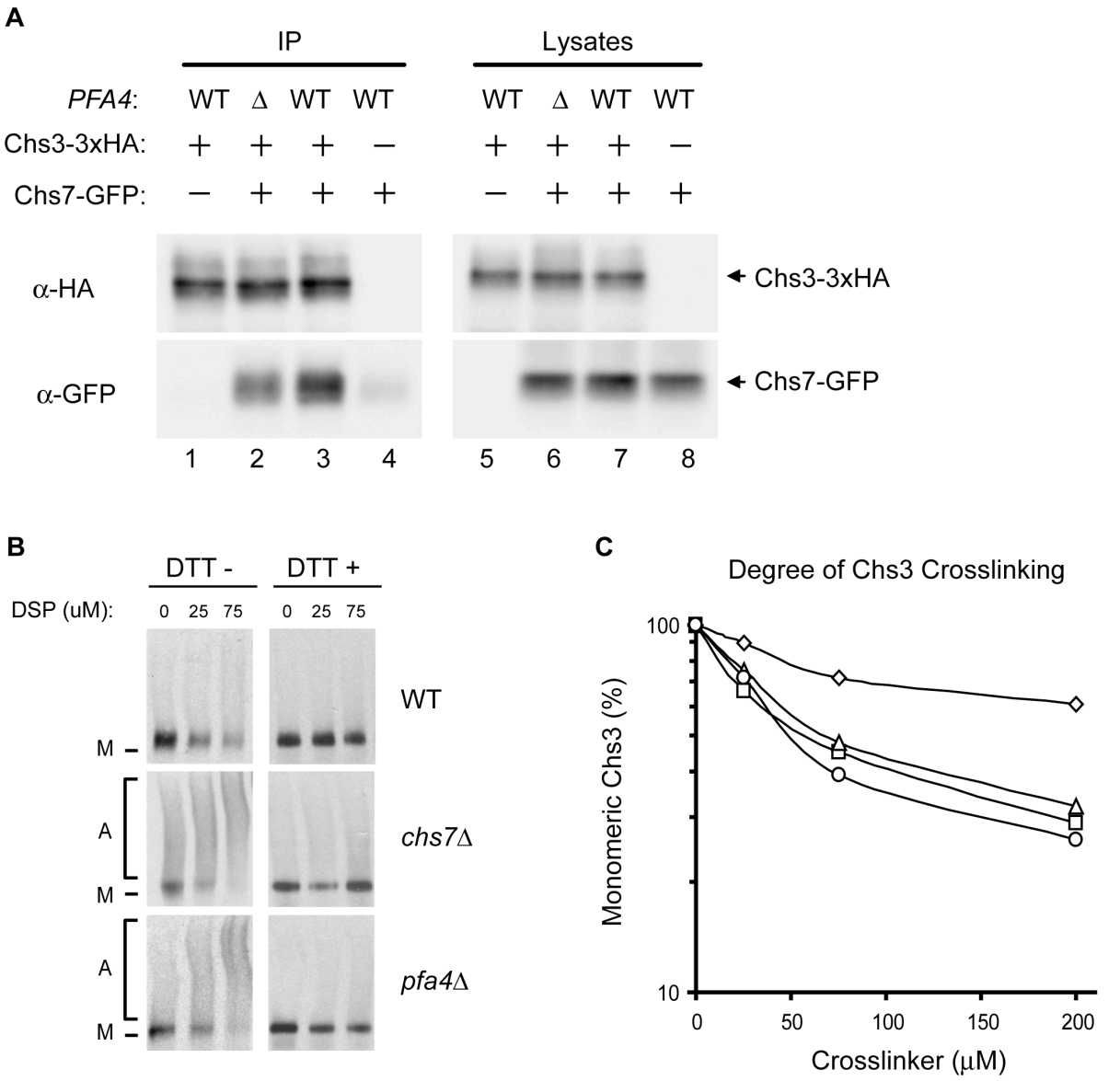

Figure 2.5. Loss of *PFA4* causes Chs3 aggregation in the ER.
(A) Cells expressing Chs3-3xHA and Chs7-GFP were subjected to immunoprecipitation with α-HA antiserum and analyzed by Western blotting with α-HA and α-GFP mAbs. Strains used were KLY46 (lanes 1, 5), KLY45 + pTM15 (lanes 2, 6), KLY46 + pTM15 (lanes 3, 7), and KLY14 + pTM15 (lanes 4, 8). (B) Lysates of wild-type, *chs7*Δ, and *pfa4*Δ cells transformed with plasmid-borne Chs3-3xHA (pHV7) were crosslinked using increasing concentrations of DSP; DTT was added to duplicate samples to reverse crosslinks. Chs3 was detected by Western blotting with α-HA mAb. M: monomer, A: aggregates. (C) Crosslinking of chromosomally encoded Chs3-3xHA in wild-type (KLY41) (◇), *chs7*Δ (KLY46) (△), *pfa4*Δ (KLY43) (□), and *chs7*Δ*pfa4*Δ (KLY45) (○) strains was carried out as for (B). Disappearance of monomeric Chs3 as a function of crosslinker concentration was quantified by densitometry.

2.3. EXPERIMENTAL PROCEDURES

General molecular biology methods were as described (Conibear and Stevens, 2000; Conibear and Stevens, 2002). Primer sequences are available on request. Protein topologies were predicted by the Simple Modular Architecture Research Tool (http://smart.embl-heidelberg.de/).

2.3.1. CALCOFLUOR WHITE GENOMIC SCREEN

Three yeast knockout collections (in strain backgrounds BY4741, BY4742 and BY4743) were obtained from Open Biosystems (Huntsville, AL) and pinned four times in 1536-array format onto YPD plates containing 50 µg/mL Calcofluor white (Sigma, St Louis, MO), using a Virtek automated colony arrayer (BioRad, Hercules, CA). After incubation at 30°C for 3d, 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). The open-source spot-finding program GridGrinder (http://gridgrinder.sourceforge.net) was used for densitometry of digital images. Average growth and fluorescence values from two independent screens were calculated for each strain using Microscoft Excel; slow growing strains were removed from the analysis.

2.3.2. STRAIN CONSTRUCTION

The BY4741 (*MAT* a *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) knockout strains *akr1*Δ, *akr2*Δ, *chs3*Δ, *chs6*Δ, *chs7*Δ, *erf2*Δ, *pfa3*Δ, *pfa4*Δ, *pfa5*Δ, *swf1*Δ, and BY4742 (*MAT*α *his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0)* knockout strains *chs7*Δ and *pfa4*Δ used in this study were obtained from Open Biosystems. Deletion mutants used for palmitoylation assays were in the BY4742 background. Other strains are listed in Table I.

KLY14 was created by transforming a 3.5 kb EcoRV fragment from pTM10 (Cos et al., 1998) into wild-type BY4741. To create KLY45, a Nat^R cassette amplified from p4339 (Tong et al., 2001) using primers with flanking homology to 5' and 3' *PFA4* sequences was transformed into KLY14. C-terminal tags (GFP, 3xHA) were integrated at the *CHS3* locus as described (Longtine

et al., 1998). C-terminal tagging of *SEC61* with RFP was carried out as described (Sheff and Thorn, 2004).

2.3.3. PLASMID CONSTRUCTION

A PCR fragment containing *PFA4*-3xHA was created using a two step PCR method (Conibear and Stevens, 2000) and co-transformed into *pfa4*Δ yeast together with XhoI-cut pRS415 or XhoI-cut pRS426, creating pKL1 and pKL3, respectively. Plasmids were rescued and tested for complementation of *pfa4*Δ CW resistance phenotypes and expression of Pfa4-3xHA by Western blotting. pKL6 was created by Quickchange mutagenesis of pKL1, using primers designed to change Cys108 to alanine. The resulting mutant was confirmed by sequencing. For construction of pND2115, *GAL1$_P$-CHS3/3xHA/FLAG/His* carried on pRS316, the *CHS3* ORF was PCR-amplified from yeast genomic DNA was used to replace the *AKR1* ORF of a *GAL1$_P$-AKR1/3xHA/FLAG/His* plasmid construct (Roth et al., 2002). pHV7 (*CHS3*-3xHA), pTM10 (*chs7*::*HIS3*) (Cos et al., 1998), and pTM15 (*CHS7*-GFP) (Trilla et al., 1999) were a gift from C. Roncero.

2.3.4. MICROSCOPY

For fluorescence microscopy of GFP- or RFP-tagged strains, log phase cells grown in minimal media were observed directly. Indirect immunofluorescence microscopy was carried out as previously described (Conibear and Stevens, 2000; Conibear and Stevens, 2002). For DAPI staining, cells were mounted in buffered glycerol containing 1mg/mL p-phenylenediamine and 0.05 µg/mL DAPI. Cells were viewed using a 100x oil-immersion objective on a Zeiss Axioplan2 fluorescence microscope, and images were captured with a CoolSnap camera using MetaMorph 6.2r6 software. Images were adjusted for brightness and contrast with Adobe Photoshop CS2.

2.3.5. CROSSLINKING

Crosslinking of yeast cell lysates with DSP was performed as described (Kota and Ljungdahl, 2005) with slight modifications. DSP was added to 10ug 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.

2.3.6. PALMITOYLATION ASSAY

Chs3 palmitoylation was assessed by acyl-biotinyl exchange (Politis et al., 2005), using a modified version of Drisdel and Green method (Drisdel and Green, 2004). Denatured protein extracts, prepared from Chs3-3xHA/FLAG/His-expressing cells (2h galactose-induced expression period) by glass bead lysis, were subjected to the three steps of acyl-biotinyl exchange protocol as previously described (Politis et al., 2005). The epitope-tagged Chs3 was immune precipitated with M2 anti-FLAG-agarose and then Western blotted with either anti-biotin-HRP or anti-HA-HRP.

2.3.7. COIMMUNOPRECIPITATIONS

Co-precipitation was performed as previously described (Conibear and Stevens, 2000). 20 OD_{600} of spheroplasts were resuspended in 1mL lysis buffer (1% CHAPSO, 50mM KPO_4 pH7.5, 50mM NaCl, protease inhibitors), and incubated with 3uL of rabbit α-HA antiserum for 1h at 4°C. 30uL of Protein G sepharose (Amersham) was added for 1h at 4°C. Beads were washed twice in lysis buffer, and subjected to SDS PAGE. Co-immunoprecipitated proteins were analyzed by Western blotting with antibodies to HA or GFP.

Table 2.1. Yeast strains used in Chapter 2

Strain	Genotype
KLY1	BY4741 *chs6*::kan^R *CHS3*-GFP::*HIS3*
KLY3	BY4741 *chs7*::kan^R *CHS3*-GFP::*HIS3*
KLY5	BY4741 *pfa4*::kan^R *CHS3*-GFP::*HIS3*
KLY9	BY4741 *CHS3*-GFP::*HIS3*
KLY14	BY4741 *chs7*::*HIS3*
KLY41	BY4741 *CHS3*-3xHA::kan^R
KLY43	BY4741 *pfa4*::nat^R *CHS3*-3xHA::kan^R
KLY45	BY4741 *chs7*::*HIS3 pfa4*::nat^R *CHS3*-3xHA::kan^R
KLY46	BY4741 *chs7*::*HIS3 CHS3*-3xHA::kan^R
KLY55	BY4741 *pfa4*::kan^R *CHS3*-GFP::*HIS3 SEC61*-RFP::*URA3*
KLY60	BY4741 *CHS1*-GFP::*HIS3*
KLY62	BY4741 *CHS2*-GFP::*HIS3*
KLY65	BY4741 *CHS2*-GFP::*HIS3 pfa4*::nat^R
KLY66	BY4741 *CHS1*-GFP::*HIS3 pfa4*::nat^R
KLY67	BY4741 *chs7*::kan^R *pfa4*::nat^R *CHS3*-GFP::*HIS3*

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CHAPTER 3. THE BRE5/UBP3 DEUBIQUITINATION COMPLEX MEDIATES ER QUALITY CONTROL OF PALMITOYLATION-DEFICIENT CHS3 [2]

[2] A version of this chapter will be submitted for publication: **Lam, K.K.Y.**, Pagant, S., Davey, M., Roth, A., Davis, N.G., Miller, E.A., and Conibear, E. Data that will be submitted as supplemental figures or unpublished observations have been included in the Appendices of this thesis.

3.1. INTRODUCTION

The endoplasmic reticulum (ER) is a major site of quality control in the cell, where newly synthesized proteins are properly folded and assembled before entering the secretory pathway. Unfolded or aberrantly folded proteins are often aggregated and retained, and may be targeted for proteasomal degradation by a quality control process known as ER associated degradation (ERAD) (Ellgaard and Helenius, 2003). Various chaperones and other ER-resident proteins are central to the ER quality control machinery, acting generally or substrate-specifically to assist in folding, as well as to retain misfolded species for refolding or eventual degradation (Brodsky, 2007). Unlike soluble proteins, whose misfolding lesions are recognized primarily within the ER lumen, multispanning (or "polytopic") membrane proteins can display misfolding lesions in the cytosol as well as transmembrane domains. As such, folding monitors that are both substrate- and lesion-specific must be employed. Because many polytopic proteins are plasma membrane transporters crucial to cellular function, the quality control mechanisms for such proteins are intensely studied.

ER retention of misfolded proteins can happen through two mechanisms. It is often thought that many non-native species are retained in a static manner without export from the ER. However, quality control can also be a dynamic process involving active vesicle transport between the ER and the Golgi apparatus. Both misfolded soluble and membrane proteins have been shown to be exported from the ER in COPII-coated vesicles followed by COPI-mediated retrograde transport at the early Golgi. These include the misfolded yeast carboxypeptidase Y (CPY*), the Kar2-hemmagglutinin neuraminidase chimera KHN_t (Vashist et al., 2001), and the unassembled iron transporter Fet3 (Sato et al., 2004). Mammalian cells have evolved an ER-to-Golgi-intermediate-compartment (ERGIC) that is now hypothesized to play a part in ER retrieval and protein quality control (Appenzeller-Herzog and Hauri, 2006). Such post-ER checkpoints provide an additional safeguard should non-native species escape the initial quality control, but the precise role of vesicle transport in these processes remains unclear.

Components of ERAD also participate in recognition and retention of misfolded proteins. In yeast, misfolded forms of the polytopic plasma membrane transporters Gap1 and Yor1 are ER-retained, form molecular aggregates, and activate ERAD (Kota et al., 2007; Pagant et al., 2007). ERAD appears to be coupled to vesicular export machinery to act together as molecular

determinants of retention. Upon inhibition of ERAD, ER-retention of misfolded Gap1 can be relieved and the protein is transported to the plasma membrane (Kota et al., 2007). This does not apply to misfolded Yor1, however, which is not packaged into COPII vesicles despite ERAD disruption (Pagant et al., 2007). The different trafficking fates may be due to the specific nature of the misfolding defects displayed, which can differentially affect the exposure of an ER export signal and/or the interaction with chaperones.

We have previously reported that the polytopic yeast chitin synthase Chs3 is ER-retained when not palmitoylated by one of the seven yeast DHHC protein acyltransferases (PATs), Pfa4 (Lam et al., 2006). In *pfa4*Δ 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). Aggregation is similarly observed for Gap1 when its cognate chaperone, Shr3, is absent (Kota and Ljungdahl, 2005). It is hypothesized that Chs7 and several other Shr3-like proteins act as substrate-specific chaperones to assist in polytopic protein membrane insertion and folding (Kota and Ljungdahl, 2005). Recent work suggests a role for palmitoylation in contributing to protein folding at the ER also exists in mammalian cells. For example, palmitoylation of the Wnt signaling receptor LRP6 may facilitate ER export by affecting both transmembrane domain tilting and recognition by degradation machinery (Abrami et al., 2008). Although palmitoylation is known to have various functions in vesicle transport by acting as a membrane anchor, precise mechanisms of how it regulates ER quality control and export are not well understood.

In this study, we sought to decipher how ER surveillance mechanisms monitor unlipidated and misfolded polytopic proteins by using Chs3 as a model. We performed a genome-wide screen for suppressors of the *pfa4*Δ mutation to identify potential ER retention factors of palmitoylation-deficient Chs3. We observed no major involvement of several known ERAD components in the ER retention of unpalmitoylated Chs3. Instead, we found that deletion of the Bre5/Ubp3 deubiquitination complex rescued *pfa4*Δ mutant phenotypes, and restored Chs3 transport to the cell surface through palmitoylation-independent mechanisms. We show that unpalmitoylated Chs3 is subject to static retention through exclusion from COPII exit vesicles, as well as dynamic retrieval from the early Golgi via COPI. It is likely that factors in these two pathways are regulated by ubiquitination and deubiquitination cycles that involve the Bre5/Ubp3 complex, and the disruption of this complex hampers these quality control pathways, resulting in cell surface transport of unpalmitoylated Chs3. Together, our findings indicate that, instead of a

reliance on the classical ERAD pathways for retention, unpalmitoylated Chs3 may be recognized by alternative surveillance mechanisms.

3.2. RESULTS

3.2.1. MISFOLDED CHS3 IS NOT RETAINED BY KNOWN ERAD PATHWAYS

Many misfolded polytopic proteins are ER retained and activate ER associated degradation (Pety de Thozee and Ghislain, 2006). The inhibition of ERAD pathways is known to stabilize misfolded Yor1 (Pagant et al., 2007) and restore plasma membrane expression of misfolded Gap1 (Kota et al., 2007). To determine if the misfolded pools of Chs3 in *pfa4*Δ and *chs7*Δ are also retained by ERAD, we disrupted the *UBC6* and *UBC7* genes that encode for the redundant E2 ubiquitin-conjugating enzymes. We also deleted either one of the E3 ubiquitin ligases *HRD1* or *DOA10,* which are specific for ERAD lesions located in lumenal/transmembrane (ERAD-L/ERAD-M) or cytosolic (ERAD-C) domains, respectively. Together, these ubiquitination enzymes recognize most known ERAD substrates (Kostova et al., 2007). If ERAD is involved in ER retention of Chs3, the disruption of these pathways may restore its cell surface localization, which can be assessed using a Calcofluor white (CW) fluorescence assay we have previously developed (Lam et al., 2006). CW is a dye that binds the chitin produced by Chs3 when it is present on the cell surface. The fluorescence of yeast colonies grown on CW media in either a spot test or an array assay can be imaged using a fluoroimager and quantified by densitometry methods (Lam et al., 2006; Burston et al., 2008). Deletion of *PFA4* or *CHS7*, which causes ER retention of Chs3, leads to a dim phenotype on CW media (Fig. 3.1A). The *pfa4*Δ mutant has slightly higher fluorescence than *chs7*Δ due to the fact that a small amount of Chs3 can be observed at the budneck of some *pfa4*Δ mutants (Fig. 3.1B). This shows that in *pfa4*Δ cells, retention is not complete and some Chs3 escapes the ER and reaches the cell surface. Disruption of *UBC6* and *UBC7*, or either *HRD1* or *DOA10,* failed to rescue the CW-dim phenotypes of *pfa4*Δ or *chs7*Δ cells (Fig. 3.1A; Appendix Fig. A.1, Table G.1), which indicates that loss of these ERAD pathways does not restore Chs3 transport to the cell surface. A possible interpretation of this data is that Chs3 is present at the cell surface but inactive, and therefore unable to produce chitin. However, we confirmed by fluorescence microscopy that genomically tagged Chs3-GFP was indeed still ER-retained in *pfa4*Δ*ubc6*Δ*ubc7*Δ and *chs7*Δ*ubc6*Δ*ubc7*Δ mutants (Fig. 3.1B). These results show that, unlike other mutant transmembrane proteins in yeast (Hill and Cooper, 2000; Huyer et al., 2004; Kota et al., 2007; Pagant et al., 2007), the

misfolded forms of Chs3 in *pfa4*Δ and *chs7*Δ cells are not subjected to retention by known ERAD pathways.

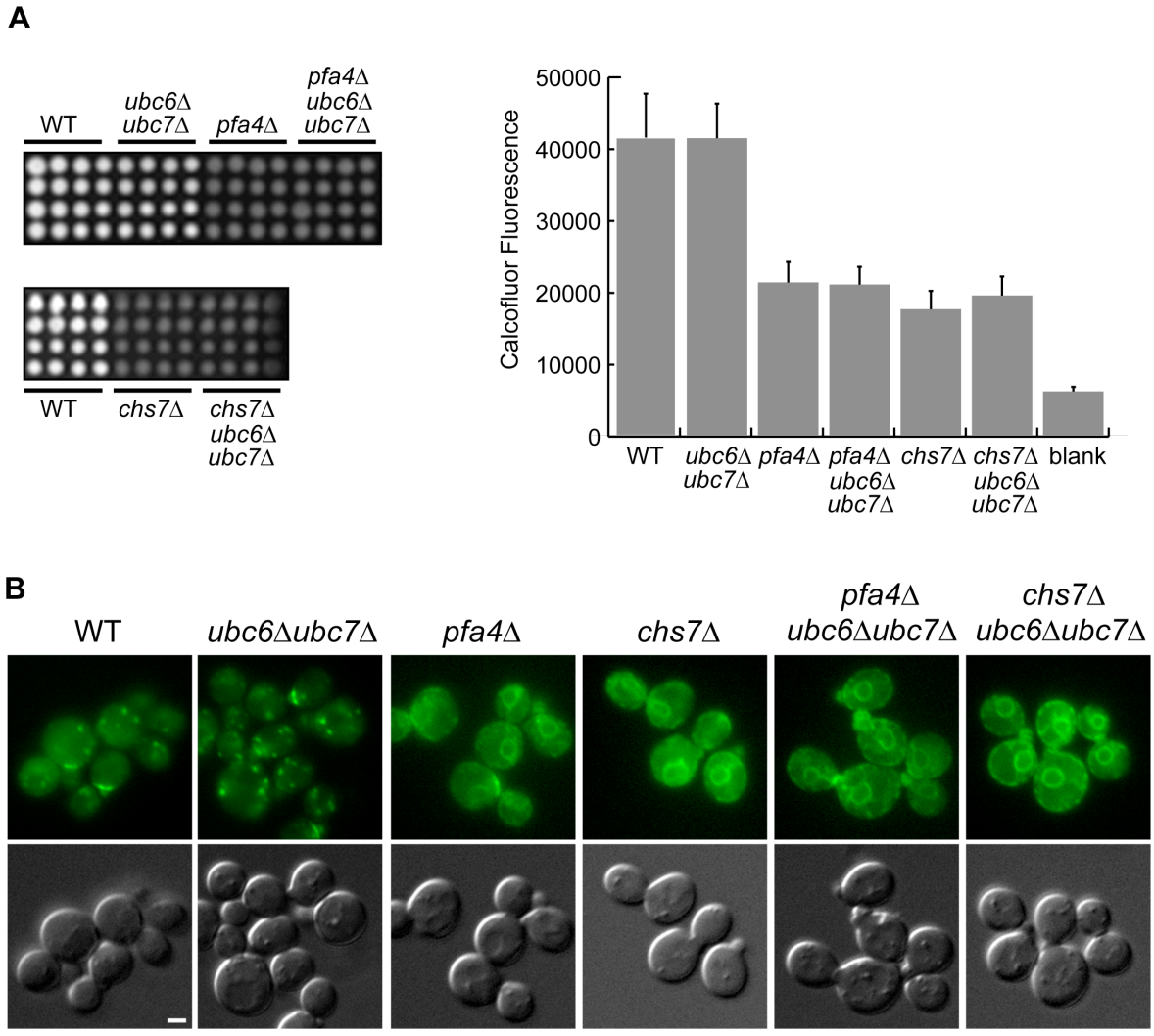

Figure 3.1. Inhibition of ERAD pathways does not suppress *pfa4*Δ or *chs7*Δ.
(A) CW fluorescence of yeast colony array grown on YPD media containing 50µg/mL CW; each strain is represented by a 4x4 quadrant of 16 spots. The two groups of cells shown are cropped images from different regions of the same plate. Fluorescence was observed using a fluoroimager, and quantified by densitometry. The values depicted are averaged over six randomly arranged 4x4 quadrants on the array, with error bars corresponding to standard deviations. (B) Log-phase cells expressing Chs3-GFP WT BY4742 (KLY70), *ubc6*Δ*ubc7*Δ (KLY73), *pfa4*Δ (KLY71), *chs7*Δ (KLY72), *pfa4*Δ*ubc6*Δ*ubc7*Δ (KLY74), and *chs7*Δ*ubc6*Δ*ubc7*Δ (KLY75) observed by DIC (bottom panel) and fluorescence microscopy (top panel). Bar: 2µm.

3.2.2. LOSS OF THE BRE5/UBP3 COMPLEX SUPPRESSES *PFA4*Δ

Besides ERAD components, the loss of other quality control proteins or retention factors may restore cell surface transport of misfolded, palmitoylation-deficient Chs3 in *pfa4*Δ mutants. To systematically uncover such suppressors of *pfa4*Δ, we introduced the *pfa4*Δ mutation into the genome-wide knockout collection of nonessential ORFs using Synthetic Genetic Array analysis (Tong et al., 2001). The resulting *pfa4*Δ*xxx*Δ double mutants were assayed for CW fluorescence in an array-style CW screen that we had previously developed (Lam et al. 2006; Burston et al., 2008). We found that deletion of the cytosolic deubiquitination enzyme Ubp3 or its obligate subunit Bre5 rescued the CW-dim fluorescence of *pfa4*Δ cells to levels comparable to wild-type (Fig. 3.2A). Chs3-GFP in *pfa4*Δ*ubp3*Δ or *pfa4*Δ*bre5*Δ double mutants also displayed the typical budneck and chitosomal localization observed in wild-type cells (Fig. 3.2B). Moreover, there was no residual ring-like ER staining as seen in *pfa4*Δ cells, and Chs3-GFP showed little colocalization with the ER-marker Sec61-RFP in these double mutants (Fig. 3.2B, Appendix Fig. A.2).

The *pfa4*Δ suppression screen also identified factors with suggested roles at the ER that, if absent, may be expected to lead to defective ER retention. These include the two members of the 14-3-3 family of proteins (Bmh1, Bmh2) that are known to bind arginine-based ER-retention signals (Michelsen et al., 2005), a member of the GET (Golgi-ER trafficking) complex (Get3/Arr4) involved in insertion of tail-anchored proteins into the ER membrane (Schuldiner et al., 2008), and the Erd1 protein, which is hypothesized to act similarly to the Erd2 receptor in the retention of lumenal ER proteins (Hardwick et al., 1990). Deletion of these factors rescued the CW fluorescence of *pfa4*Δ to near wild-type levels as assayed by our screen; however, Chs3-GFP remained ER localized in these double mutants (Appendix Fig. A.3). Similarly, deletions of downstream trafficking proteins identified from the screen were also unable to rescue the ER retention of Chs3-GFP in *pfa4*Δ mutants (Appendix Fig. A.3). These factors include 1) components of endocytosis machinery, such as subunits of the clathrin adaptor protein AP-2 complex (Apl1, Apl3), and the clathrin binding Yap1801 (Kirchhausen, 2000; Maldonado-Baez et al., 2008); 2) proteins involved in trans-Golgi network trafficking, such as a subunit of the adaptor protein AP-3 complex (Apl6) involved in Golgi-to-vacuole transport (Bonifacino and Traub, 2003), and Sys1, a membrane protein involved in Golgi targeting (Graham, 2004). Since

deletion of these factors increased cell surface chitin levels (as assayed by the CW screen) but did not restore ER export of unpalmitoylated Chs3 (as assayed by fluorescence microscopy), it is likely that other pathways of increasing cell surface levels of unpalmitoylated Chs3 exist. This could occur, for example, through the inhibition of Chs3 endocytosis, or through indirect effects on Chs3 trafficking at post-ER compartments. Alternatively, deletion of these factors could also increase synthesis or cell surface delivery of one or both of the other *S. cerevisiae* chitin synthases (Chs1 and Chs2). Because a clear rescue of Chs3 ER retention was observed in *pfa4*Δ*bre5*Δ and *pfa4*Δ*ubp3*Δ mutants, this suggested that Bre5 and Ubp3 affect ER-specific retention mechanisms. Therefore, these proteins were chosen for further study.

We next developed a protease-accessibility assay to measure the amount of cell-surface localized Chs3 more quantitatively. Treatment of cell lysates with Pronase, a broad-specificity protease that cleaves cell-surface exposed proteins, produced two Chs3-specific degradation bands in wild-type cells (Fig. 3.2C, white arrows). These bands were not present in *pfa4*Δ mutants where most Chs3 was ER-retained and inaccessible to protease (black arrow), but reappeared in the *pfa4*Δ*ubp3*Δ or *pfa4*Δ*bre5*Δ double mutants, indicating that Chs3 cell surface levels were restored to wild-type levels (Fig. 3.2C). The level of protease-inaccessible Chs3 in wild-type corresponds to approximately 75% of total Chs3, corroborating previous observations that up to 70% of Chs3 is normally stored in internal, chitosomal compartments (Santos and Snyder, 1997). In *pfa4*Δ, only about 10% of Chs3 is present at the cell surface and is protease-accessible (Fig. 3.2C). Interestingly, the *bre5*Δ and *ubp3*Δ single mutants displayed higher cell surface levels of Chs3, indicating that this complex may have a more general role of Chs3 upregulation or may act on the chitosomal pools of Chs3.

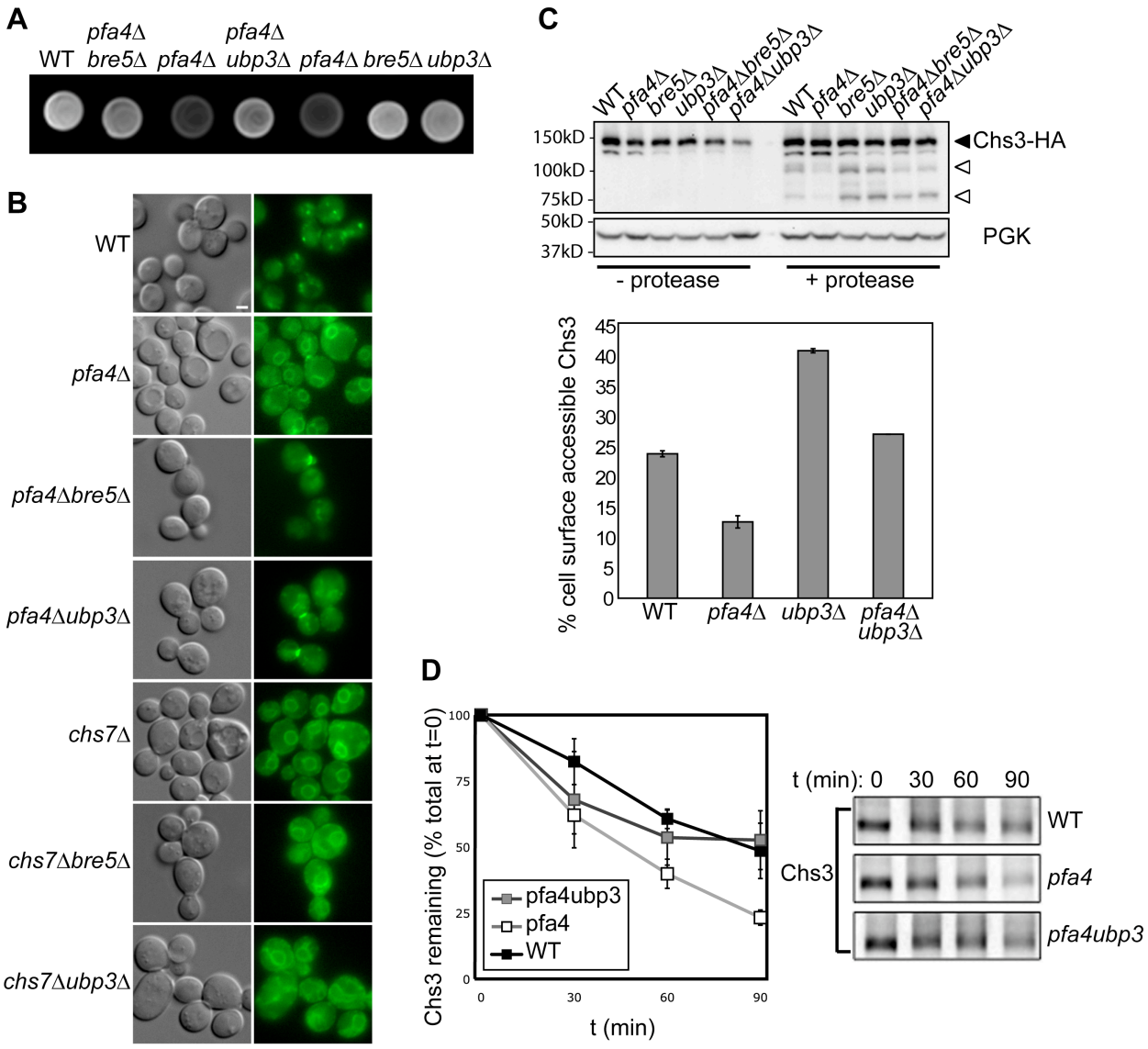

Figure 3.2. Deletion of *BRE5* or *UBP3* restores ER export of palmitoylation-deficient Chs3. (A) Fluorescence phenotypes of yeast colony dot spots grown on YPD with 50μg/mL CW. 4μL of saturated overnight yeast cultures were grown for two days at 30°C, and the resulting fluorescence was observed using a fluoroimager. (B) Log-phase cells expressing Chs3-GFP in WT BY4741 (KLY9), *pfa4*Δ (KLY5), *pfa4*Δ*bre5*Δ (KLY85), *pfa4*Δ*ubp3*Δ (KLY86), *chs7*Δ (KLY3), *chs7*Δ*bre5*Δ (KLY105), and *chs7*Δ*ubp3*Δ (KLY106) were observed by DIC (left) and fluorescence (right) microscopy. Bar: 2μm. (C) Protease-accessibility assay of WT (KLY87), *bre5*Δ (KLY89), *ubp3*Δ (KLY90), *pfa4*Δ (KLY88), *pfa4*Δ*bre5*Δ (KLY91), and *pfa4*Δ*ubp3*Δ (KLY92) mutants expressing Chs3-3xHA. Protease treated (+ protease) and parallel samples mock-treated with buffer (- protease) were subjected to SDS-PAGE and immunoblotting with α-HA, and α-PGK as a loading control. Levels of Chs3 degradation products relative to total were quantified by densitometry. The resulting graph represents the average of three independent experiments with error bars corresponding to standard deviations. (D) Pulse chase of Chs3 in WT (KLY87), *pfa4*Δ (KLY88), and *pfa4*Δ*ubp3*Δ (KLY92) mutants. Strains expressing Chs3-3xHA were pulse-labeled for 5 min with (35S)methionine/cysteine and chased for the indicated times. Chs3 was immunoprecipitated from cell lysates and analyzed by SDS-PAGE. The results represent the average of three independent experiments with error bars corresponding to standard deviations.

Another interpretation of the lack of ER-staining in *pfa4*Δ*ubp3*Δ or *pfa4*Δ*bre5*Δ mutants could be that the existing ER pools of Chs3 were degraded. Pulse chase studies show that Chs3 is slightly destabilized in *pfa4*Δ, and this is suppressed in the *pfa4*Δ*ubp3*Δ mutant (Fig. 3.2D). The latter observation confirms that the lack of ER-localized Chs3-GFP in *pfa4*Δ*ubp3*Δ mutants is not due to degradation of Chs3. The increased cell surface transport in *pfa4*Δ*ubp3*Δ strains is also not likely to be due to an increased rate of Chs3 synthesis, as overexpression of Chs3 does not result in higher cell surface expression without a concomitant overexpression of the Chs7 chaperone required for ER exit (Trilla et al., 1999). These observations further indicate that unpalmitoylated Chs3 may be subjected to degradation, and this is averted in the absence of *UBP3*.

We also found that deletion of *BRE5* or *UBP3* failed to restore the ER export of Chs3 in *chs7*Δ mutants by GFP microscopy as well as protease accessibility (Fig. 3.2B; Appendix Figs. A.4, A.5). This suggests that the cellular response to Chs3 misfolding is different when the Chs7 chaperone is absent, and the Bre5/Ubp3 complex has a role in ER retention of the palmitoylation-deficient form of Chs3.

3.2.3. PALMITOYLATION OF CHS3 IS NOT RESTORED IN *PFA4*Δ*UBP3*Δ

It is conceivable that the restoration of Chs3 ER export in *pfa4*Δ*bre5*Δ and *pfa4*Δ*ubp3*Δ is due to a repalmitoylation event, perhaps through upregulation of one of the remaining six DHHC PATs. We therefore examined the palmitoylation status of Chs3 using the acyl-biotin exchange assay (Drisdel and Green, 2004; Politis et al., 2005; Lam et al., 2006), in which potential palmitoyl-thioester linkages are cleaved by hydroxylamine, and the resulting exposed cysteinyl thiols are replaced by biotin. The amount of incorporated biotin can then be assessed by immunoblotting after immunoprecipitation of epitope-tagged Chs3. As we have previously found, Chs3 palmitoylation is highly Pfa4-dependent, and was completely abolished when this DHHC PAT was absent (Fig. 3.3, lanes 1 and 2). Chs3 remains similarly unlipidated in *pfa4*Δ*ubp3*Δ and *pfa4*Δ*bre5*Δ double mutants (Fig. 3.3, lanes 3 and 4), indicating that the restored ER export of Chs3 in these cells was not due to repalmitoylation. Bre5/Ubp3 also did not affect Chs3 palmitoylation normally, as we could not detect a change in the amount of palmitoylated Chs3

by deleting either gene in the wild-type background (Fig. 3.3, lanes 5 and 6). Taken together, these results show that loss of the Bre5/Ubp3 complex does not restore Chs3 palmitoylation, and Chs3 in *pfa4*Δ*bre5*Δ and *pfa4*Δ*ubp3*Δ mutants can be functional and properly targeted to the cell surface without being palmitoylated.

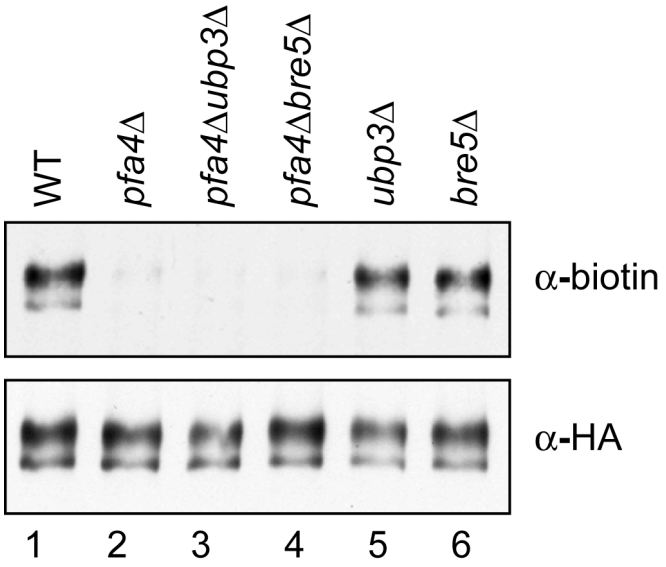

Figure 3.3. Palmitoylation of Chs3 is not restored in *pfa4*Δ*bre5*Δ and *pfa4*Δ*ubp3*Δ cells. BY4741 WT, *pfa4*Δ, *bre5*Δ, *ubp3*Δ, *pfa4*Δ*bre5*Δ (KLY83), and *pfa4*Δ*ubp3*Δ (KLY84) cells carrying the pND2115 plasmid encoding *CHS3*-3xHA-FLAG-His were subjected to the acyl-biotin exchange assay. Protein extracts 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.

3.2.4. COPI RETRIEVAL AS A MECHANISM OF ER RETENTION

Ubiquitination is a major post-translational modification affecting protein stability and trafficking at various cellular compartments. Therefore, enzymes responsible for ubiquitin conjugation and removal, or deubiquitination, have important roles in regulating these processes (Millard and Wood, 2006). The deubiquitinating enzyme Ubp3 and its cofactor Bre5 are known

to act on many substrates that are implicated in processes such as autophagy, mitogen-activated protein kinase (MAPK) signaling, and transcription (McCullock et al., 2006; Kraft et al., 2008; Wang et al., 2008). It was recently shown that deletion of *UBP3* leads to increased stability of Pkc1, the protein kinase of the yeast cell wall integrity signaling pathway (Wang et al., 2008). The upregulation of this MAPK pathway increases transcription of cell wall related genes, and is also known to increase the transport of Chs3 to the cell surface (Valdivia and Schekman, 2003). We considered the possibility that deletion of Bre5/Ubp3 could increase cell surface delivery of Chs3 through this signaling pathway, and therefore tested this by transforming wild-type, *pfa4*Δ, and *chs7*Δ cells with plasmids expressing an activated allele of Pkc1 ($Pkc1^{R398A}$) under the *GAL1* promoter. Upon shifting cells to galactose media, Chs3 ER staining was still observed in *pfa4*Δ and *chs7*Δ (Appendix Fig. A.6). A slight increase in vacuolar Chs3 was also observed, but this was true for wild-type, *pfa4*Δ, and *chs7*Δ strains, indicating that this may be an effect from Chs3 overexpression in general. Additionally, upregulation of the pathway through activated $Mkk1^{S386P}$, the downstream kinase of the Mpk1 MAPK, was unable to rescue ER export when expressed from its endogenous promoter (Appendix Fig. A.6). These results show that loss of Bre5/Ubp3 does not affect Chs3 trafficking at the ER through the Pkc1 MAPK pathway.

Loss of either *BRE5* or *UBP3*, which results in the loss of the entire complex, causes destabilization of COPII and COPI, the vesicular coats which respectively govern anterograde and retrograde transport between the ER and Golgi (Cohen et al., 2003a; Cohen et al., 2003b). Because disruption of COPII forward transport would not restore ER export of Chs3, we considered that the *ubp3*Δ suppression of *pfa4*Δ is caused by a destabilization of COPI. If palmitoylation-deficient Chs3 is capable of reaching the early Golgi, quality control mechanisms at this compartment could potentially retrieve Chs3 back to the ER via COPI retrograde transport, and failure to do so could result in plasma membrane transport. Indeed, we found that palmitoylation-deficient Chs3 is no longer ER-retained in the temperature-sensitive COPI mutant *sec21-1* at non-permissive temperatures (Fig. 3.4A). As verified by the protease accessibility assay, the level of Chs3 located at the cell surface in *pfa4*Δ*sec21-1* mutants is comparable to wild-type (Fig. 3.4B). We also saw suppression by deleting the only nonessential COPI subunit, Sec28 (Appendices Fig. A.3, Tab. G.1). Similar effects on *chs7*Δ mutants were not observed (Appendix Figs. A.4, A.5), corroborating the *ubp3*Δ suppression data that the misfolded Chs3 in *chs7*Δ is subject to different quality control mechanisms.

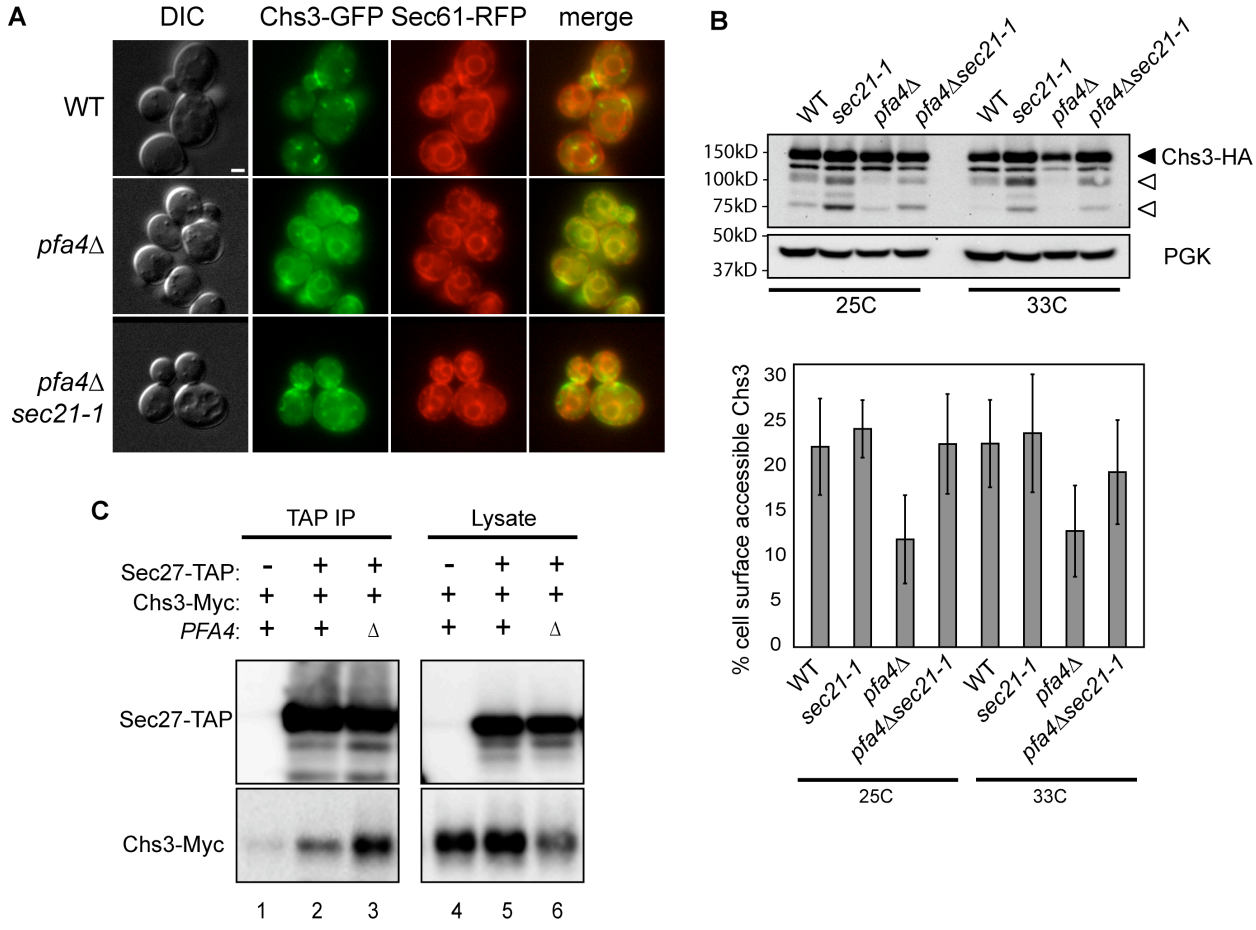

Figure 3.4. Inhibition of COPI retrograde transport restores cell surface expression of palmitoylation-deficient Chs3.

(A) Log phase cells co-expressing genomically integrated Chs3-GFP and the ER marker Sec61-RFP in WT (KLY93), *pfa4*Δ (KLY94), and *pfa4*Δ*sec21-1* (KLY96) strains were observed by DIC and fluorescence microscopy after 45 min shift to the restrictive temperature of 33°C. Bar: 2μm. (B) Protease-accessibility assay of WT (KLY97), *pfa4*Δ (KLY98), and *sec21-1* (KLY99), and *pfa4*Δ*sec21-1* (KLY100) strains expressing genomically integrated Chs3-3xHA was performed as in Fig. 3.2C, at permissive (25°C) and after 1 hr of shifting to non-permissive (33°C) temperatures. The resulting graph is an average of three independent experiments with error bars corresponding to standard deviations. Black arrow: protease-inaccessible Chs3. White arrows: protease-accessible Chs3 degradation products. (C) Cells co-expressing genomically integrated Sec27-TAP and Chs3-13xMyc were subjected to TAP immunoprecipitation using IgG-Sepharose, and analyzed by Western blotting with α-Myc and α-TAP antibodies. Strains used were KLY107 (Lanes 1, 4), KLY108 (Lanes 2, 5), and KLY (Lanes 3,6). The amount of IP loaded relative to lysate was approximately 65-fold.

An analogous retrieval mechanism has been observed for the misfolded iron transporter Fet3, which is ER retained through dynamic retrieval from the early Golgi (Sato et al., 2004). This is mediated by an adaptor, Rer1, which recognizes misfolded Fet3 at the early Golgi (Sato et al., 2004). By binding distinct transmembrane motifs, Rer1 is also known to be required for Golgi-to-ER transport of misfolded versions of yeast Gas1 and Ste2 (Letourneur and Cosson, 1998), and the mammalian γ-secretase component Pen2 (Kaether et al., 2007; Spasic et al., 2007). Loss of the Rer1 adaptor resulted in cell surface transport of misfolded substrates. However, we found no evidence that Rer1 affects trafficking of misfolded Chs3, as Rer1 was not isolated in the *pfa4*Δ suppression screen (Appendix Tab. G.1), and Chs3-GFP was still ER-retained in *pfa4*Δ*rer1*Δ (Appendix Fig. A.3). Therefore, we tested whether COPI binds misfolded Chs3 by assessing for the presence of Myc-epitope tagged Chs3 in immunoprecipitates of TAP-epitope tagged COPI strains. We found that Chs3 can be co-immunoprecipitated with the *β*'-COP subunit Sec27 in wild-type strains (Fig. 3.4C, lane 2). This interaction was stronger in *pfa4*Δ mutants (Fig. 3.4C, lane 3), which suggests that there may be increased retrieval or COPI-binding of unpalmitoylated Chs3. We also detected similar interactions between Chs3 and other COPI subunits (Sec26, Sec28, Ret1, and Ret2) (Appendix Fig. A.7).

Together, these results show that COPI retrieval is involved in ER retention of palmitoylation-deficient Chs3. Moreover, they suggest that the ER-retained pool of Chs3 in *pfa4*Δ mutants can be dynamically maintained through transport to the early Golgi and subsequent retrieval back to the ER.

3.2.5. COPII EXPORT OF UNPALMITOYLATED CHS3 IS IMPAIRED

Having examined COPI retrieval as one retention mechanism, we wanted to investigate the possibility that loss of Ubp3 can affect export of misfolded Chs3 from the ER. We therefore examined the incorporation of Chs3 into COPII coated vesicles using an *in vitro* COPII budding assays (Pagant et al., 2007). In this assay, microsomes containing ER membranes were prepared from wild-type, *pfa4*Δ, and *pfa4*Δ*ubp3*Δ mutants expressing HA-epitope tagged Chs3, then incubated with purified COPII coat proteins (Sar1, Sec23/Sec24, Sec13/31). The addition of GTP drives the COPII budding reaction, with samples supplemented with GDP representing negative controls (Pagant et al., 2007). The vesicles generated from this reaction were isolated

by differential centrifugation, and the presence of Chs3-HA, corresponding to the levels of Chs3 incorporated into COPII vesicles, was monitored by immunoblotting. We found that Chs3 was not detectably present in COPII vesicles in either *pfa4*Δ or *chs7*Δ cells (Fig. 3.5). Such a complete block of COPII export in *pfa4*Δ was not expected because a small amount of Chs3 can still be found on the plasma membrane in this mutant (Fig. 3.1B, 2B). Therefore, this likely reflects the lack of sensitivity of the assay in detecting low amounts of *in vivo* budding. Nevertheless, Chs3 was efficiently incorporated into COPII in *pfa4*Δ*ubp3*Δ double mutants, and as expected, the *chs7*Δ budding defect was not suppressed in a *chs7*Δ*ubp3*Δ mutant. All strains were also able to incorporate the ER-Golgi SNARE protein Sec22 into COPII vesicles, showing they were not generally defective in COPII budding (Fig. 3.5).

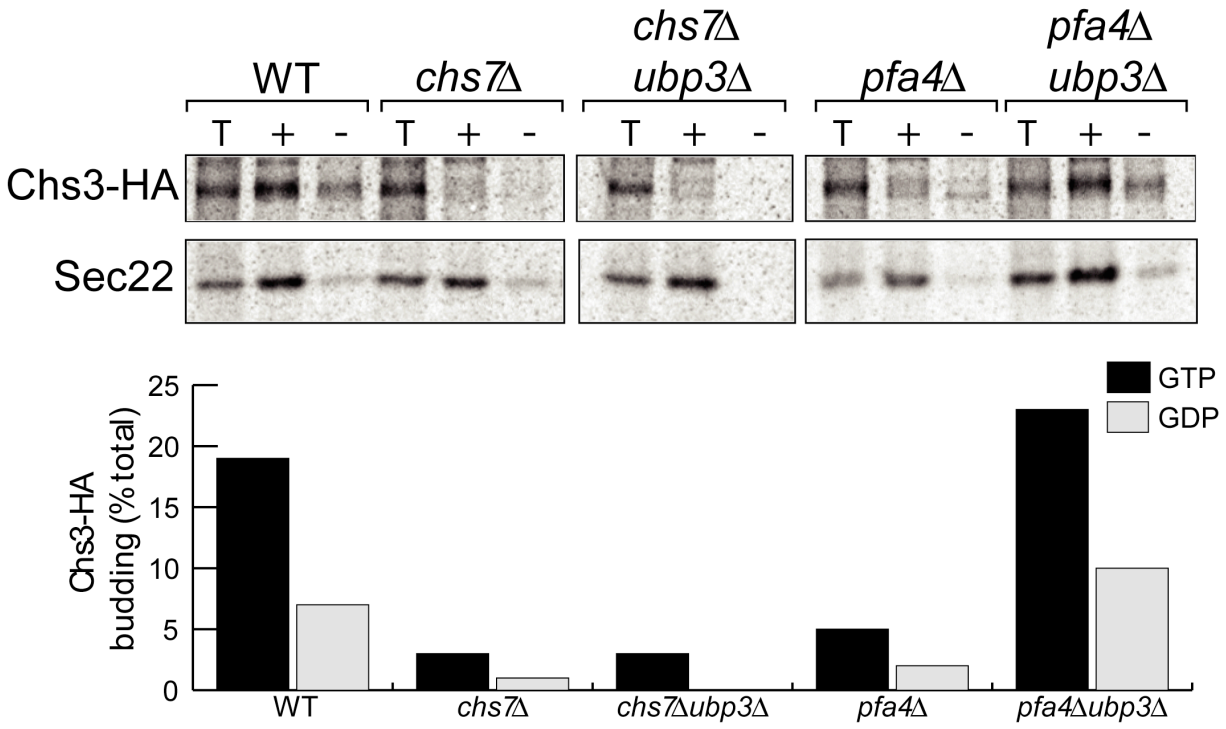

Figure 3.5. Chs3 is exported in COPII coated vesicles in *pfa4*Δ*ubp3*Δ.
In vitro COPII budding reactions were performed on ER membranes isolated from WT (KLY87), *chs7*Δ (KLY101), *pfa4*Δ (KLY89), *pfa4*Δ*ubp3*Δ (KLY92), and *chs7*Δ*ubp3*Δ (KLY103) expressing genomically tagged Chs3-3xHA. Cells were chased with 35S-methionine/cysteine for 5 min, permeabilized, and incubated with COPII proteins in the presence of GTP (+ lanes) or GDP (- lanes). Percentage of Chs3 budding is relative to the total Chs3 from the donor membranes (T). Chs3-3xHA and the ER/Golgi SNARE Sec22 were immunoprecipitated and analyzed by SDS-PAGE and PhosphorImage analysis.

Although Chs3 cell surface localization was also restored in the *pfa4*Δ*sec21-1* mutant (Fig. 3.4A, B), Chs3 was not incorporated into COPII vesicles in this genetic background (E. Miller, personal communication). Since the Sec61-RFP marker shows a largely normal ER morphology in *pfa4*Δ*sec21-1* (Fig. 3.4A), the restored cell surface transport of Chs3 in these mutants is unlikely due to an indirect effect on ER structure or secretion, although the ER export of other secreted proteins in these mutants should be examined to substantiate this conclusion. These findings support the observations that COPI suppression happens only at a post-ER level influencing retrieval, whereas loss of *UPB3* affects an ER-localized retention event involving COPII export, as well as the retrieval by COPI.

3.2.6. UNPALMITOYLATED CHS3 ESCAPES AGGREGATION IN *UBP3*Δ CELLS

When cell surface expression of the misfolded polytopic Gap1 was rescued by alleviating ERAD-mediated ER retention, there was a partial rescue of its misfolding defect (Kota et al., 2007). Misfolded Chs3 forms molecular aggregates when ER retained in *chs7*Δ and *pfa4*Δ, but not wild-type cells (Lam et al., 2006), suggesting that the cell-surface localized Chs3 in *pfa4*Δ*ubp3*Δ is refolded. We therefore examined the aggregation state of Chs3 by chemical crosslinking with the crosslinker dithiobis[succinimidyl] propionate (DSP) (Kota and Ljungdahl, 2005; Lam et al., 2006). As expected, Chs3 formed high molecular weight smears in *pfa4*Δ lysates treated with DSP (Fig. 3.6). These aggregates were absent in *pfa4*Δ*ubp3*Δ double mutants, and Chs3 maintained its wild-type, monomeric form despite increasing crosslinker, similar to wild-type cells (Fig. 3.6). Although Chs3 aggregates to a similar degree in both *chs7*Δ and *pfa4*Δ mutants (Lam et al, 2006), *UBP3* deletion did not resolve aggregation in the *chs7*Δ background (Fig. 3.6). This corroborates our earlier observations that the nature of Chs3 misfolding – and hence the mechanism of ER retention – differs between *chs7*Δ and *pfa4*Δ mutants. The restored COPII export of Chs3 in *pfa4*Δ*ubp3*Δ cells is likely due to alleviation from aggregation that is independent of palmitoylation.

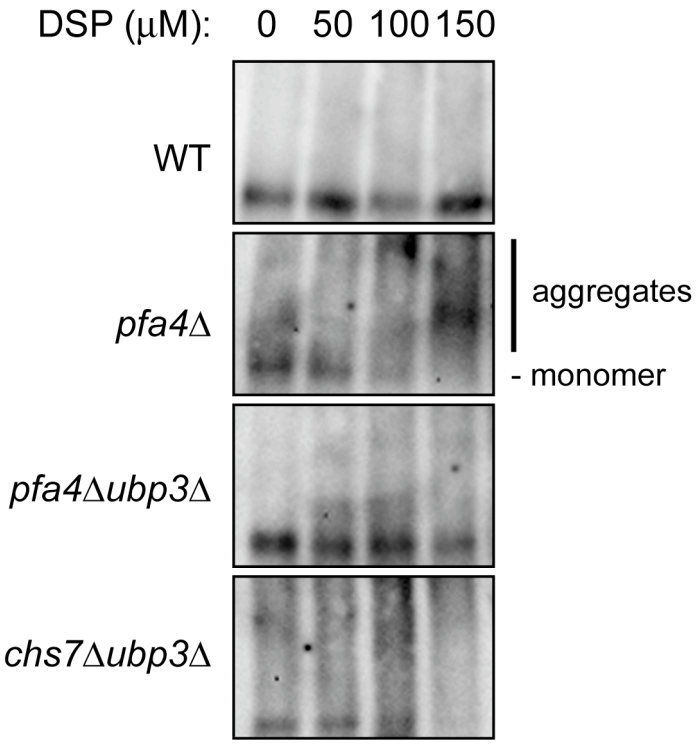

Figure 3.6. Chs3 aggregation in *pfa4*Δ is relieved by deletion of *UBP3*.
Lysates from WT (KLY87), *pfa4*Δ (KLY88), *pfa4*Δ*ubp3*Δ (KLY92), and *chs7*Δ*ubp3*Δ (KLY103) cells expressing Chs3-3xHA were crosslinked using the indicated concentrations of DSP. Treated lysates were subjected to SDS-PAGE and Chs3 was detected by Western blotting with α-HA mAb.

3.3. DISCUSSION

Polytopic proteins, many of which are cell surface transporters and receptors crucial to cell survival, are highly scrutinized for folding and assembly at the ER. The quality control mechanisms for these proteins are specific to both substrates and folding defects involved, and at present are still not well characterized.

Here, we report that ER retention of the misfolded, palmitoylation-deficient Chs3 is not mediated by the common ERAD machinery that recognizes misfolded membrane proteins. Instead, we present evidence this process is influenced by the Bre5/Ubp3 deubiquitination complex. Deletion of this complex restores ER export of palmitoylation-deficient Chs3 and wild-type cell surface expression. Our results suggest that ER retention of unmodified Chs3 happens through two mechanisms that can be affected by *ubp3*Δ: First, unlipidated Chs3 is statically retained by exclusion from COPII export vesicles, which is likely due to protein aggregation that is rescued in *ubp3*Δ mutants. Second, a COPI retrieval pathway dynamically returns unpalmitoylated Chs3 to the ER. Since loss of Ubp3 deubiquitination decreases COPI levels (Cohen et al., 2003b), it therefore also indirectly inhibits this Chs3 retrieval pathway. These quality control mechanisms are specific to palmitoylation-deficient Chs3, as they do not appear to play a role in retention of similarly aggregated Chs3 in *chs7*Δ.

3.3.1. THE BRE5/UBP3 DEUBIQUITINATION COMPLEX IN CHS3 TRANSPORT

Through its role in removing ubiquitin from various substrates, the Bre5/Ubp3 complex is implicated in pathways such as the regulation of yeast pheromone signaling (Wang and Dohlman, 2002), ER-Golgi transport (Cohen et al., 2003a; Cohen et al., 2003b), cytoplasm-to-vacuole trafficking (Baxter et al., 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 (Cohen et al., 2003a; Cohen et al., 2003b; Kraft et al., 2008; Wang et al., 2008); in others, the ubiquitinated protein is not destabilized but has altered function (Baxter et al., 2005). Conversely, *UBP3* deletion stabilizes the Ste7 kinase in the pheromone response pathway, and

the accumulation of polyubiquitinated Ste7 is correlated with increased activity (Wang and Dohlman, 2002). The differences observed likely reflect the diverse effects that ubiquitination can have on each of these specific substrates.

We considered the Ubp3 substrates that could affect trafficking of unpalmitoylated Chs3 at the ER. Specifically, loss of Ubp3 leads to an accumulation of ubiquitinated forms of the COPI subunit Sec27 (β'-COP), resulting in increased proteasomal degradation (Cohen et al., 2003b). Although Ubp3 is also known to affect anterograde transport through regulation of COPII (Cohen, 2003a), inhibition of forward transport does not seem a plausible mechanism for restoring cell surface targeting of Chs3. Our results support a model in which misfolded Chs3 is exported from the ER at low levels and subjected to later quality control at the Golgi. Therefore, the loss of COPI-mediated Golgi-to-ER retrieval owing to *UBP3* deletion results in restored cell surface transport of Chs3. ER retrieval as a quality control mechanism for polytopic proteins has been a controversial matter. It was originally thought that misfolded soluble proteins such as CPY*, and proteinase A (PrA*) require ER-to-Golgi transport and retrieval for ER quality control, whereas misfolded transmembrane proteins (Ste6-166p, Sec61-2p, Vph1*) are statically retained (Caldwell et al., 2001; Vashist et al., 2001). However, later work showed that a misfolded version of the iron transporter Fet3, a type I transmembrane protein, is transported out of the ER and retrieved (Sato et al., 2004). Our results suggest retention of unpalmitoylated Chs3 is in part a dynamic process maintained by ER export and subsequent retrieval from the early Golgi by COPI. Since palmitoylation is catalyzed in the cytosol (Linder and Deschenes, 2004), it is likely that lack of Chs3 palmitoylation culminates in a cytosolic lesion. The retrieval of Chs3 may be different from previous observations that membrane proteins with cytosolic lesions are statically retained while those with luminal lesions undergo retrieval (Vashist and Ng, 2004).

Although Rer1 is the only known retrieval receptor for misfolded polytopic proteins at present, it does not appear to be involved in the retrieval of Chs3. That COPI preferentially binds palmitoylation-deficient Chs3 indicates it recognize misfolded proteins directly. Chs3 does not harbour any of the canonical signals for COPI retrieval, but lack of palmitoylation could change the conformation of Chs3 to expose a novel COPI signal. Currently, however, we cannot exclude the possibility that additional chaperones or COPI adaptors exist at the early Golgi to recognize misfolded Chs3.

In wild-type cells, an interaction between Chs3 and COPI could still be observed (albeit at much reduced levels compared to *pfa4*Δ). This suggests that COPI may serve as a quality control mechanism even in wild-type cells by recognizing newly synthesized Chs3 that has escaped palmitoylation and returning it to the ER for another round of lipidation. Thus, the purpose of exporting misfolded, unlipidated Chs3 may be to provide a detour from degradative fates until the protein has a chance to be repalmitoylated at the ER. A similar function has been suggested for the retrieval of misfolded Fet3 (Sato et al., 2004).

3.3.2. MISFOLDED CHS3 IS SUBJECTED TO NON-CLASSICAL, ERAD-INDEPENDENT RETENTION MECHANISMS

The observation that *ubp3*Δ suppresses the COPII export defect in *pfa4*Δ indicates that, in addition to dynamic retrieval by COPI, a static retention mechanism also exists. One possibility is that loss of Ubp3 destabilizes an ER retention component. Although ERAD machinery appears to retain misfolded forms of Gap1 (Kota et al., 2007) and the soluble carboxypeptidase Y (CPY) (Kincaid and Cooper, 2007), inhibiting ERAD does not result in restored export of misfolded forms of Sec61 (Kincaid and Cooper, 2007) or Yor1 (Pagant et al., 2007). It has also been reported that the unassembled vacuolar ATPase Vph1, which is ER-retained, is degraded in a manner independent of the Ubc6 and Ubc7 E2 enzymes, or the Hrd1 complex (Hill and Cooper, 2000). Alternate pathways are thought to exist but are currently unknown. One alternative retention mechanism, at least in the case of Chs3, may be monitored by the Ubp3 complex. Similar to unassembled Vph1, ER export of misfolded Chs3 is not affected by disruption of the known ERAD pathways involving Ubc6 and Ubc7, or the E3 ligases Hrd1 or Doa10. Although we cannot rule out a redundant function of the E3s, it is likely that Chs3 is not retained through the Hrd1-specific ERAD-L/ERAD-M or the Doa10-specific ERAD-C pathways for lumenal/transmembrane and cytosolic lesions, respectively. Thus, Ubp3 may be regulating the levels of a novel ER retention or quality control component through deubiquitination. Alternatively, Ubp3 could affect chaperones that expose COPII export signals or restore folding of Chs3. In either case, this results in the stabilization of unpalmitoylated Chs3 by diverting it from degradation.

The specific misfolding defect caused by the lack of palmitoylation may contribute to the different retention mechanisms used for Chs3 compared to other misfolded polytopic proteins. These differences also highlight that ER retention mechanisms demonstrate high substrate and lesion specificity. Further, a role for Ubp3 in ER quality control has not been previously reported; it would therefore be interesting to investigate whether this deubiquitination complex has a more general function in ERAD-independent quality control of other proteins.

3.3.3. LACK OF PALMITOYLATION AND LACK OF CHAPERONE ASSOCIATION PRODUCE DIFFERENT MISFOLDING LESIONS

Previous work demonstrated that Chs3 palmitoylation might promote folding, as Chs3 forms similar molecular aggregates in both *chs7*Δ and *pfa4*Δ mutants (Lam et al., 2006). Like the Gap1-specific chaperone Shr3, Chs7 is hypothesized to be a Chs3-specific folding factor that prevents aberrant interactions between transmembrane domains as the polytopic protein is inserted into the ER membrane (Kota and Ljungdahl, 2005). Unlike palmitoylation, chaperone association appears to be absolutely required for ER export: while a small proportion of Chs3 can be observed at the budneck in *pfa4Δ* strains, Chs3 is localized to the ER exclusively in *chs7*Δ mutants. Our data now suggest that misfolded Chs3 in *pfa4*Δ strains is ER retained by different mechanisms than in *chs7*Δ strains. Although still palmitoylated (Lam et al., 2006), Chs3 may be much more severely misfolded in cells lacking *chs7*Δ, while the unpalmitoylated Chs3 in *pfa4*Δ cells may represent a more stable form. It is also possible that lack of palmitoylation in *pfa4*Δ cells creates a misfolding lesion that is recognized by ER surveillance machinery that is regulated by Ubp3, but misfolding due to lack of Chs7 involves different retention factors. Chs3 could also be excluded at ER export sites in *chs7*Δ mutants, contributing to its failure to be recognized by later quality control factors. The precise molecular function of Chs7 is unknown, and, due to its potentially greater contribution to folding and the involvement of differing retention pathways, warrants further study.

3.3.4. DECOUPLING PALMITOYLATION AND FOLDING

Palmitoylation assists in folding and ER export of Chs3 most likely by regulating transmembrane domain tilting (or hydrophobic matching) in the ER membrane (Lam et al., 2006). A similar function has been demonstrated for the Wnt signaling protein, LRP6 (Abrami et al., 2008). However, our data indicate that an export competent structure can be achieved without this lipidation event, at least in the context of the *UBP3* deletion. The effects of Ubp3 are two-fold in that its loss restores COPII-dependent export of unpalmitoylated Chs3, and inhibits a retrieval pathway through deubiquitination of COPI. However, in *pfa4*Δ*sec21-1* mutants, unpalmitoylated Chs3 is both aggregated and not packaged into COPII vesicles (E. Miller, personal communication), suggesting that restored COPII export is tightly coupled to a properly folded or non-aggregated conformation. The stringency of ER quality control for what is "export competent" may not be absolute, as long as a protein is sufficiently – but not necessarily completely – folded. We propose a model where palmitoylation-deficient Chs3 is subject to two levels of quality control: the first being an ER-localized, Ubp3-dependent step that monitors aggregation and COPII export, and a second post-ER COPI retrieval step that is distinct from refolding, and indirectly regulated by Ubp3.

Aggregation and ER retention resulting from the misfolding of polytopic proteins have been implicated in many human diseases (Hebert and Molinari, 2007). Our findings are important because they show that aggregation caused by the absence of palmitoylation can be overcome through the inhibition and/or activation of other pathways, such as those regulated by Bre5/Ubp3 deubiquitination. They also point to the existence of a novel ER retention mechanism that is independent of known ERAD pathways. For disease proteins that misfold due to a lack of palmitoylation or other lipidation event, suppression of these other quality control mechanisms may provide strategies for of lipid-independent refolding.

3.4. EXPERIMENTAL PROCEDURES

3.4.1. STRAINS, PLASMIDS, AND MEDIA

General molecular biology methods were as described in (Conibear and Stevens, 2000; Conibear and Stevens, 2002). 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. Activated alleles of PKC1 and MKK1 (pRS316:*GAL1-PKC1*^{R398A} and pRS316:*MKK1*^{S386P}) were gifts from David Levin.

Integration of C-terminal tags (GFP, 3xHA, or 13xMyc) into the *CHS3* locus was by the PCR tagging method and primer design 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 knockout strains (*pfa4*Δ::nat^{R} or *chs7*Δ::nat^{R}) were created by PCR amplification of the nat^{R} cassette from the p4339 pCRII-TOPO::nat^{R}MX4 plasmid (Tong et al., 2001) with gene-specific primers. The resulting PCR products were transformed into yeast and confirmed by PCR. The C-terminal TAP (Tandem Affinity Purification) tagged *SEC27-TAP-HIS3MX6* strain in the BY4741 background was obtained from the yeast TAP-fusion Library (Open Biosystems, Huntsville, AL). The temperature sensitive *sec21-1* mutant CKY69 (MATa *ura3-52 sec21-1*) and its congenic wild-type CKY10 (MATa *ura3-52 leu2-3,112*) were gifts from Chris Kaiser.

3.4.2. CW FLUORESCENCE SCREEN FOR *PFA4*Δ SUPPRESSORS

The Synthetic Genetic Array method (Tong et al., 2001) was used to generate genome-wide haploid *pfa4*Δ*xxx*Δ double mutants. The array-style CW fluorescence screen on this double mutant collection was performed and analyzed as described in Lam et al., 2006 and Burston et al., 2008. Briefly, the BY4741 *MATa* collection were pinned on YPD + 50μg/mL CW plates by a Virtek automated colony arrayer (BioRad, Hercules, CA) in a condensed 1536-array format. For fluorescence spot tests on CW, 4μL of saturated overnight yeast culture in YPD was spotted directly onto YPD media containing 50μg/mL CW. Colonies were grown in the dark at 30°C for 5 days (for arrays), and 2 days (for spots), and growth and fluorescence images obtained using

digital scanner and a Fluor S Max Multi-imager (BioRad), respectively. The resulting images were analyzed by a densitometry program.

3.4.3. 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.

3.4.4. BIOCHEMICAL ASSAYS

Protease assessibility

The protease assessibility assay was modified from a described protocol (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 NaN_3, 10mM NaF). Cells were resuspended in 350uL PB and incubated for 30 min at 30°C. Half were treated with a final concentration of 2mg/mL Pronase (Calbiochem-Novabiochem, La Jolla, CA) at 37°C for 1h and the other half mock treated with equal volume PB. Cells were collected at 6500rpm and resuspended in 225uL PB and 50uL of 100% TCA. Glass beads were added and samples were vortexed for 10 min. The supernatant was collected and remaining glass beads were washed twice with 5%TCA, with the supernatant from the washes added to the samples. Samples were spun at 4°C for 10min at 14000rpm. Pellets were resuspended in SDS-PAGE sample buffer and analyzed by Western blotting by α-HA (1:1000, Covance) or α-PGK (1:800) mAbs followed by HRP-labeled anti-mouse secondary antibody (1:10000, Jackson ImmunoResearch). Blots were developed with ECL and imaged on a Fluor S Max Multi-imager.

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).

COPII Budding

In vitro COPII budding assays was 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).

Pulse-chase studies

Pulse-chase analysis was described previously (Pagant et al., 2007). Briefly, log phase cells expressing Chs3-3xHA were harvested and resuspended in synthetic medium lacking methionine/cysteine, and incubated for 15min at 30°C. Cells were metabolically labeled for 5 min by adding 30µCi of Express protein labeling mix (MP Biomedicals) per OD_{600} unit of cells. 2OD of cells were chased with 10 mM L-cysteine, 50 mM L-methionine, 4% yeast extract, and 2% glucose. At indicated times, cells were harvested in chilled tubes and treated with 20mM of sodium azide. Cells were washed once with 20mM sodium azide, respuspended in 100uL 1% SDS, and lysed 15 min with glass beads at 4°C. Lysates were heated at 55°C for 5 min, diluted with 5 volumes of 50 mM Tris, pH 7.5, 160 mM NaCl, 1% Triton X-100, and 2 mM NaN_3, and cleared by centrifugation. Chs3-3xHA was immunoprecipitated from the cleared lysate and analyzed by SDS-PAGE as described above.

3.4.5. 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 30 μL of a 75% slurry of IgG–Sepharose (GE Healthcare) for 1 h at 4°C to immunoprecipitate TAP-tagged proteins. Sepharose beads were washed twice in lysis buffer, resuspended in sample buffer, and analyzed by SDS-PAGE. Coimmunoprecipitated proteins were detected by Western blotting with rabbit α-TAP (CAB1001, 1:2000, Open Biosystems) and mouse α-Myc (1:1000 Upstate) antibodies.

3.4.6. PROTEIN CROSS-LINKING

Cross-linking of yeast cell lysates with DSP was performed as previously described (Kota and Ljungdahl, 2005; Lam et al., 2006) with the exception that DSP was added to 20 μg of protein in 40 μL PBS.

Table 3.1. Yeast strains used in Chapter 3

Strain	Genotype	Source
BY4741	*MATa his3-1 leu2-0 met15-0 ura3-0*	Open Biosystems
BY4741-2835	BY4741 *chs7*Δ::kan^R	Open Biosystems
BY4741-1694	BY4741 *pfa4*Δ::kan^R	Open Biosystems
BY4741-6078	BY4741 *bre5*Δ::kan^R	Open Biosystems
BY4741-6148	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
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
KLY76	BY4742 *CHS3*-3xHA::kan^R	This study
KLY77	BY4742 *CHS3*-3xHA::kan^R *pfa4*Δ::nat^R	This study
KLY78	BY4742 *ubc6*Δ::*HIS3 ubc7*Δ::*LEU2 CHS3*-3xHA::kan^R	This study
KLY79	BY4742 *ubc6*Δ::*HIS3 ubc7*Δ::*LEU2 pfa4*Δ::nat^R *CHS3*-3xHA::kan^R	This study
KLY83	BY4741 *bre5*Δ::kan^R *pfa4*Δ::nat^R	This study
KLY84	BY4741 *ubp3*Δ::kan^R *pfa4*Δ::nat^R	This study
KLY85	BY4741 *bre5*Δ::kan^R *pfa4*Δ::nat^R *CHS3*-GFP::*HIS3*	This study
KLY86	BY4741 *ubp3*Δ::kan^R *pfa4*Δ::nat^R *CHS3*-GFP::*HIS3*	This study
KLY87	BY4741 *CHS3*-3xHA::*HIS3*	This study
KLY88	BY4741 *pfa4*Δ::nat^R *CHS3*-3xHA::*HIS3*	This study
KLY101	BY4741 *chs7*Δ::nat^R *CHS3*-3xHA::*HIS3*	This study
KLY89	BY4741 *bre5*Δ::kan^R *CHS3*-3xHA::*HIS3*	This study
KLY90	BY4741 *ubp3*Δ::kan^R *CHS3*-3xHA::*HIS3*	This study
KLY91	BY4741 *bre5*Δ::kan^R *pfa4*Δ::nat^R *CHS3*-3xHA::*HIS3*	This study
KLY92	BY4741 *ubp3*Δ::kan^R *pfa4*Δ::nat^R *CHS3*-3xHA::*HIS3*	This study
KLY103	BY4741 *ubp3*Δ::kan^R *chs7*Δ::nat^R *CHS3*-3xHA::*HIS3*	This study
KLY105	BY4741 *bre5*Δ::kan^R *chs7*Δ::nat^R *CHS3*-GFP::*HIS3*	This study
KLY106	BY4741 *ubp3*Δ::kan^R *chs7*Δ::nat^R *CHS3*-GFP::*HIS3*	This study
KLY93	MATa *ura3-52 leu2-3,112 CHS3*-GFP::kan^R *SEC61*-RFP::*URA3*	This study
KLY94	MATa *ura3-52 leu2-3,112 pfa4*Δ::nat^R *CHS3*-GFP::kan^R *SEC61*-RFP::*URA3*	This study
KLY95	MATa *ura3-52 sec21-1 CHS3*-GFP:: kan^R *SEC61*-RFP::*URA3*	This study
KLY96	MATa *ura3-52 pfa4*Δ::nat^R *sec21-1 CHS3*-GFP:: kan^R *SEC61*-RFP::*URA3*	This study
KLY97	MATa *ura3-52 leu2-3,112 CHS3*-3xHA::kan^R	This study
KLY98	MATa *ura3-52 leu2-3,112 pfa4*Δ::nat^R *CHS3*-3xHA::kan^R	This study
KLY99	MATa *ura3-52 sec21-1 CHS3*-3xHA::kan^R	This study
KLY100	MATa *ura3-52 pfa4*Δ::nat^R *sec21-1 CHS3*-3xHA::kan^R	This study
KLY107	BY4741 *CHS3*-13xMyc::kan^R	This study
KLY108	BY4741 *CHS3*-13xMyc::kan^R *SEC27*-TAP::*HIS3*	This study
KLY109	BY4741 *CHS3*-13xMyc::kan^R *SEC27*-TAP::*HIS3 pfa4*Δ::nat^R	This study

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CHAPTER 4. CONCLUSIONS AND FUTURE DIRECTIONS

4.1. OVERVIEW

The overall goal of this work was to develop the chitin synthase Chs3 as a model for studying intracellular transport of polytopic membrane proteins using high-throughput techniques. This yeast protein was chosen due to the trafficking pattern and machinery it shares with mammalian counterparts such as cell surface transporters. At the start of this project, several transport factors essential for Chs3 cell surface targeting had been characterized. However, little was known about factors that mediate regulated transport. This prompted a systematic approach to uncover additional factors on a genome-wide basis. A new assay for cell surface Chs3 levels was therefore developed to screen the yeast gene knockout arrays. This led to the discovery that a novel gene, *PFA4*, encoding for a protein containing a well-conserved DHHC domain, was required for Chs3 transport to the plasma membrane. At the time, very few DHHC proteins had been characterized, and it was hypothesized that these proteins may represent a family of protein acyltransferases (PATs) which influence protein trafficking through palmitoylation. The findings described in Chapter 2 demonstrated that Pfa4-mediated palmitoylation of Chs3 is a requisite for ER export, and this modification assists in proper folding. These were novel findings for the roles of a previously uncharacterized PAT and for palmitoylation at the ER.

At the time this work was carried out, the diverse regulatory functions of this newly discovered family of PATs and their substrates were just being realized. Several other DHHC proteins have since been studied, but the functions of many remain unidentified, especially those in the mammalian genome. The unknowns of this exciting and growing field motivated further studies on the role of palmitoylation in protein folding, trafficking and quality control at the ER. A second genetic screen was performed, this time to find factors that inhibit export of palmitoylation-deficient Chs3. Chapter 3 describes the discovery and characterization of the role of the Ubp3 deubiquitination enzyme (DUB) in ER retention. This subsequent work suggested Ubp3 may retain misfolded Chs3 at the ER through at least two trans-acting pathways: exclusion from COPII exit vesicles (static retention), and retrieval in COPI vesicles from the early Golgi (dynamic retention). Moreover, in the absence of Ubp3, ER export of Chs3 can be restored without repalmitoylation, possibly through a refolding event. These latter findings point to the existence of potentially new ER quality control (QC) pathways that are mediated through Ubp3, which can be targeted to restore cell surface function of Chs3.

The contribution of this work to the present knowledge of palmitoylation and ER transport has been discussed separately in Chapters 2 and 3. In this closing chapter, attention will be given to the new questions and avenues of research in light of the data gathered, possible future experimental directions, and implications for understanding pathologies attributable to protein misfolding and retention.

In particular, the focus will be on the following areas:

1) The precise molecular roles of Pfa4-mediated palmitoylation in ER export,
2) The ways in which ubiquitination may affect Chs3 trafficking,
3) QC stringency and the contribution of retrieval mechanisms in ER retention,
4) Therapeutic targets for misfolding diseases, with an emphasis on palmitoylation and ubiquitination.

4.2. PALMITOYLATION

4.2.1. MECHANISMS OF PALMITOYLATION-MEDIATED FOLDING

This work reported a new function for palmitoylation in the ER export of Chs3, but aside from precluding aggregation, it is not clear how palmitoylation contributes to export mechanistically. To understand why palmitoylation-deficient Chs3 is subjected to ER retention mechanisms, it will be important to determine how it is misfolded, as differing structural defects recruit distinct retention factors and can thus bring about different trafficking and degradative fates.

4.2.1.1. Hydrophobic mismatch

The most efficient export in COPII vesicles occurs when proteins have an optimal transmembrane domain (TMD) length of 21 amino acids, a length favored by a large majority of single spanning membrane proteins (Nufer et al., 2003; Abrami et al., 2008). Proteins with longer TMDs than the membrane in which they are situated are expected to expose hydrophobic regions, a phenomenon known as "hydrophobic mismatch" (Bowie, 2005). At the ER, this is anticipated to occur frequently for membrane proteins designed to be expressed at the plasma membrane, as the eukaryotic ER bilayer is approximately 5Å thinner than that of the plasma membrane (Mitra et al., 2004). Exposed hydrophobic regions are expected to form inappropriate interactions with each other, or may be recognized by chaperones. Matching may be achieved through various structural rearrangements such as oligomerization, "tilting" of TMDs along the bilayer plane, or incorporation into different lipid environments. At present, many studies on hydrophobic mismatch involve synthetically manufactured transmembrane helices and membranes (for example, see Krishnakumar and London, 2007).

Recent work by the van der Goot group provided one of the first convincing demonstrations that palmitoylation at juxtamembrane cysteines affected the tilt of the Wnt signaling protein LRP6, resulting in hydrophobic matching of its single, 23 amino acid-long TMD to the ER bilayer and proper COPII export (Abrami et al., 2008). The observation that palmitoylation of Chs3 prevented aggregation suggested that it could also assist in a conformational change or TMD tilting, protecting Chs3 from aberrant hydrophobic interactions. Unfortunately, the topology of

Chs3 is unclear at present, so precise structural changes cannot be easily determined. However, our preliminary data suggest that Chs3 can be palmitoylated at one or more cysteines located close to a putative TMD (Appendix B), suggesting a possible function in TMD rearrangement. For a large, polytopic protein like Chs3, it is feasible that palmitoylation affecting one TMD may also lead to rearrangement of multiple TMDs.

Palmitoylated Chs3 could also be targeted to lipid-rich rafts at the ER, which can potentially provide confined regions of matching for membrane proteins through selective incorporation of lipids with longer acyl chains. Proper cell surface transport of several yeast polytopic proteins, such as Pma1 and the Tat2 tryptophan permease, are raft-dependent (Bagnat et al., 2001; Umebayashi and Nakano, 2003), although they do not require raft association for ER export. Palmitoylation may increase the hydrophobicity of Chs3 and thus promote incorporation into these microdomains. However, density gradient fractionation methods could not detect cofractionation of palmitoylated Chs3 with the known raft marker Pma1 in Triton-X 100 detergent-resistant membranes. Instead, cofractionation with a non-raft marker, the vacuolar alkaline phosphatase ALP, was observed (Appendix C), suggesting that Chs3 is not a raft-associated protein.

4.2.1.2. "Thiol-mediated" retention

Another possible cause of Chs3 misfolding is that palmitoylation deficiency exposes free cysteines, resulting in aberrant interactions. It is well known that disulfide bond formation between cysteine residues, also called oxidative folding, is vital to cell survival. Formation of disulfide bridges between two cysteine residues of the gonadotropin hormone-releasing hormone receptor GnRHR, a G protein-coupled receptor implicated in hypogonadotropic hypogonadism, is important for both folding and ER export (Conn et al., 2007). Oxidative folding occurs in the ER lumen almost exclusively, and is catalyzed by the protein disulfide isomerase Pdi1 (Tu and Weissman, 2004). Exposed thiol groups from unpaired cysteines are recognized by ER quality control (ERQC), and can form mixed disulfides with proteins in the PDI family, which leads to thiol-mediated retention (van Anken and Braakman, 2005). A single exposed cysteine is often sufficient to recruit ERQC as well as change protein conformation (Kim and Arvan, 1998).

Because the DHHC domains that catalyze palmitoylation and the modified cysteines are cytosolic (Linder and Deschenes, 2004), the unpalmitoylated Chs3 cysteine is not expected to be exposed to lumenal quality control factors such as PDI. However, the determination of the final topology of polytopic membrane proteins is by TMD interactions and charge distribution. During translocation, topology changes allow inter-TMD loops to "sense" whether they are on the appropriate side of the ER membrane, such that the positive charges will be lumenal (van Anken and Braakman, 2005). Whether a loop is cytosolic or lumenal, therefore, determines the factors with which it interacts. If the lack of palmitoylation causes sufficient changes in Chs3 topology to rearrange the unmodified cysteine into the lumen of the ER, it may be recognized by lumenal chaperones and retained. However, this would imply that palmitoylation occurs relatively early during the folding or translocation process, thereby having a major contribution to Chs3 structure. This possibility is not supported by our data, as some non-palmitoylated Chs3 can leave the ER, and it also appears to be functional once at the cell surface.

Free cysteines could also be modified by other post-translational moieties, which may cause structural changes or recruit additional binding factors. A recent report showed that the E2 enzyme Ubc7, which is an ERAD target when misassembled, can be polyubiquitinated at a cysteine residue instead of the classical lysine (Ravid and Hochstrasser, 2007). A similar finding was reported for the major histocompatibility complex class I molecule, where monoubiquitination of a cysteine mediates endocytosis (Cadwell and Coscoy, 2005). It is conceivable that an unpalmitoylated cysteine on Chs3 could be a target for ubiquitination, and recognized by quality control factors. Thus, Chs3 could be subjected to thiol-mediated retention but not in the classic sense, in that cytosolic rather than lumenal chaperones may recognize or bind directly to the unpalmitoylated cysteine, which could in turn produce aberrant interactions and aggregation.

These different possibilities merit further studies on how palmitoylation affects Chs3 folding, which may elucidate the link between palmitoylation and ER export at a molecular level. Future work could examine topological and structural properties of unpalmitoylated Chs3, and also investigate the binding of factors that recognize exposed cysteines, such as Pdi1 and related proteins.

4.2.2. PFA4 SPECIFICITY

Since the discovery of the first DHHC PATs, several other groups including our own have reported the functions and *in vivo* substrates of additional DHHC proteins in both yeast and mammalian cells (Table 4.1). In addition to Pfa4, there are two other ER-localized DHHC PATs in yeast (Swf1 and Erf2), and twelve in the mammalian genome (Ohno et al., 2006). The ER-localized Erf2 and its mamalian homolog are responsible for palmitoylation and translocation of Ras to the plasma membrane, which happens through a non-classical secretory pathway (Zhao et al., 2002; Swarthout et al., 2005). Several human and yeast PATs have roles in regulating trafficking in post-ER compartments, specifically at the Golgi and cell surface (Zhao et al., 2002; Roth et al., 2002; Singaraja et al., 2002; Keller et al., 2004). No other DHHC protein besides Pfa4 has described roles in ER quality control. Since Chs3 palmitoylation is completely abolished in *pfa4*Δ mutants, and deletion of other yeast DHHC PATs did not significantly affect cell surface levels of Chs3, this corroborates other reports that PATs demonstrate high specificities towards the substrates they modify. Pfa4 may therefore be the only DHHC PAT with a specialized role in protein folding and export at the ER. It is important to note here that a few recent studies have suggested that overexpression of some PATs may compensate for the loss of another (Roth et al., 2006; Meiringer et al., 2008). Although there is no evidence that Swf1 or Erf2 can substitute for Chs3 palmitoylation under normal conditions, future experiments may want to investigate the overexpression of these PATs in *pfa4*Δ (and, conversely, overexpression of Pfa4 in other PAT-deficient backgrounds) to explore specificities of ER PATs.

PAT function is well conserved from yeast to humans. For example, heterologous expression of the huntingtin PAT HIP14 functionally rescues a mutant yeast strain deleted for the HIP14 homolog, Akr1 (Singaraja et al., 2002). The mammalian homolog of Pfa4, DHHC6, is localized to the ER (Ohno et al., 2006), but is presently uncharacterized. The complementation of *pfa4*Δ by DHHC6 has not been attempted, but it would be a worthwhile experiment to elucidate whether this putative mammalian PAT also has roles in folding or transport at the ER. Another closely related ER DHHC protein, Aph2 (DHHC16), regulates apoptosis through ER stress pathways by interacting synergistically with the c-Abl signaling kinase (Li et al., 2002) and the c-Jun activating binding protein JAB1 (Zhang et al., 2006). Although these interactions require the DHHC domain, it has not been demonstrated whether Aph2 acts as a PAT for either substrate, or that these proteins require lipid modification for ER export. Therefore, it may also

Table 4.1. Substrates, localization and functions of yeast and characterized mammalian DHHC PATs

	DHHC PAT	Localization[1]	Selected *in vivo* substrates[2]	Function of palmitoylation	Reference
YEAST	Akr1	Golgi	Yck2	PM targeting	Roth et al., 2002
	Erf2-Erf4[3]	ER	Ras2	PM targeting	Lobo et al., 2002
	Pfa3	Vacuole	Vac8	Vacuole targeting	Smotrys, et al., 2005
	Swf1	ER	SNAREs (eg. Tlg1)	Prevents ubiquitin-mediated vacuolar degradation	Valdez-Taubas and Pelham, 2005
	Pfa4	ER	• Amino acid permeases • Chs3	• Unknown • ER export	• Roth et al., 2006 • Lam et al., 2006
	Akr2	Golgi	Unknown		
	Pfa5	PM	Unknown		
MAMMALIAN	**DHHC PAT/Alias** *(yeast counterpart[1])*	**Localization[1]**	**Selected *in vivo* substrates[2]**	**Function of palmitoylation**	**Reference**
	HIP14 / DHHC17 *(Akr1)*	Golgi	neuronal proteins (eg. PSD-95 SNAP-25 huntingin)	PM targeting	Huang et al., 2004
	GODZ / DHHC7 *(Akr1)*	Golgi	$GABA_A$ γ subunit	Golgi transport	Keller et al., 2004
	DHHC9-GCP16[3] *(Erf2-Erf4)*	ER	Ras	PM targeting	Swarthout et al, 2005
	DHHC15 *(Pfa3)*	Golgi	PSD-95	PM targeting	Fukata et al., 2005
	DHHC3 *(Pfa3)*	Golgi	Cysteine-string protein	Membrane attachment	Greaves et al., 2008
	DHHC21 *(Pfa5)*	PM	eNOS	Targeting and activation	Fernandez-Hernando et al., 2006

[1] From Ohno et al., 2006.
[2] Based on first characterization of PAT.
[3] Form obligate PAT complexes. Erf2 is the DHHC-containing protein.

be interesting to test other closely related ER-localized mammalian DHHC proteins for complementation of *pfa4*Δ export defects. Such a complementing DHHC protein could well be the currently unidentified PAT of the aforementioned LRP6, which requires palmitoylation for ER exit (Abrami et al., 2008).

4.2.3. POST-ER FUNCTIONS OF PFA4

One direction this work did not pursue was to examine additional roles of Chs3 palmitoylation in post-ER compartments. For example, although the main function of LRP6 palmitoylation is ER export and quality control, it may be additionally required for cell surface function, possibly by mediating the binding of LRP6 interaction partners (Abrami et al., 2008).

A study by the Davis group demonstrated that Pfa4 is responsible for palmitoylation of the conserved family of amino acid permeases (AAPs) at a C-terminal consensus sequence, FWC (Roth et al., 2006). The consequences of lipidation on AAP trafficking are not known. Furthermore, one of the palmitoylated AAPs, the Gap1 general amino acid permease, can be properly localized to the plasma membrane when its C-terminus is truncated (Hein and Andre, 1997). Interestingly, an independent genome-wide screen in our laboratory suggested that *pfa4*Δ mutants lack cell surface activity of the Tat2 tryptophan permease, a palmitoylated AAP (M. Davey, L. Conibear, personal communication). However, palmitoylation did not appear to affect trafficking of Tat2, as Tat2-GFP was properly localized to the plasma membrane in *pfa4*Δ mutants (Appendix Fig. D.1). These results suggest that the cell surface localized population of unpalmitoylated Tat2 may be functionally defective. It may be that Pfa4-mediated lipidation of Tat2 regulates permease structure or function, but not ER export. However, since Gap1 functions without its FWC sequence, future experiments should determine permease function (and localization) of other AAPs with FWC truncations to resolve whether Pfa4-mediated palmitoylation has a general role in regulating AAPs.

It seems unlikely that palmitoylation could affect post-ER transport of Chs3. The work described in Chapter III has shown that unpalmitoylated Chs3, at least in the *pfa4*Δ*ubp3*Δ background, can achieve wild-type plasma membrane targeting, budneck localization, and chitosomal/late Golgi

staining. The last observation suggests that recycling between Golgi and endosomal compartments, a process mediated by the clathrin adaptor AP-1, is also unlikely to be affected by palmitoylation. Nevertheless, since unpalmitoylated Chs3 can exit the ER in *pfa4*Δ*ubp3*Δ (or COPI-defective *pfa4*Δ) cells, these double mutants offer an opportunity to study potential effects of palmitoylation, or the lack thereof, on post-ER Chs3 trafficking. For example, if other post-translational modifications such as phosphorylation or ubiquitination are important at the Golgi or cell surface, whether they can be influenced by palmitoylation deficiency can be studied in a *pfa4*Δ*ubp3*Δ mutant background.

4.2.4. PALMITOYL-THIOESTERASES IN REGULATION

Dynamic regulation of reversible post-translational modifications is influenced by proteins that catalyze the addition, as well as the removal, of the functional group. The enzymes responsible for the removal of palmitate, acyl-protein thioesterases (APTs), were discovered a decade before PATs, with the purification of palmitoyl-protein thioeseterase-1 (PPT1) which deacylates H-Ras *in vitro* (Camp and Hofmann, 1993). It was soon established that deficiency of this lysosomal APT causes infantile neuronal ceroid lipofuscinosis (INCL), a type of childhood neurodegenerative disease characterized by faulty lysosomal degradation and accumulation of palmitoylated proteins in neurons (Vesa et al., 1995; Calero et al., 2003). It has been recently proposed that another possible mechanism of INCL disease progression could be ER structural abnormalities resulting from accumulation of palmitoylated proteins in the ER (Kim et al., 2006; Zhang et al., 2006). Increased levels of one of these proteins, the soluble neuronal growth-associated protein GAP-43, appears to trigger the unfolded protein response, increasing caspase-12 mediated apoptosis and, subsequently, neurodegeneration (Kim et al., 2006; Zhang et al., 2006). It is not clear if PPT1 is the thioesterase directly deacylating GAP-43, but these observations show that aberrant palmitoylation in the early secretory pathway can also bring about ER defects that lead to disease presentation. GAP-43 palmitoylation has been shown to take place at the ER-to-Golgi intermediate compartment (ERGIC) (McLaughlin and Denny, 1999), possibly by a putative Golgi-localized PAT, DHHC15 (Fukata and Fukata, 2005). Although PPT1 is unlikely to be the *in vivo* APT for GAP-43 due to its localization to the lysosome lumen, inhibition of putative APTs or induction of DHHC15 may prove to be useful

therapeutic targets for restoring GAP-43 cell surface levels and treating INCL cases in which ER abnormalities are detected.

PPT1 has no homolog in yeast; the only known yeast acyl-protein thioesterase, Apt1, is responsible for deacylating the Gα protein subunit, Gpa1 (Duncan and Gilman, 2002). Acylation of Gα is important for plasma membrane association and subsequent G protein-coupled receptor signaling (Duncan and Gilman, 2002). It is also known that the trafficking of some proteins is regulated by cycles of palmitoylation and depalmitoylation, hence necessitating the action of both PATs and APTs. One such example involves the H- and N-Ras isoforms, for which acylation cycles mediate recycling between the plasma membrane and Golgi (Rocks et al., 2005). The PAT for H- and N-Ras has been identified as the DHHC9/GCP16 complex (Swarthout et al., 2005); the yeast homologs of these PATs also modify yeast Ras (Lobo et al., 2002). Ras can be deacylated *in vitro* by mammalian APT1, which may demonstrate broad *in vitro* substrate activity (Duncan and Gilman, 1998). Ras deacylation by APT1 has not been confirmed by *in vivo* studies; therefore, the existence of other Ras-specific APTs cannot be excluded. The search for putative APTs using high-throughput yeast genomics can thus be of great value, and is one of the current pursuits by others in the Conibear laboratory.

Preliminary data suggest that Chs3 may undergo depalmitoylation (N. Davis, L. Conibear, personal communication), implying this modification could be regulated for later trafficking events. However, more tests comparing the lifetime of the palmitate group to that of Chs3 turnover are needed to confirm these initial observations. These experiments, if also performed in mutants deleted for transport genes to trap Chs3 in internal compartments such as the trans-Golgi network (eg. *chs6*Δ), can determine the precise location of depalmitoylation. If Chs3 is found to be deacylated, this would also merit a search for the responsible APT. Further implications and applicability of regulating palmitoylation will be revisited in Section 4.5.

4.2.5. PALMITOYLATION AND OTHER POST-TRANSLATIONAL MODIFICATIONS

Not all palmitoylated membrane proteins require the modification for ER export, post-ER trafficking, or cell surface activity. These include single spanning proteins such as the influenza C virus CM2 and M_2 (Holsinger et al., 1995; Li et al., 2001), as well as polytopic proteins such as the CD9 tetraspannin (Kovalenko et al., 2004). On the other hand, for some proteins, palmitoylation at the ER is linked to other post-translational modifications that may facilitate export. For example, glycosylation of the secreted soluble Wnt-3a appears to precede and augment palmitoylation, and both modifications are required for ER export of Wnt-3a (Komekado et al., 2007). Conversely, palmitoylation of the polytopic V_2 vasopressin receptor (V_2R), which is required for membrane insertion, is not dependent on glycosylation (Sadeghi et al., 1997). For secreted proteins like Wnt-3a, palmitoylation mechanisms may differ and may be mediated by another class of protein acyltransferases (Linder and Deschenes, 2004). These differences could account for why glycosylation and lumenal chaperones can affect palmitoylation for soluble proteins, but not transmembrane ones. Chs3 is a glycoprotein, and it is properly core-glycosylated when ER-retained in *chs7*Δ mutants (Trilla et al., 1999). Later (Golgi-localized) glycosylation events and the site of this modification on Chs3 have not been characterized, and the function of glycosylation is presently unknown. Since some unpalmitoylated Chs3 can exit the ER, lectin chaperones such as calnexin may have already allowed the dissociation of Chs3 from glycosylation QC cycles, signifying that glycosylation of unlipidated Chs3 is mostly normal. However, the possibility that glycosylation steps occurring in the Golgi could be affected by lack of palmitoylation cannot be excluded.

Ubiquitination and palmitoylation have also been linked. At the ER, unpalmitoylated LRP6 is ubiquitinated and targeted for degradation in a lysosomal-independent manner (Abrami et al., 2008). It is unclear if ERAD is involved, or which ubiquitination enzymes are important in recognition of unpalmitoylated LRP6. However, an ubiquitination-deubiquitination cycle that functions as an ERQC system for newly synthesized LRP6 has been proposed (Abrami et al., 2008). At downstream compartments, palmitoylation of the Tlg1 SNARE in yeast (normally a target of the DHHC PAT Swf1) prevents its recognition and ubiquitination by a Golgi-localized ubiquitin ligase, thus averting vacuolar degradation (Valdez-Taubas and Pelham, 2005).

Similarly, palmitoylation of the anthrax toxin receptor inhibits ubiquitination through an unexpected physical segregation from an ubiquitin ligase that is localized in plasma membrane rafts (Abrami et al., 2006). The involvement of the deubiquitination enzyme Ubp3 in ER retention of unpalmitoylated Chs3 suggests the two post-translational modifications may also be directly coupled in regulating Chs3 transport. The following section will discuss this and other possibilities.

4.3. UBIQUITINATION

4.3.1. UBIQUITIN-MEDATED ER ASSOCIATED DEGRADATION

Whether ER retention occurs through a static or dynamic fashion, almost all misfolded polytopic proteins characterized to date (Ste6*, Yor1, Pma1-D378N, Gap1, Vph1, Fur4–430Np) ultimately activate ERAD (Galan et al., 1998; Wang and Chang, 1999; Hill and Cooper, 2000; Huyer et al., 2004; Kota et al., 2007; Pagant et al., 2007). Wild-type CFTR, when expressed in yeast, is ER-retained and recognized by the cytosolic chaperones Ssa1 (an Hsp70), Ydj1 and Hlj1 (Hsp40s), but not the lumenal BiP. Subsequently, its degradation is mediated by the E2 ubiquitin enzymes Ubc6 and Ubc7, but not the Hrd1 E3 ubiquitin ligase (Zhang et al., 2001; Youker et al., 2004). Likewise, the misfolded version of the yeast CFTR homolog, Ste6*, which displays a cytosolic lesion, has the same ERAD requirements (Huyer et al., 2004). Although the downstream requirements for unassembled Vph1 differ in that it is independent of Ubc6, Ubc7, and Hrd1, Vph1 is also recognized by Ssa1, and undergoes ubiquitin-mediated proteasomal degradation (Hill and Cooper, 2000). The ubiquitination machinery for Vph1 is not known, and the involvement of the other E3 ligase, Doa10, has not been reported.

Like Vph1, ER retention of misfolded Chs3 does not require the known ERAD pathways involving Ubc6 and Ubc7. Deletion of either the Hrd1 or Doa10 E3 ligases also did not suppress the CW fluorescence dim phenotypes of *chs7*Δ or *pfa4*Δ (Appendix Fig. A.4, Table G.1). Although these E3's have some overlapping functions, they appear to act specifically towards misfolded transmembrane proteins, in that lumenal and transmembrane lesions require Hrd1 (ERAD-L, ERAD-M), and cytosolic lesions require Doa10 (ERAD-C). Since neither misfolded form of Chs3 (in *chs7*Δ nor *pfa4*Δ) is retained by ERAD, it is likely that Chs3 is subjected to substrate-specific retention pathways. These mechanisms may also demonstrate lesion specificity, in that the Ubp3-mediated effects were only observed in *pfa4*Δ, and not *chs7*Δ, mutants. Prior to the pulse-chase studies described in Chapter III, we could not observe a decrease in steady state Chs3 levels when it is misfolded in either *chs7*Δ or *pfa4*Δ mutants. Moreover, the destabilization of misfolded Chs3 is not dramatic as observed by pulse chase experiments. Thus, even if the protein enters degradative pathways, it may be doing so inefficiently, and may rely on alternative mechanisms. At the time of writing, work is still

ongoing to address the possible redundant functions of Hrd1 and Doa10 with *chs7*Δ*hrd1*Δ*doa10*Δ and *pfa4*Δ*hrd1*Δ*doa10*Δ triple mutants, and the involvement of the proteasome for Chs3 degradation.

If Chs3 is indeed subjected to proteasomal degradation, previously unidentified ubiquitination machinery may be involved. As seen for Vph1, downstream components of ERAD could be divergent from those used for other polytopic proteins, but Chs3 may still engage similar upstream recognition chaperones. Chs3 is a large 130kD protein predicted to have at least six transmembrane segments (Appendix B, Table B.1) and large extracellular loops like Ste6. As a folding model for CFTR, the topology of Ste6 and the misfolding defect in Ste6* are well mapped, as are those for Pma1 and Fur4. On the contrary, a weakness of the Chs3 model is that its precise topology has not been defined, and the crosslinking assay is also not diagnostic enough to reveal the locations of misfolding defects. Despite this, however, palmitoylation is known to occur in the cytosol (Linder and Deschenes, 2004), and the unpalmitoylated cysteine of Chs3 is expected to face the cytosol unless major topological changes have occurred due to palmitoylation-deficiency. Interestingly, the cytosolic heat shock chaperone Hlj1 was identified in the *pfa4*Δ suppressor screen (Rank #63) (Appendix G). To test if Hlj1 can act as a Chs3 chaperone, the localization of Chs3-GFP in *hlj1*Δ*pfa4*Δ will first have to be tested for a restoration of ER export. These mutants may additionally show a stabilization of unpalmitoylated Chs3 by pulse chase if Chs3 is no longer targeted for disposal. A triple mutant of *hlj1*Δ*ydj1*Δ*pfa4*Δ may also be required due to redundancy of these Hsp40 chaperones (Youker et al., 2004). Because wild-type Chs3 would not be expected to engage the cytosolic chaperone machinery, binding of Chs3 to Hlj1 and Ydj1 can also be compared between wild-type, *pfa4*Δ, and *chs7*Δ by co-immunoprecipitation.

Our present knowledge of the factors for recognition and degradation of transmembrane proteins bearing different misfolded lesions is still vague. A better definition of these processes for misfolded Chs3 will contribute to understanding the specificity of the ER quality control system.

4.3.2. UBP3-MEDIATED DEUBIQUITINATION

Deubiquitination can regulate surface expression of a protein in a multifaceted fashion, affecting substrates directly and indirectly, through both degradation and trafficking pathways. Multiple deubiquitination enzymes could also collaborate in controlling the fate of a protein. The Ubp3 mammalian homolog, Usp10, interacts with the β' subunit of bovine COPI by yeast two-hybrid (Cohen et al., 2003b), suggesting a conserved function in Golgi and ER transport. One substrate of Usp10 is the sorting nexin Snx3 (Boulkroun et al., 2008), which is important for retrieval of proteins from the prevacuolar compartment to the Golgi (Voos and Stevens, 1998). Snx3 regulates cell surface expression of the epithelial sodium channel ENaC, a process that is induced by the hormone vasopressin. Usp10-mediated deubiquitination, which is also vasopressin-induced, prevents proteasomal degradation of Snx3, thus increasing cell surface levels of ENaC (Boulkroun et al., 2008). Interestingly, cell surface levels of ENaC can be further affected through direct deubiquitination by two other DUBs: UCH-L3 and USP2-45. Both appear to prevent the internalization of ENaC from the cell surface through antagonizing the effects of the ubiquitin ligase Nedd4-2 (Rsp5 in yeast) (Butterworth et al., 2007; Fakitsas et al., 2007). Thus, in the case of ENaC, deubiquitination upregulates cell surface levels in both trans-acting (through Snx3-mediated retrieval from the vacuole) and cis-acting (endocytosis) mechanisms.

The effects of Ubp3 on Chs3 trafficking appear opposite to those of Usp10 on ENaC, in that in the absence of Ubp3, Chs3 cell surface expression is increased. The elevated steady state levels of ubiquitinated protein conjugates observed in *ubp3*Δ mutants indicate it has many *in vivo* substrates (Baxter and Craig, 1998). Like its homolog Usp10, Ubp3 may also regulate Chs3 trafficking in various ways; these possibilities will be examined in the following sections.

4.3.2.1. Cis-regulation: Chs3 deubiquitination

The ubiquitination status of Chs3 still requires *in vivo* confirmation. However, Chs3 was found to be ubiquitinated in a high-throughput study (Peng et al., 2003), and loss of the Ubp3 DUB increases cell surface levels of unpalmitoylated Chs3. One straightforward model is that unpalmitoylated Chs3 is directly deubiquitinated by Ubp3, and increased Chs3 ubiquitination in

*ubp3*Δ strains causes ER exit by either acting as a positive signal for COPII packaging, or refolding Chs3. However, there is currently no evidence to support such direct effects of ubiquitination on COPII sorting or protein folding. Instead, one study reported that an increase, and not decrease, of Ubp3 levels and deubiquitination may in fact stabilize slowly folding proteins in cells lacking cytosolic Hsp70 chaperones (Baxter and Craig, 1998). In contrast, it has been observed that increased polyubiquitination of Ste7 (a MAPK kinase) in *ubp3*Δ mutants increases Ste7 levels and activity (Wang and Dohlman, 2002). These data suggest the effects of Ubp3-mediated deubiquitination likely vary from substrate to substrate. We therefore cannot yet exclude the possibility that *UBP3* deletion causes an increase of Chs3 ubiquitination, which leads to the observed stability in *pfa4*Δ*ubp3*Δ mutants, and allows Chs3 the time to complete its folding at the ER.

If ubiquitination of Chs3 is a signal for disposal, loss of Ubp3 would result in degradation, and not increased cell surface levels or ER export. Loss of a DUB can also deplete the available pools of cellular ubiquitin, since DUBs are crucial for recycling of ubiquitin molecules after substrates have been targeted for degradation (Millard and Wood, 2006). If Chs3 is subjected to ubiquitin-mediated degradation, a global depletion of cellular ubiquitin levels could potentially avert disposal and restore ER export of unpalmitoylated Chs3. However, deletion of the ubiquitin-encoding gene, *UBI4*, which is also known to deplete free ubiquitin pools (Baxter and Craig, 1998), did not alter the localization of unpalmitoylated Chs3-GFP (Appendix Fig. A.3). Given these considerations, direct Chs3 deubiquitination by Ubp3 is therefore unlikely. Nevertheless, determining the ubiquitination status of Chs3 in the absence of Ubp3 is still a current focus at the time of writing.

4.3.2.2. Trans-regulation: Other Ubp3 substrates

4.3.2.2.1. Trafficking substrates

For the reasons stated above, a model in which Ubp3 is indirectly affecting Chs3 transport is favored over a direct role for this DUB in regulating Chs3 ubiquitination. Since many proteins, in particular components of the trafficking machinery (Millard and Wood, 2006), can be targets for deubiquitination, Ubp3 could stabilize other factors that inhibit Chs3 transport to the cell surface. We now know that at least one such target is the COPI coat for Golgi-to-ER retrograde

transport. Although no other Ubp3 substrate besides COPI and COPII is known to be trafficking machinery, Ubp3 may well regulate post-Golgi trafficking events, since *UBP3* deletion also increases cell surface levels of wild-type Chs3. This observation also challenges the model in which Chs3 is a direct Ubp3 substrate: if Chs3 requires ubiquitination for plasma membrane internalization, for example, increased ubiquitination of Chs3 in *ubp3*Δ is expected to result in increased endocytosis. Instead, it is more likely that proteins required for Chs3 endocytosis or recycling may be regulated by Ubp3-mediated deubiquitination, as seen for the Snx3 and ENaC example.

In light of such observations, the next prudent investigation would be to find other Ubp3 substrates that may affect Chs3 trafficking. Like COPI, unpalmitoylated Chs3 may recruit different trafficking proteins, and these factors could be Ubp3 substrates. A mass spectrometry approach to compare *pfa4*Δ and wild-type cells could reveal the identity of these binding partners. Another systematic search would be to use yeast genomic techniques to create *pfa4*Δ*ubp3*Δ*xxx*Δ triple mutants, and assay for the return of the *pfa4*Δ CW dim phenotype (ie. *pfa4*Δ*ubp3*Δ suppressor screen). Conversely, assaying for a CW bright phenotype through introduction of the *pfa4*Δ mutation into an over-expression ORF library could identify substrates that are potentially stabilized by loss of Ubp3.

4.3.2.2.2. Folding and quality control substrates

Restored ER export by *UBP3* deletion appears to be correlated with two effects on unpalmitoylated Chs3: stabilization and refolding. Accordingly, other candidate Ubp3 substrates besides trafficking factors would be folding factors or QC proteins. In this case, two possibilities can be envisioned. First, Ubp3 could normally inhibit a chaperone responsible for Chs3 folding. In the absence of Ubp3, this chaperone refolds unpalmitoylated Chs3, resulting in its export. The Chs7 chaperone may be a natural candidate; however, overexpression of Chs7 does not rescue *pfa4*Δ defects, indicating that this chaperone cannot refold unpalmitoylated Chs3. The Ubp3 target in this case might be a novel Chs3 folding factor. Indeed, the reported observations that increased Ubp3 may aid protein folding in the Hsp70 mutant *ssa1ssa2*, and that *ubp3*Δ*ssa1ssa2* triple mutants shows a synthetic growth inhibition phenotype (Baxter and Craig, 1998), point to a possibility that Ubp3 could affect additional folding pathways.

Another likelihood is that Ubp3 could inhibit a QC protein that recognizes misfolded Chs3 and targets it for degradation, which would lead to the Chs3 stabilization observed in *pfa4*Δ*ubp3*Δ mutants. Because the deletion of known ERAD machinery does not rescue ER export of unpalmitoylated Chs3, the Ubp3 target could be a previously uncharacterized QC component, or an upstream recognition protein that binds to misfolded Chs3. A likely candidate in this case would be the cytosolic chaperone Hlj1, for example. This is supported by the observation that deubiquitination by Ubp3 has been shown to affect protein interaction. Specifically, the Ubp3 substrate Sec23, a COPII subunit, dissociated less from the ER and also failed to interact with another COPII subunit, Sec24 in a *ubp3*Δ mutant (Cohen et al., 2003a). Future mass spectrometry experiments should therefore also compare binding factors of misfolded Chs3 in the presence and absence of *UBP3* (ie. *pfa4*Δ and *pfa4*Δ*ubp3*Δ).

4.3.3. SUBSTRATE SPECIFICITY OF DUBS

Besides Ubp3, no other DUB in the yeast genome has a characterized role in vesicular trafficking steps at the ER, and no additional DUBs were identified in the *pfa4*Δ suppression screen. A membrane-bound form of the DUB Ubp1 was found to be ER localized, but does not appear to play a role in ERAD, as neither its overexpression nor deletion altered turnover of the ERAD substrate CPY* (Schmitz et al., 2005). Given the specificity demonstrated by most characterized DUBs, and that CPY* is a soluble protein, a role for Ubp1 in ERAD of membrane proteins cannot be excluded. This study also showed that, although the soluble form of Ubp1 stabilizes turnover of Ste6 and increases its cell surface expression, Ubp3 overexpression did not affect Ste6 levels (Schmitz et al., 2005). Overexpression of Ubp3 in *pfa4*Δ mutants should also be tested, as it may alter the stability of unpalmitoylated Chs3, and decrease Chs3 ubiquitination levels if Chs3 is an Ubp3 substrate. However, such future studies should take into account that, since Bre5 and Ubp3 act together as an obligate complex, overexpression of both subunits may be required to increase deubiquitination activity. Since Ubp3 interacts with its known substrates, co-immunoprecipitation experiments to test interaction between Ubp3 and Chs3 may help resolve whether Chs3 is a direct Ubp3 target.

At present, the main unanswered questions are whether Chs3 undergoes ubiquitination-mediated ER degradation, and whether it is subjected to deubiquitination. The key substrate of Ubp3, if it is not Chs3, also needs to be identified. In terms of Ubp3 function, the best approach would be to use the genomic tools available in yeast for systematic discovery of potential substrates as mentioned above, with a subsequent focus on those that could affect ER retention of unlipidated Chs3. Such investigations will likely reveal novel substrates for a DUB with already diverse cellular functions.

4.4. TRANSPORT QUALITY CONTROL

4.4.1. MULTIPLE ROUTES TO EXPORT COMPETENCY

Deletion of the Ubp3 DUB restores COPII packaging of Chs3 in *pfa4*Δ cells without restoring its palmitoylation, which calls into question the contribution and necessity of this lipid modification in Chs3 export. Unpalmitoylated Chs3 appears to regain its export competency by escaping aggregation in *ubp3*Δ strains. This could reflect 1) an alternative folding route taken by Chs3 to achieve a conformation acceptable by QC standards, or 2) different interactions with ER chaperones. In the first scenario, the role of the palmitate moiety is to impart a structural contribution without necessarily serving as a binding signal for requisite folding factors. Protein folding is a stochastic process that is highly dependent on thermodynamics and kinetics, and experimental models have shown that folding can occur through multiple routes (Shimada and Shakhnovich, 2002; Wright et al., 2003). Larger proteins may be especially prone to sampling several energetically similar pathways to reach the native state (Chavez et al., 2006). These multiple routes may have evolved for the acclimatization to genetic mutations or environmental alterations (Udgaonkar, 2008). The "palmitoylation route" may thus be the most energetically favourable route for Chs3 to reach the native state, but another pathway may exist to reach a similarly folded state in *pfa4*Δ*ubp3*Δ mutants that is not contingent upon palmitoylation. QC may therefore be surveying overall structural conformation. It has also been noted that, for at least some proteins, aggregation represents an intermediate and transient form during folding before the native state is reached (Ellgaard et al., 1999). Therefore, the lack of palmitoylation, although leading to aggregation, may not necessarily culminate in terminal misfolding.

In the second scenario, palmitoylation could signal the binding of chaperones that accelerate folding. Here, palmitoylation-deficient Chs3 may be taking the same folding route as wild-type Chs3, but display slower folding kinetics. This could prompt premature recognition by retention factors. Since palmitoylation of Chs3 has a protective effect on protein stability as shown by pulse chase studies, it is very likely that QC retention factors are also responsible for targeting unpalmitoylated Chs3 for disposal. Aggregation of unpalmitoylated Chs3 could reflect binding to these factors, and not necessarily misfolding itself. Ubp3 may regulate the levels of these QC

proteins, such that in their absence, unpalmitoylated Chs3 is diverted from degradation, given the chance to slowly achieve the native state, and become incorporated into COPII vesicles.

Export competency of the native state is often thought of as being determined by the display of a COPII exit signal, or the concealment of a retention signal upon proper folding (Ellgaard et al., 1999). Current research supports the coexistence of both situations to some extent. Early in its residence at the ER, a nascent protein is likely retained due to major structural defects such as exposed hydrophobic patches, which are often recognized by primary QC factors. Once properly folded, these "retention signals" become masked, allowing dissociation from retention machinery. However, the protein may then rely on motifs inherent in its protein sequence to direct subsequent transport. These motifs often recruit more tailored secondary QC proteins such as chaperones like Chs7, or dedicated transport escorts. Since some misfolded membrane proteins can leave the ER if strong export signals are present, while others cannot (Kincaid and Cooper, 2007; Pagant et al., 2007), ER exit is likely determined by competitive binding by both retention and export factors. The dynamics of these interactions may be distinct for different substrates, misfolding defects, and protein sequences. Additionally, the order in which these factors bind may also be determined by differences in the folding pathways taken.

Comparisons between wild-type, palmitoylation-deficient (*pfa4*Δ), and chaperone-deficient (*chs7*Δ) states of Chs3 would be most informative in deciphering these dynamics of folding. The COPI retrieval data suggests palmitoylation-deficient Chs3 can leave the ER whereas chaperone-deficient Chs3 cannot. Therefore, signals for COPII (or COPII adaptor) binding may still be at least partially intact in palmitoylation-deficient Chs3. Besides Chs7 and Pfa4, no other factors are known to act in ER export of Chs3. We also have not found another mutant from the initial CW screen with significantly low Chs3 cell surface activity as well as ER mislocalization. Therefore, if Chs3-specific ERQC or transport proteins are involved, they may be essential and/or show some level of redundancy. Future screens for Chs3 cell surface activity using the yeast temperature-sensitive mutant collections and overexpression libraries should be considered. More quantitative assays will also have to be developed to distinguish between misfolded and partially folded states, as well as rates of folding. At present, the DSP crosslinking assay used to study Chs3 aggregation neither distinguishes between different misfolded states nor measures degree of "misfoldedness" (eg. no difference is observed between *chs7*Δ and *pfa4*Δ). As mentioned above, retention machinery binding to unpalmitoylated Chs3 at the ER

can also phenocopy aggregation in the cross-linking assay without necessitating misfolding events. Other *in vivo* assays, such as trypsin sensitivity, are currently being developed in our laboratory to address these issues.

Despite over 25 years of progress in the field of protein folding, our comprehension of membrane protein folding, compared to that of soluble ones, is extremely inadequate (Radford, 2000). The state of *in vitro* biophysical studies is especially underdeveloped, limited to only a handful of model proteins mostly of bacterial origin (Stanley and Fleming, 2008). Therefore, the study of *misfolding* events, such as those seen for Chs3, is particularly informative for not only the structural requirements of folding, but also the roles of post-translational modifications and chaperones in the biological context. It may also be advantageous to identify alternate folding pathways that may not necessarily restore the wild-type native state, but nonetheless produce a QC acceptable form.

4.4.2. STRINGENCY OF ERQC

Because some unpalmitoylated Chs3 is still found at the cell surface even in the presence of Ubp3, this could also mean the ER surveillance system may accommodate for a low level of export of slowly folding forms. It is believed that the high stringency of ERQC evolved due to the potentially costly consequences of allowing aberrant proteins the time to eventually fold instead of rapidly disposing of such forms (Milojevic et al., 2006). However, if the QC system is too inflexible, stresses that slow protein folding could lead to extensive degradation of partially folded proteins. Indeed, both soluble and membrane proteins in mammalian cells have also been observed to leave the ER despite being misfolded (Molinari et al., 2004; Welch, 2004; Milojevic et al., 2006). Notably, ΔF508-CFTR can escape QC machinery at lowered growth temperatures, and can function as a chloride channel once at the cell surface, albeit slightly less efficiently (Welch, 2004). Likewise, unpalmitoylated Chs3 is enzymatically functional; it retains capability to form chitin as assayed by both Calcofluor White solid media fluorescence and cell wall/bud scar staining microscopy (Appendix Fig. E.1). This unlipidated form of the protein probably does not suffer from major structural defects, or at least from defects that significantly hamper catalytic activity or cell surface targeting. The QC system has perhaps evolved this flexibility

such that a very low basal level of unpalmitoylated but functional Chs3 is still exported, to ensure proper cell wall construction. In the absence of Ubp3, the QC system is overcome, allowing increased export. This apparent "reduction" in QC stringency is not unrestricted, however, and appears to apply only to some forms of partially folded Chs3 (misfolded Chs3 is completely ER retained in *chs7*Δ, for example).

Under certain stress conditions, ER degradative processes can sometimes be compromised, leading to increased export of misfolded conformers (Hebert and Molinari, 2007). Cell wall stress induces the cell integrity MAP kinase pathway in yeast, which can upregulate Chs3 transcription (Heinisch et al., 1999). Thus, it may be expected that increased expression of unpalmitoylated Chs3 under cell wall stress could potentially "flood" ERQC and allow increased export. However, preliminary data suggest that neither cell wall stress nor induction of the MAPK pathway through constitutively activated Pkc1 can induce unregulated export of unpalmitoylated Chs3 from the ER. One explanation is that the COPI retrieval pathway is still actively counteracting export (see below, Section 4.4.4); this possibility can be tested in a conditional COPI-deficient background. Another is that ERQC is not significantly affected by cell wall stress. However, it was very recently reported that the Pkc1 pathway can upregulate the unfolded protein response (UPR) (Scrimale et al., 2008). Since ERAD is one of the targets of UPR, QC functions should be intensified, not diminished, upon induction of the cell wall integrity pathway. In this case, Chs3 export may be subjected to even greater scrutiny. Future work could therefore include targeting the ER more specifically with known ER stress inducers such as DTT and tunicamycin (Travers et al., 2000), to explore whether impaired ERQC could allow increased export of misfolded Chs3.

4.4.3. QUALITY CONTROL THROUGH COPI RETRIEVAL

The saturability of some ERQC processes may have necessitated a second layer of defense at the early Golgi (Ellgaard et al., 1999). Some misfolded proteins use the COPI retrieval pathway, and this is also the case for Chs3. The export-retrieval pathway may be accessed by only a subset of misfolded proteins, and in some cases, a subset of lesions. For example, ER-retained Chs3 in *chs7*Δ mutants is not exported and therefore does not undergo COPI retrieval, while the

unpalmitoylated version is subjected to both retention and retrieval. Indeed, certain misfolded mutants of yeast Ste6 are segregated into ER proliferations known as ER associated compartments, which are hypothesized to sequester non-native proteins from ER exit sites (Ferreira et al., 2001; Huyer et al., 2004). Similarly, a subset of G protein coupled V_2 vasopressin receptor (V_2R) mutants are statically retained at the ER, while other mutants are exported and retrieved from the ER-Golgi intermediate compartment (ERGIC) through a COPI retrieval motif, RXR (Hermosilla et al., 2004). Other examples have also implicated such a QC role for the ERGIC, and it has been proposed that this intermediate compartment has evolved in mammalian cells to increase QC efficiency (Appenzeller-Herzog and Hauri, 2006).

Because unpalmitoylated Chs3 is retrieved by COPI and unlipidated Chs3 was found to bind COPI more strongly than wild-type Chs3, lack of palmitoylation may have caused a conformation change in Chs3 to expose a COPI binding signal, such as RXR or a dilysine KKXX motif. The latter, however, would have to be located at the C terminus, and no such sequence can be found in the vicinity of the Chs3 C-terminus. Mutation of all possible cytosolic RXR motifs of the ER-retained ΔF508-CFTR can result in diversion from ERAD and restored cell surface transport (Chang et al., 1999), suggesting a role of these motifs in dynamic, COPI-mediated retention. Due to the uncharacterized topology of both wild-type and misfolded Chs3, however, such a signal may be difficult to isolate. Moreover, direct or random mutagenesis of the putative recognition region on unpalmitoylated Chs3 could also trigger further misfolding, causing the protein to be statically retained. In this case, *in vitro* binding studies between mutant Chs3 and COPI would be required.

There is no indication in the literature that COPI can directly bind biophysical misfolded lesions such as hydrophobic patches. Instead, such misfolding lesions appear to be recognized by Golgi retrieval adaptors that also bind COPI (Sato et al., 2004). Neither preliminary observations of Chs3-GFP localization, nor the *pfa4*Δ suppression screen, showed involvement of Rer1 – the only characterized adaptor for misfolded membrane proteins to date – in retrieval of unpalmitoylated Chs3. An interaction between Chs3 and the KDEL retrieval receptor for soluble proteins (Erd2) was also not detected by co-immunoprecipitation (Appendix Fig. F.1). If a novel adaptor exists, its deletion should rescue the *pfa4*Δ retention defect, which warrants a careful survey of the uncharacterized genes from the *pfa4*Δ suppression screen, especially those that are localized to the early Golgi.

Misfolding of Chs3 in *pfa4*Δ and *chs7*Δ mutants clearly differ such that only palmitoylation-deficient forms undergo recycling. The Ubp3-mediated pathways for ER retention are thus specific for unpalmitoylated Chs3, most likely because chaperone-deficiency leads to the binding of additional, or alternative retention machinery. This contributes to our current model that the nature of misfolding lesions determines entry into different pathways of export and retrieval (Figure 4.1). More work is required to determine possible QC factors and chaperones that may recognize different misfolded forms of Chs3 and direct sorting into these pathways.

4.4.4. EFFICIENCY OF COPI RETRIEVAL AND THE TWOFOLD CONTRIBUTION OF UBP3

Inhibition of the COPI retrieval pathway alone can sufficiently restore cell surface transport of unpalmitoylated Chs3 to wild-type levels. The only non-essential COPI subunit, Sec28, was also identified from the *pfa4*Δ suppression screen (Rank #29, Appendix G), indicating that this screen is generally diagnostic for genes acting in the retrograde pathway such as retrieval adaptors. However, the effectiveness of this pathway may also have been a caveat in that it could mask the actions of potential retention factors. That is, deletion of retention factors may not result in strong suppression of *pfa4*Δ if the COPI pathway is intact and ensuring any unpalmitoylated Chs3 is returned to the ER. The clear phenotypic rescue observed in *pfa4*Δ*ubp3*Δ mutants could likely be due to the twofold effect of *UBP3* deletion in restoring COPII export as well as blocking COPI retrieval. It is therefore possible that many potential retention factors were not identified in our initial suppression screen because they do not inhibit COPI function. To counteract this effect and more specifically target export-inhibitory factors, a second screen for *pfa4*Δ suppression should be performed in a COPI-deficient background, either through the temperature sensitive *sec21-1* allele, or deletion of the non-essential *SEC28* (ie. create *pfa4*Δ*sec21-1 xxx*Δ or *pfa4*Δ*sec28*Δ*xxx*Δ mutants).

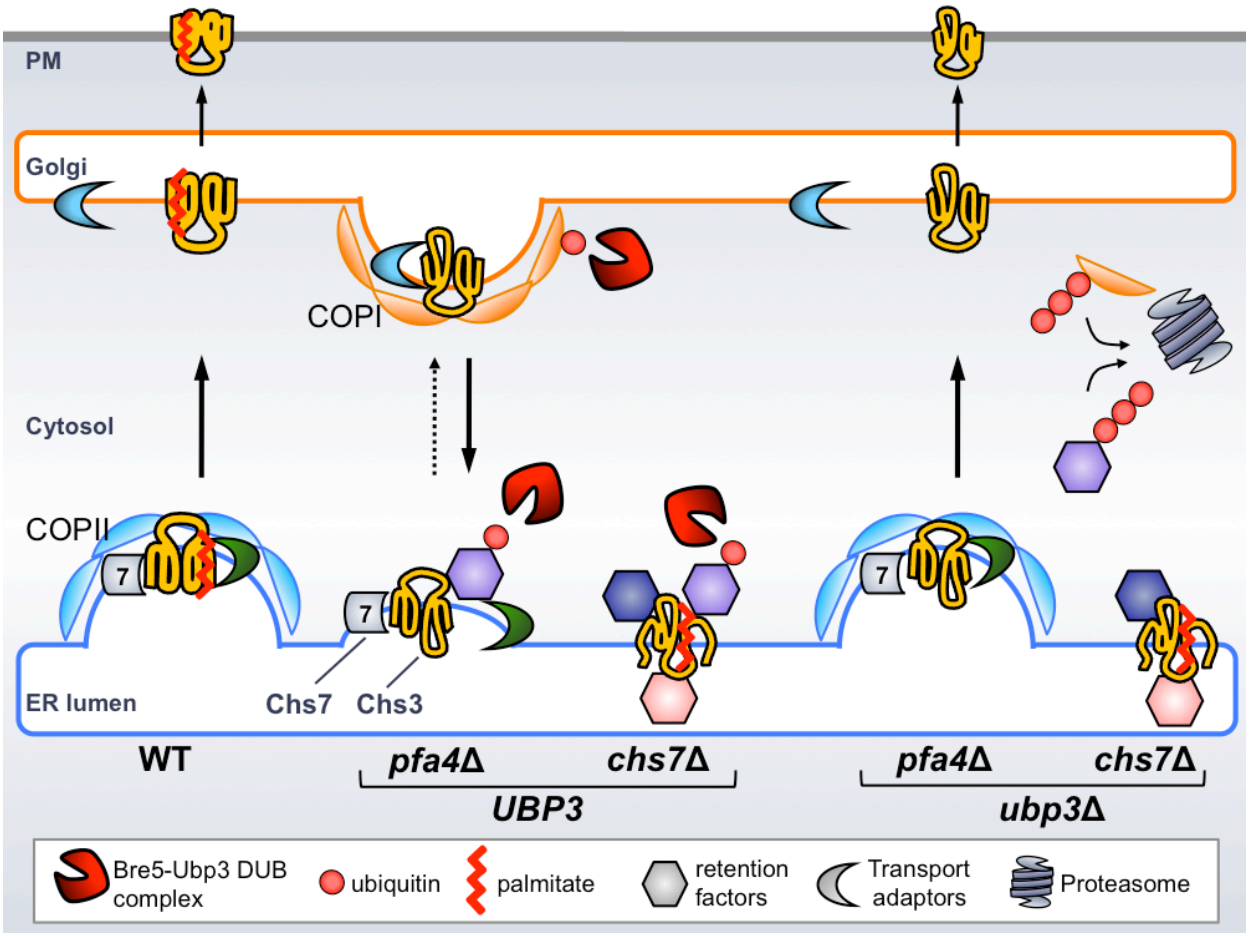

Figure 4.1. Model of Chs3 ER quality control.
A current model of how palmitoylation, chaperone association, and Ubp3-mediated deubiquitination control ER export of Chs3. In wild-type cells, Chs3 (yellow) requires palmitoylation by Pfa4 and association with Chs7 to be properly folded and exported in COPII coated vesicles. COPII adaptors (green) may also bind to export signals on Chs3 to facilitate exit. When not lipidated in *pfa4*Δ mutants, Chs3 does not fully fold, and is recognized by ER retention factors (purple), which may further impede folding. Ubp3 may be responsible for ensuring the continued presence of these proteins through their deubiquitination. In *pfa4*Δ cells, some unpalmitoylated Chs3 can escape to the Golgi, but is quickly recognized by cis-Golgi quality control factors and retrieved by COPI. Ubp3 has a twofold effect on the retention of unpalmitoylated Chs3 in that, in its absence, ubiquitination of both COPI and the putative ER retention factors are increased, resulting in their degradation. The absence of retention factors may allow Chs3 to refold and gain export competency, escape post-ER quality control mechanisms, and be transported to the cell surface. Similar factors can also bind to Chs3 in *chs7*Δ cells, but additional retention machinery is likely engaged since Chs3 may be more severely misfolded. Therefore, *UBP3* deletion is ineffective in restoring Chs3 export in *chs7*Δ mutants.

4.4.5. DOWNSTREAM QC PATHWAYS

Since COPI is also localized to late Golgi compartments where it mediates retrograde transport through Golgi stacks (Teasdale and Jackson, 1996), it is unclear how far Chs3 traverses the Golgi before its retrieval. Because almost no Chs3-GFP can be detected in punctate Golgi/endosomal compartments or the vacuole in *pfa4*Δ mutants, retrieval most likely takes place once Chs3 reaches the early Golgi. However, it is still possible for unpalmitoylated Chs3 to be recognized by Golgi quality control factors and routed for vacuolar degradation.

Indeed, Golgi pathways for quality control of membrane proteins have been described. Notably, proteins with exposed polar residues, presumably misfolded proteins, can be ubiquitinated and transported to the vacuole from the Golgi through the Tul1 E3 ubiquitin ligase (Reggiori and Pelham, 2002). Vacuolar degradation has also been proposed as an alternative – and perhaps competing – pathway for ER retention, at least for proteins with exposed polar transmembrane domains (Hettema et al., 2004). The Bsd2 adaptor is hypothesized to bind strongly polar TMDs and sort proteins for vacuolar degradation at the Golgi, and may compete with the Rer1 Golgi-to-ER receptor (Hettema et al., 2004). Rer1 and Bsd2 could represent a two-layered system for recognition of misfolded proteins: one at the ER retrieval level and another for vacuolar degradation, respectively. Interestingly, Bsd2 was identified in the *pfa4*Δ suppressor screen to have increased cell surface levels of Chs3 (Rank #86). Other key components important for vacuole targeting were also identified, including Bul2 (Rank #4), a binding partner of the Rsp5 E3 ubiquitin ligase that mediates vacuolar degradation (Helliwell et al., 2001), and Doa1 (Rank #51), an ubiquitin binding protein that captures and sorts ubiquitinated membrane proteins into the multivesicular body (MVB) pathway (Ren et al., 2008) (Appendix G). These observations suggest unpalmitoylated Chs3 could be recognized for post-ER degradation.

Membrane proteins that are ER-retained can also undergo vacuolar degradation instead of ERAD. For the Pma1-G381A mutant (considered to be an intermediate between the strongly ER retained Pma1-D378N and wild-type), ER retention is only temporary and a vacuolar pathway is used for degradation, which may explain why this particular mutant does not activate ERAD (Ferreira, 2001). Similarly, the misfolded, single-span CPY chimera CFs' is ER-retained, but its disposal is independent of Hrd1 and Doa10, and instead requires the Rsp5 ligase for vacuolar degradation (Kincaid and Cooper, 2007). It is presently unclear whether the destabilization of

unpalmitoylated Chs3 is due to ER or vacuolar degradation, or a contribution from both. One criticism of our current pulse-chase studies is that the site of degradation has not been distinguished. To overcome this flaw and examine only ER-specific degradative pathways, an ER-to-Golgi block in the mutants studied would be required. This can be done by impairing COPII export, for example by introduction of the *sec12* temperature sensitive mutation. Alternatively, a deletion of the vacuolar protease Pep4 would inhibit any supplemental effects from the vacuolar pathway. This is presently being pursued in the laboratory.

If unpalmitoylated Chs3 is subject to post-Golgi QC pathways, it could be expected that deletion of these genes would increase cell surface levels of Chs3 by diversion from vacuolar degradation. Whether they could affect Chs3 directly or indirectly through transport components remains to be explored, but the suppression data suggest that unpalmitoylated Chs3 could potentially be subjected to some post-ER quality control. Vacuolar targeting mutants were also identified in other Chs3 trafficking screens in which Chs3 is palmitoylated (C. Tam, L. Conibear, personal communication), suggesting that they may not necessarily act as QC for aberrant forms, but instead serve a more general regulatory function. Future investigations into the *pfa4*Δ-suppressing mutants mentioned above will not only shed light on Chs3 quality control, but also post-Golgi and vacuolar trafficking pathways that may well involve ubiquitination.

4.5. PROTEIN REFOLDING AND THERAPEUTIC STRATEGIES: SIGNIFICANCE AND APPLICATIONS

Protein misfolding, which has detrimental effects on cell survival, can arise from inherited mutations, faulty chaperone or degradative activities, as well as cellular or environmental attacks. For misfolding and ER retention diseases, amelioration of the disease phenotype can occur through two main methods: 1) inhibiting interaction or access of the QC machinery to mutant proteins, or 2) changing or improving the folding environment (Welch, 2004). The essential outcome of either method is a wild-type, or at least functional, conformation that escapes retention and degradation. In terms of energy expenditure, it may be more favorable to refold mutant proteins rather than to dispose of them, the latter of which requires unfolding, retrotranslocation, proteolysis, and sometimes resynthesis (Liberek et al., 2008). Physiologically, if refolding is possible, it may also represent a less lethal route for the cell. Cells have evolved the system of molecular chaperones to not only assist folding, but also refolding, to counteract aggregation either constitutively or under stress conditions (Liberek et al., 2008). These chaperones have been intensively exploited as pharmacological therapies for folding diseases.

4.5.1. REFOLDING: MANIPULATING CHAPERONES

In inherited folding diseases like CF and NDI, the pathological mechanisms are sometimes due to slower folding dynamics of mutant proteins that are otherwise functional, or partially so. This is also observed for unpalmitoylated Chs3, which appears to be functional in chitin synthesis despite being misfolded. For these diseases, much attention has been given to enhancing the actions of chaperones to improve folding (Hebert and Molinari, 2007). Pioneering work focused on lowering levels of endogenous chaperones such as BiP to increase secretion of proteins otherwise retained by ERQC (Dorner et al., 1988). We now know that reducing the activities of classical chaperones, which regulate the maturation of many proteins, can have deleterious effects (Welch, 2004). Instead, increasing chaperone levels or manipulating specific chaperones may be better alternatives.

Chemical chaperones, ie. those that are not naturally occurring like proteins, are often protein-stabilizing agents used to correct protein folding. These include small molecules such as dimethylsulfoxide, deuterated water, glycerol, and trimethlamine-N-oxide, which have been shown to rescue folding of mutant CFTR (Welch, 2004). Although a few have been approved for clinical use, chemical chaperones have restricted therapeutic value because they generally have very non-specific mechanisms and require high dosages (Hebert and Molinari, 2007). Using pharmacological chaperones, which resemble biological enzymes, has allowed for higher substrate specificity. Many have been used in successfully rescuing V_2R mutants (Conn et al., 2007; Dong et al., 2007). Therefore, in the development of more effective therapies, it is important to understand the action of chaperones in order to target them towards particular misfolding defects or substrates.

To offer some basic insights into chaperone function, a library of either chemical or pharmacological chaperones can be developed for use in high throughput screens to rescue misfolding of Chs3 in *pfa4*Δ or *chs7*Δ mutants. It is interesting to note here, perhaps as a possible caveat of such treatments, that overexpression of the Chs7 chaperone did not restore Chs3 export in *pfa4*Δ mutants, which may reflect a very specific action of Chs7 in folding of Chs3. In other words, just because a chaperone is required for folding of its substrate does not necessarily guarantee its success in compensating for other misfolding events. Nevertheless, the precise function of Chs7 – the only other identified ER export factor and the only Chs3-specific chaperone – is still unknown and warrants further study.

4.5.2. ESCAPING DEGRADATION: DUBS AS TARGETS

In addition to folding chaperones, the ERAD pathway has also been targeted in therapeutics. The use of proteasomal inhibitors, for instance, has been of some success in rescuing ΔF508-CFTR folding (Vij et al., 2006). However, in general, proteasomal inhibitors have very toxic effects and can often lead to accumulation of other unwanted and misfolded species (Love et al., 2007). Due to the many regulatory roles of ubiquitin especially in proteasomal targeting, DUBs are also attractive targets. This is especially true for DUBs that show high substrate specificity, such as the Usp4 DUB that enhances cell surface levels of the α_2-adrenergic receptor at the ERQC level (Milojevic et al., 2006) (Section 1.3.2.2). It is interesting to note here that, for some diseases,

sometimes an increase in degradation is required (eg. of proteins that have proliferative functions). Therefore recent effort has also focused on high-throughput assays for DUB inhibitors (Love et al., 2007; Goldenberg et al., 2008).

4.5.3. RESTORING CELL SURFACE TRANSPORT: PALMITOYL THIOESTERASES AS TARGETS

For those proteins that fail to be plasma membrane localized due to lack of palmitoylation, an appealing intervention for palmitoylation deficiency would be to inhibit thioesterases to increase cell surface levels. Therefore, since the discovery of Apt1, several studies have been undertaken to find inhibitors of Apt1 activity (Biel et al., 2006), as well as general PAT activity (Ducker et al., 2006), and DHHC PAT activity (Jennings et al., 2008). It is not clear at present whether Chs3 remains palmitoylated during its lifetime and therefore if deacylation could have regulatory functions in post-ER compartments. Moreover, no trace palmitoylation of Chs3 can be detected in *pfa4*Δ mutants, so the inhibition of putative thioesterases in this case may not change Chs3 palmitoylation status and yield informative results. Conversely, overexpression of a putative ER thioesterase has the potential to overwhelm Pfa4 function, resulting in the loss of palmitoylation and ER retention of newly synthesized Chs3. The yeast overexpression library can be used in this case, in conjunction with the CW fluorescence assay, to identify putative APTs. Finally, finding both genetic and chemical inhibitors of Pfa4 may reveal other physiologically relevant ER folding and transport pathways that may be applicable to other substrates or the Pfa4 homolog in mammalian cells.

4.5.4. PERSPECTIVES FOR DISCOVERY OF NOVEL TARGETS OF MOLECULAR THERAPIES

Substrate identification for DUBs, APTs, and PATs represents a major unresolved issue that delays the development of molecular therapies. As such, there is currently considerable focus on finding ubiquitinated and palmitoylated substrates using genome-wide methods (Ducker et al., 2006; Grillari et al., 2006). Once these substrates are known, the next sizeable task is to determine the consequences of these modifications, prior to identifying specific treatment

targets. The findings in yeast that palmitoylation facilitates protein folding and inhibition of a DUB can rescue this defect, although not immediately applicable to disease treatment, could help surmount some of these obstacles. Indeed, the yeast genomic system has been manipulated into a powerful tool to find novel drug targets (Boone et al., 2007). In particular, it has proven to be invaluable for understanding neurodegenerative disorders like Parkinson's and Huntington's diseases in which protein misfolding and aggregation are implicated (Gitler, 2008).

As with the *pfa4*Δ suppression screen, other high throughput methods could potentially identify putative refolding machinery. In addition to the genetic suppression of palmitoylation-deficient Chs3 through systematic deletion of non-essential ORFs in the *pfa4*Δ background, complementary genomic screens can also be developed to rescue misfolded Chs3. For example, the introduction of the GAL-induced overexpression library into the *pfa4*Δ background may find factors that act antagonistically to Ubp3. A chemical genomics approach, by introducing small molecule libraries (Boone et al., 2007) into *pfa4*Δ cells, could elucidate either DUB inhibitors or suppressors of Ubp3 substrates. This may be informative about the physiological relevance of Ubp3 in ER trafficking and quality control, in the same way that some PAT inhibitors were found to be anti-cancer drugs (Ducker et al., 2006). If performed in parallel in the *pfa4*Δ*ubp3*Δ double mutant background, these screens could also examine COPI-mediated retrograde transport pathways. Alternatively, screens in a *pfa4*Δ mutant that is also deficient for COPI retrieval would better pinpoint the export or retention factors affected by Ubp3. Finally, such efforts need not be restricted to Chs3. Future screens could assay for localization of different marker proteins to survey more global effects of both Pfa4 and Ubp3. Systematic analysis of the data collected could eventually produce a map of interactions of palmitoylation and ubiquitination mediated ER quality control.

4.6. POST SCRIPT: FUTURE AVENUES FOR RESEARCH IN PROTEIN QUALITY CONTROL

Through both vesicular transport and destructive pathways, the endoplasmic reticulum has a powerful potential, as well as responsibility, to regulate the quality and quantity of virtually all secretory traffic. Therefore, its function in ensuring cell survival cannot be overstated. In spite of decades of intense effort to understand ER transport and quality control, some fundamental molecular mechanisms are still poorly characterized, and the highly individualized itineraries for substrates continue to create controversies in the field. As illustrated by the findings of this work, additional players in quality control may still be undiscovered. Furthermore, many post-ER pathways for regulating cell surface expression of membrane proteins are poorly characterized.

The study of palmitoylation in ER quality control and trafficking is still in its infancy and deserves attention, especially in higher cells. Nevertheless, the budding yeast continues to be an excellent model organism not only for cell and molecular studies, but also for high-throughput discovery of novel pathways and mechanisms. Taking advantage of this powerful genomic system, future investigations should probe the specific degradation or retention machineries at the ER, perhaps with a particular focus on those that act on unlipidated proteins. A better appreciation of these underlying processes of ER quality control is crucial, especially considering this novel role for palmitoylation in protein folding, and the need to create reliable, less cytotoxic therapeutic treatments for misfolding diseases. The many unexplored and medically relevant areas of research in ER quality control continue to make this an exciting organelle to study since its discovery over half a century ago.

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APPENDIX A: CHAPTER 3 SUPPLEMENTAL DATA

(Referenced in: Section 3.2)

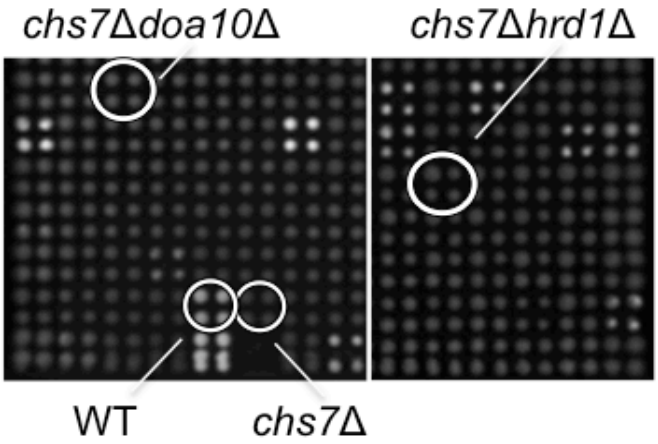

Figure A.1. Absence of either of the E3 ligases Doa10 or Hrd1 does not suppress the CW dim phenotype of *chs7*Δ mutants.

Wild-type, *chs7*Δ, and *chs7*Δ*xxx*Δ double mutants were plated on 50μg/mL CW on YPD and observed for fluorescence phenotypes as described in Chapters 2 and 3. Both *chs7*Δ*doa10*Δ and *chs7*Δ*hrd1*Δ double mutants show CW dim phenotypes similar to *chs7*Δ single mutants.

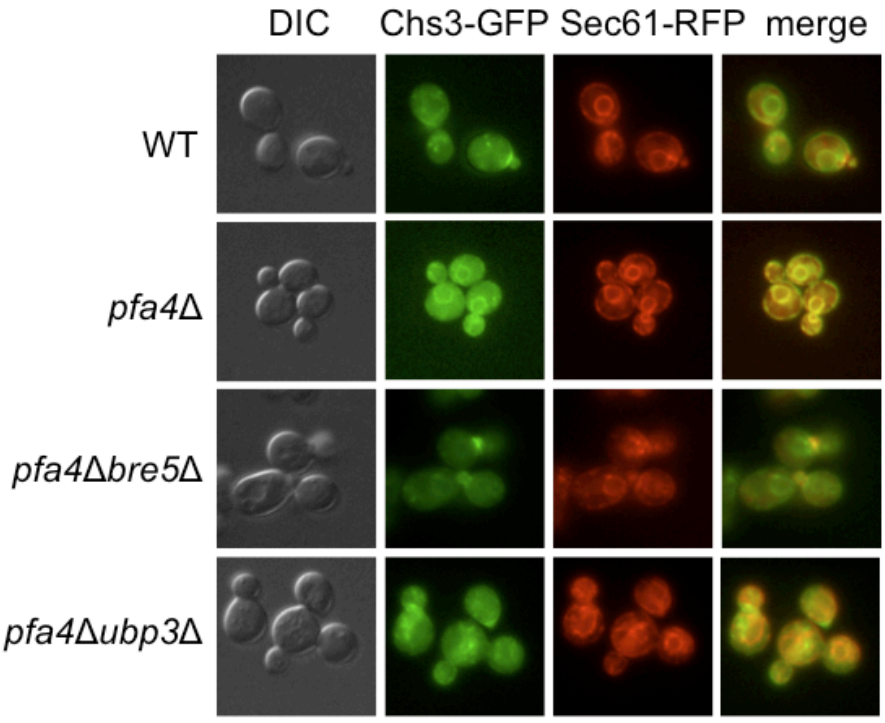

Figure A.2. ER staining in *bre5*Δ*pfa4*Δ and *ubp3*Δ*pfa4*Δ double mutants.

The indicated strains co-expressing genomically integrated Chs3-GFP and Sec61-RFP (an ER marker), were observed by DIC and fluorescence microscopy at log phase.

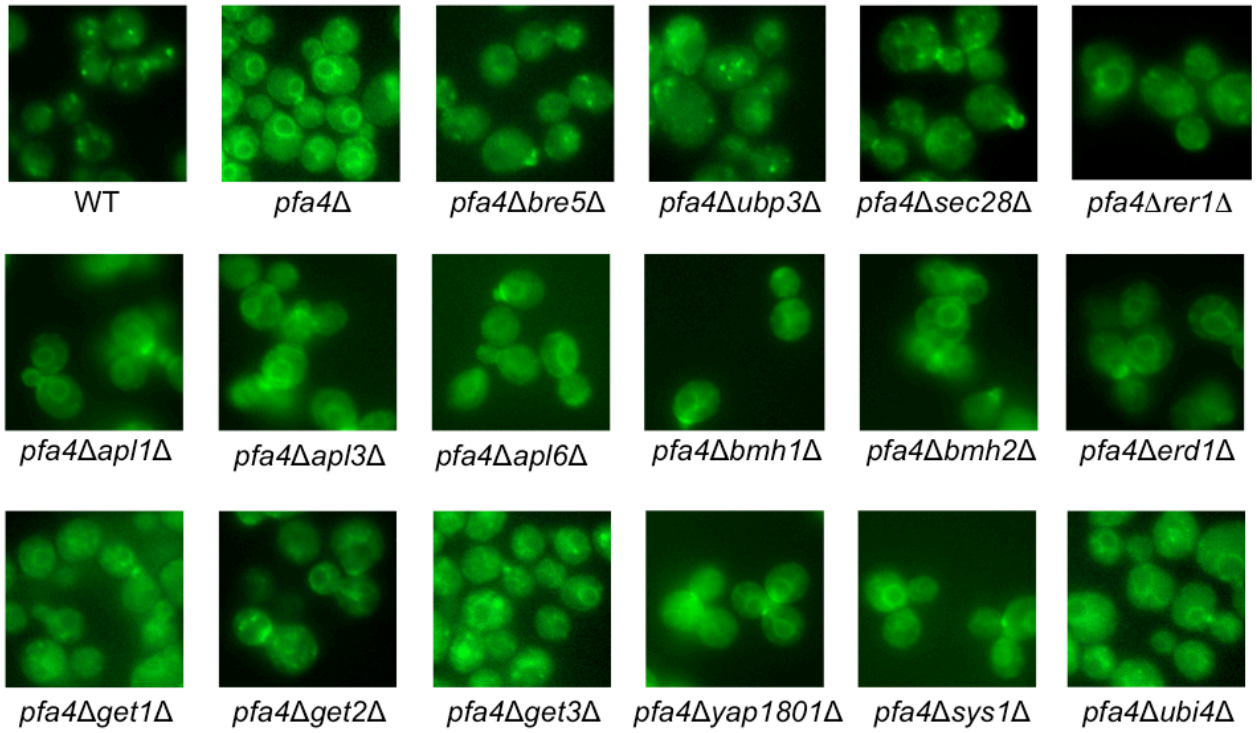

Figure A.3. Chs3-GFP localization in select *pfa4*Δ*xxx*Δ double mutants.
Log phase cells expressing genomically integrated Chs3-GFP were observed by fluorescence microscopy. These double mutants are discussed in Section 3.2, except for the *pfa4*Δ*ubi4*Δ mutant, which is discussed in Section 4.3.2.1. Deletion of each of the three members of the GET (Golgi-ER trafficking) complex (Get1, Get2, Get3) was tested here, although only Get3 (Arr4) ranked within the top 200 mutants from the suppression screen. For *pfa4*Δ*sec28*Δ mutants, which show a slight temperature sensitive mutant phenotype, cells were grown at 25ºC and shifted to 33ºC for 30 min prior to observation.

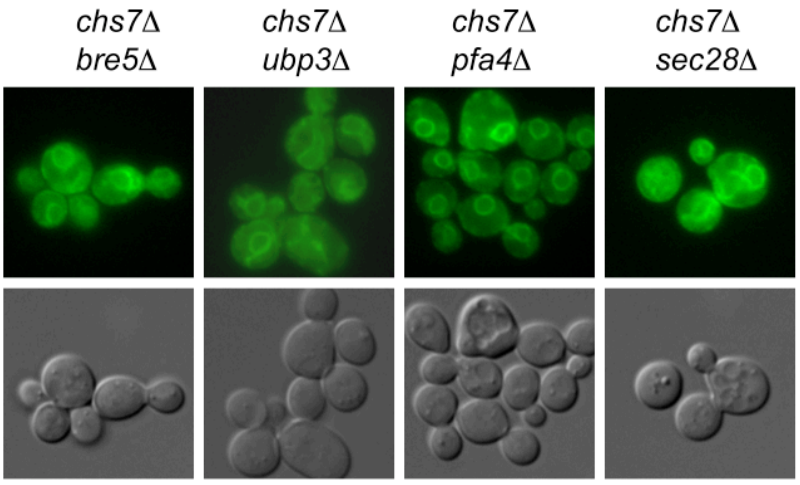

Figure A.4. Chs3-GFP remains ER retained in *chs7*Δ*bre5*Δ, *chs7*Δ*ubp3*Δ, and *chs7*Δ*sec28*Δ mutants.
Double mutants expressing genomically integrated Chs3-GFP were observed for by DIC and fluorescence microscopy at log phase.

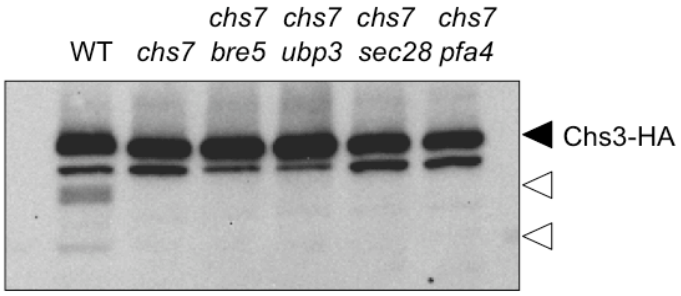

Figure A.5. Chs3 is not protease accessible in *chs7* double mutants.
The indicated strains expressing genomically integrated Chs3-3xHA were subjected to the protease-accessibility assay as described in Chapter 3. Black arrows: protease-inaccessible Chs3. White arrows: protease-accessible Chs3 degradation products.

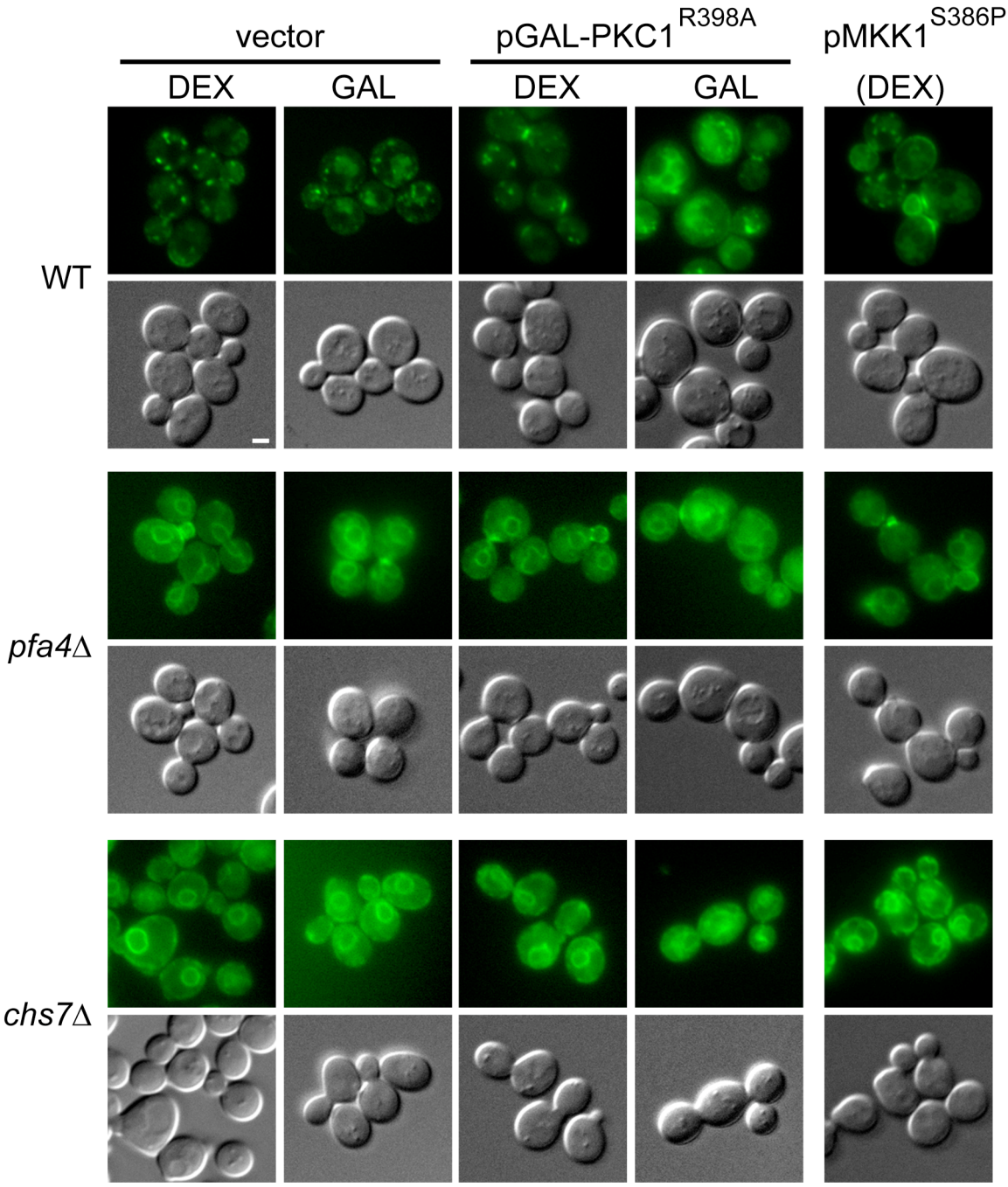

Figure A.6. Induction of the Pkc1 pathway does not suppress *pfa4*Δ or *chs7*Δ mutants.
Activated alleles of Pkc1 ($Pkc1^{R398A}$) and Mkk1 ($Mkk1^{S386P}$) carried on pRS316 vectors were transformed into WT, *pfa4*Δ, and *chs7*Δ BY4741 cells expressing endogenous Chs3-GFP. An empty pRS316 vector was also transformed as a control. For GAL induction of activated Pkc1, overnight cultures grown in minimal media with 2% dextrose were grown to log phase in minimal media containing 2% galactose. Log phase cells were observed by fluorescence microscopy as described in Experimental Procedures. Bar: 2μm.

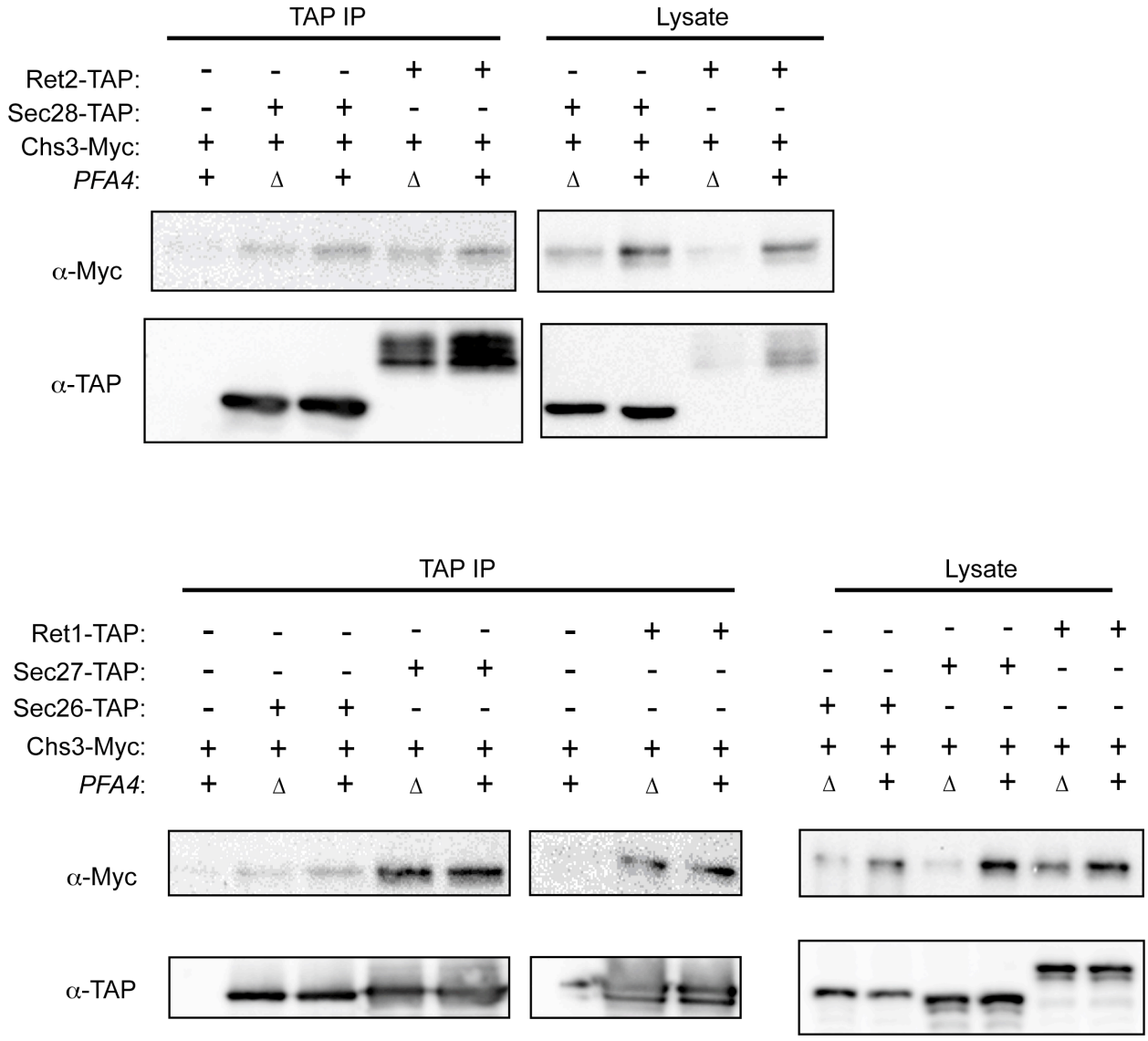

Figure A.7. Chs3 co-immunoprecipitates with different COPI subunits.
Cells co-expressing a genomically tagged COPI-TAP subunit (Sec26, Sec27, Sec28, Ret1, or Ret2) and Chs3-13xMyc were subjected to TAP immunoprecipitation using IgG-Sepharose, and analyzed by Western blotting with α-Myc and α-TAP antibodies as described in Chapter 3. Sec27 and Ret1 show stronger interactions with Chs3, while Sec26, Sec28, and Ret2 interacted less strongly with Chs3.

APPENDIX B: CHS3 PALMITOYLATION SITES

(Referenced in: Sections 4.2.1 and 4.3.1)

Table B.1. Predicted Chs3[1] transmembrane domains

TMD	Amino acid coordinates
I	168 - 190
II	203 - 224
III	454 - 476
IV	1029 - 1051
V	1055 - 1077
VI	1084 - 1106

[1]Total length of Chs3: 1165 amino acids

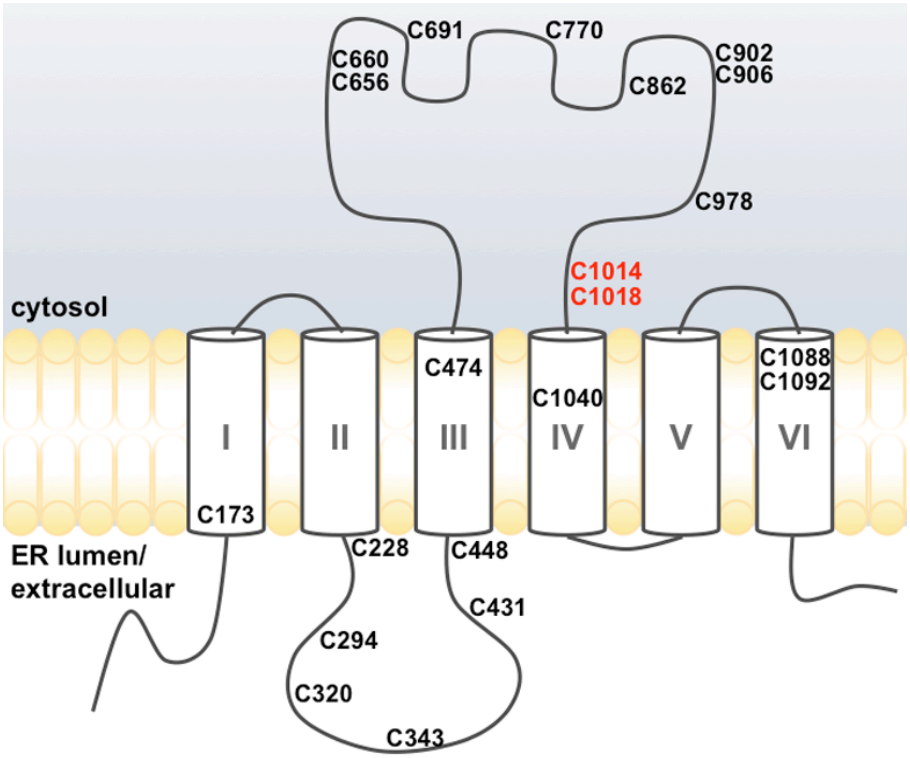

Figure B.1. Predicted Chs3 topology and location of cysteine residues.
The 21 cysteine residues of Chs3 and their approximate positions with respect to the six predicted transmembrane domains (I – VI). The putative sites of palmitoylation, cysteines 1014 and 1018, are labelled in red. Diagram not drawn to scale.

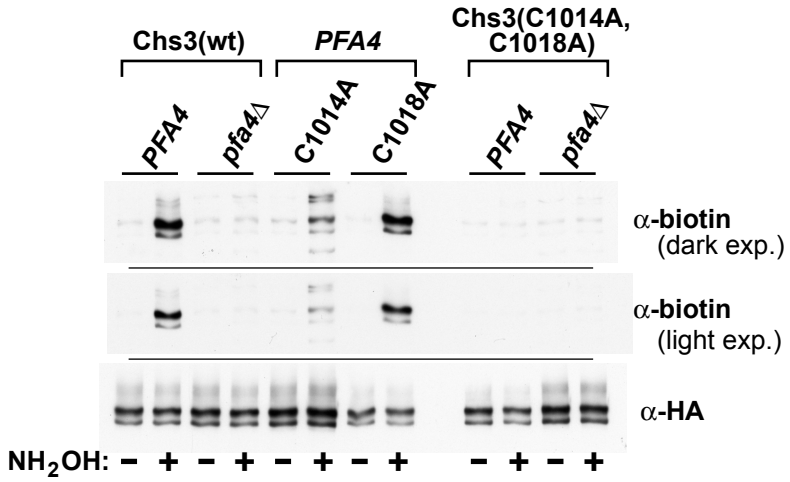

Figure B.2. Palmitoylation is abolished in Chs3 ^{C1014A,C1018A} mutants.
WT or *pfa4*Δ cells carrying the pND2115 plasmid encoding wild type *CHS3*-3xHA-FLAG-His, or versions encoding the mutations *CHS3*^{C1014A}, *CHS3*^{C1018A} or *CHS3*^{C1014A,C1018A} were subjected to the acyl-biotin exchange assay as described in Chapters II and III. Protein extracts were subjected to thioester cleavage by hydroxylamine (NH_2OH) 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 palmitoylated and total Chs3, respectively. Chs3-C1014A shows reduced palmitoylation levels, and the mutation of both C1014 and the nearby C1018 to alanine residues abolishes palmitoylation of Chs3.

Table B.2. Summary of Chs3 cysteine mutants studied to date

Cys[1]	Amino Acid Coordinate	Proximity to TMDs[2] (# residues)	GFP localization	Palmitoylation[3]
1	173	TMD I	WT	
2	228	4	WT	
3	294		linear intracellular structures	
4	320		WT	
5	343		WT	
6	431		linear intracellular structures	
7	448	6	budneck, faint ER	yes
8	474	TMD III	WT	
9	656		WT	
10	660		N/A	
9/10	656/660		N/A	
11	691		WT	
12	770		degraded	
13	862		N/A	
14	902		WT	
15	906		degraded	
14/15	902/906		WT	
16	978		WT	
17	1014	15	ER, budneck, internal dots	lowered
18	1018	11	ER, some budneck	yes
17/18	1014/1018	11/15	N/A	no
19	1040	TMD IV	N/A	
20	1088	TMD VI	N/A	
21	1092	TMD VI	WT	
20/21	1088/1092		N/A	

[1] Chs3 has 21 total cysteines, which were each singly mutated to alanine or serine residues by PCR-based site-directed mutagenesis. Cysteines that are located close together were also doubly mutated.

[2] For simplicity, only those cysteines located under 20 residues from a predicted transmembrane domain listed in Table B.2 are listed. "TMD" indicates that a cysteine is located within a transmembrane domain.

[3] Palmitoylation status was confirmed for those mutants showing ER staining.

APPENDIX C: LIPID RAFT STUDIES

(Referenced in: Section 4.2.1)

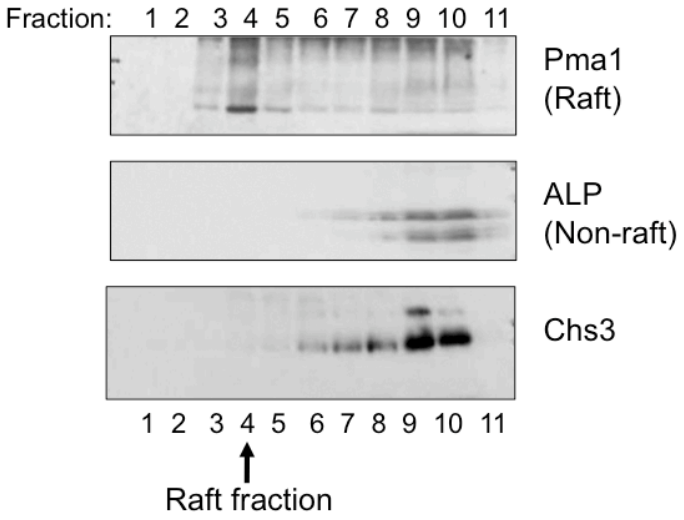

Figure C.1. Chs3 is not present in lipid rafts.
Triton-X 100 resistant membranes were isolated by Optiprep density gradient centrifugation (see Experimental Procedures). Pma1 is a raft marker and floats in fraction 4, whereas the vacuolar alkaline phosphatase (ALP) is not localized to rafts and is present mainly in fractions 9 and 10. Chs3 shows similar distribution to ALP and cannot be detected in fraction 4.

EXPERIMENTAL PROCEDURES: Isolation of Detergent Resistant Membranes

Yeast spheroplasts expressing endogenous Chs3-GFP (50ODs) were resuspended in 300uL cold TNE buffer (50mM Tris pH7.5, 150mM NaCl) supplemented with a protease inhibitor cocktail. Equal volume of glassbeads were added and samples were vortexed 2x 5 min at 4ºC, and 250uL supernatant were collected after centrifugation for 5 min at 4ºC at 3000rpm. Equal volume of TNE buffer and 1% of Triton-X 100 was added to samples and incubated on ice for 30min. 500uL was transferred to a new tube, and the volume was adjusted to 1mL with TNET (TNE plus 0.1% Triton-X 100). Two volumes of 60% Optiprep density gradient medium (Sigma, St Louis, MO) was added to create a final concentration of 40% Optiprep, mixed, and put at the bottom of a 12mL Beckman UltraClear Centrifuge tube (Beckman Coulter, Fullerton, CA). A discontinuous gradient of the following Optiprep concentrations was slowly layered over the sample: 3mL 35%, 3mL 30% Optiprep, and 2mL of TNET (0%). This four-step gradient was centrifuged for 18 hours at 4ºC at 30 000rpm in a SW41Ti Rotor of a Beckman Ultracentrifuge (Beckman Coulter, Fullerton, CA), and 1mL fractions were subsequently collected from the top. Fractions were precipitated with 100% trichloroacetic acid, resuspended in sample buffer, and subjected to SDS-PAGE and Western blotting with α-Pma1 (raft marker), α-ALP (non-raft marker), and α-GFP.

APPENDIX D: TRAFFICKING OF THE TAT2 TRYPTOPHAN PERMEASE IN *PFA4*Δ MUTANTS

(Referenced in: Section 4.2.3)

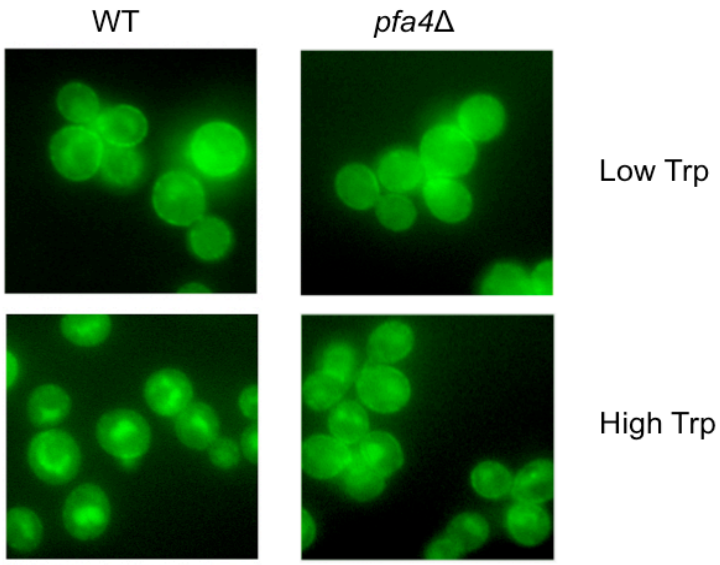

Figure D.1. Localization of the tryptophan permease Tat2-GFP in wild-type and *pfa4*Δ cells.

Wild-type and *pfa4*Δ mutants expressing genomically tagged Tat2-GFP were observed by fluorescence microscopy. Cells were grown to log phase in minimal media supplemented with either low (2.5 μg/mL) or high (200 μg/mL) levels of tryptophan (Trp), which promotes the trafficking of Tat2 permease to the cell surface or vacuole, respectively.

APPENDIX E: CALCOFLUOR WHITE STAINING OF YEAST CELL WALL AND BUD SCARS

(Referenced in: Section 4.4.2)

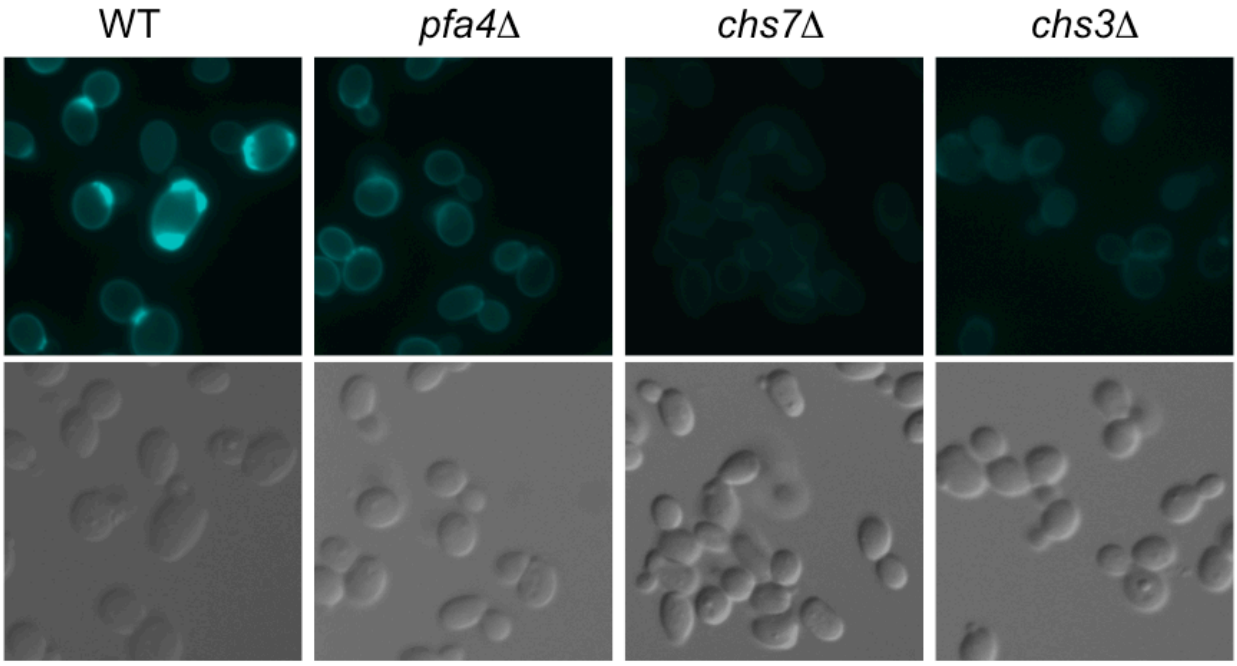

Figure E.1. Calcofluor White staining of cell surface chitin by microscopy.
Binding of the fluorescent dye Calcofluor White to yeast cell wall chitin can be visualized by fluorescence microscopy. The indicated strains (BY4741 background) were grown in fresh minimum media to log phase and fixed with formaldehylde (3.7% final concentration) for 30 min at 30ºC. 200µL of fixed cells were treated with 20µL of 1mg/mL Calcofluor white in water and incubated for 30 min at 30ºC. Wells were washed twice with water, and observed by fluorescence (top panel) and DIC (bottom panel) microscopy. Calcofluor binds to cell surface chitin that is present at the lateral cell walls and budneck in wild-type and *pfa4*Δ mutants, whereas no chitin can be detected at the cell surface in *chs3*Δ and *chs7*Δ mutants.

APPENDIX F: CO-IMMUNOPRECIPITATION OF CHS3 AND ERD2 RETRIEVAL RECEPTOR

(Referenced in: Section 4.4.3)

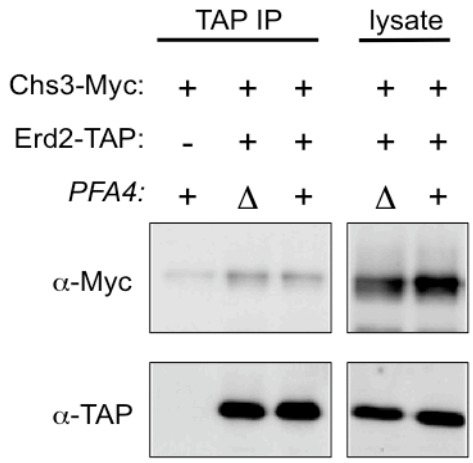

Figure F.1. The Erd2 receptor does bind significantly to Chs3.
Cells co-expressing genomically tagged Erd2-TAP and Chs3-13xMyc were subjected to TAP immunoprecipitation using IgG-Sepharose, and analyzed by Western blotting with α-Myc and α-TAP antibodies as described in Chapter 3.

APPENDIX G: CW SCREEN RESULTS FOR SUPPRESSORS OF *PFA4*Δ

(Referenced in: Sections 4.3.1 and 4.4.4)

The following table lists the top 200 suppressors of *pfa4*Δ as identified by Calcofluor White screening of the *MAT*a yeast genome deletion collection described in Chapter III, along with their annotated localizations and functions from the *Saccharomyces* Genome Database (http://www.yeastgenome.org/). A higher rank (ie. lower number in the "Pfa4 Rank" column) indicates a bright CW phenotype, or restoration of Chs3 cell surface activity, when the listed gene is deleted in the *pfa4*Δ background. Ranks from the original genome-wide CW screen for Chs3 transport factors described in Chapter II ("CW rank" column) are also included. (A lower number in this column indicates a CW dim phenotype, or decreased Chs3 cell surface activity, when the gene is deleted in the wild type background.) This list has been filtered for slow growing strains. The double *pfa4*Δ*xxx*Δ mutants in bold have been tested for Chs3-GFP localization, in addition to several others that were either below the top 200 or present prior to growth filtering, and those mentioned in the text.

Table G.1. Top 200 suppressors of *pfa4*Δ from CW genomic screen

Pfa4 Rank	CW rank	Gene	Localization	Annotated Function
1	1234	ALK1	nucleus	leucine zipper (putative), membrane protein (putative)
2	1078	VPS60	cytoplasm	vacuolar protein sorting (putative)
3	1526	IVY1	vacuolar membrane	Phospholipid-binding protein that interacts with both Ypt7p and Vps33p, may partially counteract the action
4	1443	BUL2	cytoplasm	a homologue of BUL1; (putative) ubiquitin-mediated protein degradation
5	1769	OPI1	nucleus	Transcriptional regulator of a variety of genes; phosphorylation by protein kinase A stimulates Opi1p
6	558	TIR3	cell wall (sensu Fungi)	TIP1-related
7	2767	GPX1	N/A	Phospholipid hydroperoxide glutathione peroxidase induced by glucose starvation that protects cells from
8	**35**	**ARR4**	**endoplasmic reticulum**	**ATPase, involved in resistance to heat and metal stress, active as a dimer; normally localized to**
9	1919	RUD3	Golgi apparatus	Golgi matrix protein that is involved in the structural organization of the cis-Golgi
10	**337**	**ERD1**	**membrane**	**Predicted membrane protein required for the retention of lumenal endoplasmic reticulum**
11	590	SDC25	N/A	Ras guanine nucleotide exchange factor (GEF); in the S288C strain, there is a stop codon between
12	2940	ARL3	soluble fraction	Protein similar to ADP-ribosylation factor, involved in the carboxypeptidase Y pathway
13	1355	LEM3	cytoplasm	Membrane protein of the plasma membrane and ER, involved in translocation of phospholipids and
14	1008	YOR186W	N/A	N/A
15	2668	MNN2	Golgi apparatus	Alpha-1,2-mannosyltransferase, responsible for addition of the first alpha-1,2-linked mannose to form the
16	1392	VPS74	cytoplasm	N/A
17	819	MNN5	Golgi apparatus	Alpha-1,2-mannosyltransferase, responsible for addition of the second alpha-1,2-linked mannose of the
18	1261	IZH1	endoplasmic reticulum	Membrane protein involved in zinc metabolism, member of the four-protein IZH family, direct target of the
19	1139	CLN1	cytoplasm	role in cell cycle START; G(sub)1 cyclin
20	1028	YOL050C	N/A	N/A
21	2437	SMF2	mitochondrion	Divalent metal ion transporter
22	1786	COG8	Golgi transport complex	Component of the conserved oligomeric Golgi complex, dependent on RIC1
23	2967	YPR089W	N/A	N/A
24	425	HOC1	mannosyltransferase	Alpha-1,6-mannosyltransferase involved in cell wall mannan biosynthesis; subunit of a Golgi-localized
25	1069	RPL26A	cytosolic large ribosomal	Protein component of the large (60S) ribosomal subunit, nearly identical to Rpl26Bp and has similarity to E.
26	1775	YJL051W	bud tip	N/A
27	1687	FMP16	mitochondrion	The authentic, non-tagged protein was localized to the mitochondria
28	244	VPS53	cytoplasm	Required for Vacuolar Protein Sorting; Vps53p is a hydrophilic protein that is peripherally associated with
29	**291**	**SEC28**	**COPI vesicle coat**	**Part of a heptameric protein complex that regulates retrograde Golgi-to-ER protein traffic in**
30	2578	YER084W	N/A	N/A
31	2183	LAG2	mitochondrion	affects longevity; involved in determination of longevity
32	1592	GYL1	cytoplasm	Putative GTPase activating protein (GAP) that may have a role in polarized exocytosis; colocalizes with the
33	878	SRN2	endosome	Suppressor of rna1-1 mutation; may be involved in RNA export from nucleus
34	2011	DON1	spindle	Meiosis-specific component of the spindle pole body, part of the leading edge protein (LEP) coat, forms a
35	211	BEM4	cytoplasm	Protein involved in establishment of cell polarity and bud emergence; interacts with the Rho1p small GTP-
36	**2808**	**BMH1**	**nucleus**	**14-3-3 protein, major isoform; binds proteins and DNA, involved in regulation of many**
37	2646	YGL081W	N/A	N/A
38	**441**	**BRE5**	**cytoplasm**	**Ubiquitin protease cofactor, forms deubiquitination complex with Ubp3p that coregulates**

Pfa4 Rank	CW rank	Gene	Localization	Annotated Function
39	1759	APM4	AP-2 adaptor complex	Clathrin associated protein, medium subunit
40	2292	SAP4	cytoplasm	Protein required for function of the Sit4p protein phosphatase, member of a family of similar proteins that
41	223	SWF1	integral to membrane	Spore Wall Formation
42	272	DFG5	extrinsic to plasma	Mannosidase, essential glycosylphosphatidylinositol (GPI)-anchored membrane protein required for cell wall
43	847	RPL12A	cytosolic large ribosomal	Protein component of the large (60S) ribosomal subunit, nearly identical to Rpl12Bp; rpl12a rpl12b double
44	2692	COG5	Golgi transport complex	Component of the conserved oligomeric Golgi complex
45	1744	RPS30B	cytosolic small ribosomal	Protein component of the small (40S) ribosomal subunit; nearly identical to Rps30Ap and has similarity to
46	2175	OPY1	cytoplasm	Protein of unknown function, overproduction blocks cell cycle arrest in the presence of mating pheromone
47	157	TFP3	integral to membrane	vacuolar ATPase V0 domain subunit c' (17 kDa)
48	463	YHR078W	N/A	N/A
49	2847	YMR099C	cytoplasm	N/A
50	2605	MFA2	extracellular	alpha mating factor
51	1227	DOA1	cytoplasm	WD repeat protein required for ubiquitin-mediated protein degradation, forms complex with Cdc48p, plays
52	765	YHR130C	N/A	N/A
53	2220	GCN2	cytosolic ribosome	Protein kinase, phosphorylates the alpha-subunit of translation initiation factor eIF2 (Sui2p) in response to
54	1702	YLR049C	N/A	N/A
55	1129	STF2	proton-transporting ATP	ATPase stabilizing factor
56	951	YOR235W	N/A	N/A
57	2756	YKR035C	N/A	N/A
58	2691	YPL158C	bud neck	N/A
59	1515	SUM1	nucleus	Suppresor of mar1-1 (sir2) mutation; nuclear protein involved in silencing
60	379	RAD54	nucleus	DNA-dependent ATPase, stimulates strand exchange by modifying the topology of double-stranded DNA;
61	1686	RPL6A	cytosolic large ribosomal	N-terminally acetylated protein component of the large (60S) ribosomal subunit, has similarity to Rpl6Bp
62	679	YNR005C	N/A	N/A
63	1975	HLJ1	endoplasmic reticulum	Tail-anchored ER membrane protein of unknown function, similar to the E. coli DnaJ protein
64	131	YBL046W	nucleus	N/A
65	2118	HIT1	cytoplasm	Protein of unknown function, required for growth at high temperature
66	2945	HSE1	endosome	Subunit of the endosomal Vps27p-Hse1p complex required for sorting of ubiquitinated membrane proteins
67	2004	YGR206W	endosome	N/A
68	1075	RGD2	cytoplasm	GTPase-activating protein (RhoGAP) for Cdc42p and Rho5p
69	**999**	**BMH2**	**nucleus**	**14-3-3 protein, minor isoform; binds proteins and DNA, involved in regulation of many**
70	1324	VPS51	Golgi apparatus	whiskey (whi) mutant; forms a tetramer with VPS52, VPS53, and VPS54
71	470	SGF73	SAGA complex	SaGa associated Factor 73kDa; Probable 73KkDa Subunit of SAGA histone acetyltransferase complex
72	2491	YGL231C	endoplasmic reticulum	N/A
73	1288	GLO4	mitochondrion	Mitochondrial glyoxalase II, catalyzes the hydrolysis of S-D-lactoylglutathione into glutathione and D-
74	1548	YOR385W	cytoplasm	N/A
75	185	VPS41	vacuole (sensu Fungi)	vacuolar protein sorting; component of vacuolar membrane protein complex
76	476	CYK3	cytoplasm	SH3-domain protein located in the mother-bud neck and the cytokinetic actin ring; mutant phenotype and
77	1490	FYV12	N/A	Protein of unknown function, required for survival upon exposure to K1 killer toxin
78	2043	YDL211C	vacuole (sensu Fungi)	N/A
79	408	PSP1	cytoplasm	Asn and gln rich protein of unknown function; high-copy suppressor of POL1 (DNA polymerase alpha) and

Pfa4 Rank	CW rank	Gene	Localization	Annotated Function
80	1550	YKL105C	N/A	N/A
81	2163	YGR259C	N/A	N/A
82	2660	SSP2	spore wall (sensu Fungi)	Sporulation SPecific; involved in sporulation
83	2568	GOT1	Golgi membrane	Golgi Transport; membrane protein
84	2697	RPP2A	cytosolic large ribosomal	Ribosomal protein P2 alpha, a component of the ribosomal stalk, which is involved in the interaction
85	**1432**	**UBP3**	**cytoplasm**	**Ubiquitin-specific protease that interacts with Bre5p to co-regulate anterograde and retrograde**
86	243	BSD2	endoplasmic reticulum	metal homeostasis protein; putative membrane protein; copper transporter
87	67	HCM1	nucleus	Forkhead transcription factor involved in cell cycle specific transcription of SPC110, encoding a spindle pole
88	573	KTI12	DNA-directed RNA	Protein assoctiated with the RNA polymerase II Elongator complex; involved in sensitivity to G1 arrest
89	2008	TYR1	cytoplasm	Step of tyrosine biosynthesis pathway; Prephenate dehydrogenase (NADP+)
90	2986	UGA4	vacuolar membrane	GABA-specific transport protein
91	201	YIL001W	cytoplasm	N/A
92	**468**	**YAP1801**	**actin cortical patch**	**Yeast Assembly Polypeptide, member of AP180 protein family, binds Pan1p and clathrin;**
93	766	MSG5	cytoplasm	Dual-specificity protein phosphatase required for maintenance of a low level of signaling through the cell
94	632	NGL3	N/A	DNase/RNase (putative); CCR4 C-terminal homolog; displays homology to drosophila Angel gene; homolog
95	895	YER152C	cytoplasm	N/A
96	2564	YGL118C	N/A	N/A
97	2638	RPS4A	cytoplasm	Protein component of the small (40S) ribosomal subunit; mutation affects 20S pre-rRNA processing;
98	1247	YPL077C	N/A	N/A
99	306	YNL057W	N/A	N/A
100	556	ATG1	cytosol	Protein serine/threonine kinase, required for autophagy and for the cytoplasm-to-vacuole targeting (Cvt)
101	803	YMR295C	bud	N/A
102	180	SUB1	nucleus	Suppressor of TFIIB mutations; transcriptional coactivator
103	1798	YPL236C	vacuolar membrane	N/A
104	309	CUP9	nucleus	Homeodomain-containing transcriptional repressor of PTR2, which encodes a major peptide transporter;
105	963	YOR223W	N/A	N/A
106	1088	YAL004W	N/A	N/A
107	2306	DID2	cytoplasm	Class E protein of the vacuolar protein-sorting (Vps) pathway, associates reversibly with the late
108	592	HNT1	cytoplasm	Adenosine 5'-monophosphoramidase; interacts physically and genetically with Kin28p, a CDK and TFIIK
109	1140	SER33	cytoplasm	3-phosphoglycerate dehydrogenase, catalyzes the first step in serine and glycine biosynthesis; isozyme of
110	642	YNL109W	N/A	N/A
111	863	YCK2	plasma membrane	membrane-bound casein kinase I homolog
112	1941	PCT1	nucleus	Cholinephosphate cytidylyltransferase, also known as CTP:phosphocholine cytidylyltransferase, rate-
113	814	YCK1	endoplasmic reticulum	membrane-bound casein kinase I homolog
114	1920	YOR164C	cytoplasm	N/A
115	678	YDL176W	N/A	N/A
116	931	KRE2	Golgi apparatus	Alpha1,2-mannosyltransferase of the Golgi involved in protein mannosylation
117	77	YGR272C	cytoplasm	N/A
118	2961	YDL146W	cytoplasm	N/A
119	2505	CUE2	N/A	Protein of unknown function; has two CUE domains that bind ubiquitin, which may facilitate intramolecular
120	2732	SEC72	endoplasmic reticulum	protein involved in membrane protein insertion into the ER

Pfa4 Rank	CW rank	Gene	Localization	Annotated Function
121	453	YHR087W	cytoplasm	N/A
122	595	LGE1	nucleus	Protein of unknown function; null mutant forms abnormally large cells
123	1861	HEK2	cytoplasm	RNA binding protein with similarity to hnRNP-K that localizes to the cytoplasm and to subtelomeric DNA;
124	2818	COX20	mitochondrial inner	Mitochondrial inner membrane protein, required for proteolytic processing of Cox2p and its assembly into
125	268	RPL43B	cytosolic large ribosomal	Protein component of the large (60S) ribosomal subunit, identical to Rpl43Ap and has similarity to rat L37a
126	768	BFA1	spindle pole body	Component of the GTPase-activating Bfa1p-Bub2p complex involved in multiple cell cycle checkpoint
127	848	YDL162C	N/A	N/A
128	1618	SDS24	cytoplasm	Nuclear protein with similarity to S. pombe Sds23, suppresses dis2 mutations
129	217	AVT1	vacuole	Vacuolar transporter, imports large neutral amino acids into the vacuole; member of a family of seven S.
130	2322	MLH3	nucleus	Mutl Homolog; MutL Homolog
131	1501	ARR3	integral to plasma	Arsenite transporter of the plasma membrane, required for resistance to arsenic compounds; transcription
132	1177	YLR261C	N/A	N/A
133	1799	YTA6	cell cortex	Putative ATPase of the CDC48/PAS1/SEC18 (AAA) family, localized to the cortex of mother cells but not to
134	1273	YKL200C	N/A	N/A
135	2612	RPS23B	cytosolic small ribosomal	Ribosomal protein 28 (rp28) of the small (40S) ribosomal subunit, required for translational accuracy;
136	1741	YKL136W	N/A	N/A
137	622	SPP1	nuclear chromatin	Subunit of the COMPASS complex, which methylates histone H3 on lysine 4 and is required in
138	2536	GAL2	plasma membrane	Galactose permease, required for utilization of galactose; also able to transport glucose
139	2869	YJL107C	N/A	N/A
140	1637	COG7	mitochondrion	Component of the conserved oligomeric Golgi complex
141	654	YPL182C	N/A	N/A
142	195	YDL172C	N/A	N/A
143	227	DOT5	nucleus	Nuclear thiol peroxidase which functions as an alkyl-hydroperoxide reductase during post-diauxic growth
144	737	IWR1	cytoplasm	Protein of unknown function, deletion causes hypersensitivity to the K1 killer toxin
145	2027	YOR192C	membrane	N/A
146	2234	PPQ1	cytoplasm	Putative protein serine/threonine phosphatase; null mutation enhances efficiency of translational
147	52	MUD1	snRNP U1	U1 snRNP A protein, homolog of human U1-A; involved in nuclear mRNA splicing
148	1487	CMK1	cytoplasm	Calmodulin-dependent protein kinase
149	640	YNL120C	N/A	N/A
150	280	YGR016W	N/A	N/A
151	2162	YPS7	cytoplasm	Putative GPI-anchored aspartic protease, located in the cytoplasm and endoplasmic reticulum
152	2471	YGL114W	membrane	N/A
153	1348	MGS1	nucleus	Maintenance of Genome Stability 1
154	2215	CHO2	endoplasmic reticulum	First step in the methylation pathway for phosphatidylcholine biosynthesis; Phosphatidyl-ethanolamine N-
155	1601	GAS5	cell wall (sensu Fungi)	Putative 1,3-beta-glucanosyltransferase, has similarity to Gas1p; localizes to the cell wall
156	1467	KTR4	Golgi apparatus	Putative mannosyltransferase involved in protein glycosylation; member of the KRE2/MNT1
157	564	PHO87	membrane	Low-affinity inorganic phosphate (Pi) transporter, involved in activation of PHO pathway; expression is
158	2419	CLN2	cytoplasm	role in cell cycle START; G(sub)1 cyclin
159	555	ATG5	cytosol	Involved in autophagy; involved in autophagy
160	1068	RPS17B	cytosolic small ribosomal	Ribosomal protein 51 (rp51) of the small (40s) subunit; nearly identical to Rps17Ap and has similarity to
161	1271	AYR1	cytoplasm	NADPH-dependent 1-acyl dihydroxyacetone phosphate reductase found in lipid particles and ER; involved in

Pfa4 Rank	CW rank	Gene	Localization	Annotated Function
162	789	DCC1	DNA replication factor C	Subunit of a complex with Ctf8p and Ctf18p that shares some components with Replication Factor C,
163	230	YLL014W	endoplasmic reticulum	N/A
164	2143	YGL230C	N/A	N/A
165	1766	RSA3	nucleolus	Protein with a likely role in ribosomal maturation, required for accumulation of wild-type levels of large
166	171	AZF1	nucleus	Zinc-finger transcription factor, involved in induction of CLN3 transcription in response to glucose; genetic
167	1761	RFM1	nucleus	DNA-binding protein required for repression of middle sporulation genes; specificity factor that directs the
168	1680	YJL068C	cytosol	N/A
169	2748	URE2	soluble fraction	Nitrogen catabolite repression regulator that acts by inhibition of GLN3 transcription in good nitrogen
170	2330	LRO1	endoplasmic reticulum	Acyltransferase that catalyzes diacylglycerol esterification; one of several acyltransferases that contribute
171	130	SPA2	bud neck	Component of the polarisome, which functions in actin cytoskeletal organization during polarized growth;
172	2394	YLR404W	integral to membrane	N/A
173	762	MET3	cytoplasm	ATP sulfurylase, catalyzes the primary step of intracellular sulfate activation, essential for assimilatory
174	2286	GUP2	membrane	Multimembrane-spanning protein and putative glycerol transporter that is essential for proton symport of
175	1905	HOL1	mitochondrion	Putative ion transporter similar to the major facilitator superfamily of transporters; mutations in
176	1454	GCV2	mitochondrion	P subunit of the mitochondrial glycine decarboxylase complex, required for the catabolism of glycine to
177	2332	YGL085W	mitochondrion	N/A
178	1016	SPG3	N/A	Protein required for survival at high temperature during stationary phase
179	350	YGR106C	vacuolar membrane	N/A
180	2118	HIT1	cytoplasm	Protein of unknown function, required for growth at high temperature
181	484	YML053C	cytoplasm	N/A
182	1491	PTP1	cytoplasm	phosphotyrosine-specific protein phosphatase
183	860	CRH1	cell wall (sensu Fungi)	Putative glycosidase of the cell wall, may have a role in cell wall architecture
184	1214	YPR153W	N/A	N/A
185	2048	YGL079W	endosome	N/A
186	1587	YNL324W	N/A	N/A
187	2417	GSP2	nucleus	GTP binding protein (mammalian Ranp homolog) involved in the maintenance of nuclear organization, RNA
188	575	YOR309C	N/A	N/A
189	2837	BSC6	clathrin-coated vesicle	Protein of unknown function containing 8 putative transmembrane seqments; ORF exhibits genomic
190	2654	FRM2	cytoplasm	Protein of unknown function, involved in the integration of lipid signaling pathways with cellular
191	258	CLB5	nucleus	role in DNA replication during S phase; additional functional role in formation of mitotic spindles along with
192	1357	RPS6B	cytoplasm	Protein component of the small (40S) ribosomal subunit; identical to Rps6Ap and has similarity to rat S6
193	668	CLA4	actin cap (sensu Fungi)	Involved in localizing cell growth with respect to the septin ring; protein kinase, homologous to Ste20p,
194	2081	PCL1	cyclin-dependent protein	Pho85 cyclin of the Pcl1,2-like subfamily, involved in entry into the mitotic cell cycle and regulation of
195	204	YJR054W	vacuole (sensu Fungi)	N/A
196	656	GLT1	mitochondrion	NAD(+)-dependent glutamate synthase (GOGAT), synthesizes glutamate from glutamine and alpha-
197	**302**	**SYS1**	**integral to Golgi**	**Multicopy suppressor of ypt6 null mutation**
198	1048	MSH3	nuclear chromosome	Mismatch repair protein, forms dimers with Msh2p that mediate repair of insertion or deletion mutations
199	1673	ATG23	extrinsic to membrane	Peripheral membrane protein, required for autophagy and for the cytoplasm-to-vacuole targeting (Cvt)
200	2483	DLS1	nucleus	Subunit of ISW2/yCHRAC chromatin accessibility complex along with Itc1p, Isw2p, and Dpb4p; involved in