

CHARACTERIZING THE ROLE OF PHOSPHOLIPID SYNTHESIS IN PROTEIN QUALITY
CONTROL

A THESIS SUBMITTED TO THE GRADUATE SCHOOL IN PARTIAL FULFILLMENT
OF THE REQUIREMENT FOR THE DEGREE
MASTER OF SCIENCE

BY

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JULY 2024

Acknowledgements

I would like to thank my advisor and PI, Dr. Eric “VJ” Rubenstein, for his insights into the workings of yeast, his excellent advice, his time, and his patience. I would also like to thank Dr. Douglas Bernstein and Dr. Douglas Roossien for their contributions to my thesis as part of my committee, as well as their contributions to my education. To the members of the Rubenstein lab, I would like to thank Connor Bailey, Sam Gosser, and Mary Tiña-Tragesser for their guidance in the lab, and many others for their company and insight. I would also like to thank Rheanna Walther, from the Bernstein Lab, my frequent lab partner in crime, for her fantastic support and contributions to developing my abilities as a researcher. Finally, I would like to recognize the funding obtained from the Ball State University ASPiRE grant and the NIH grant awarded to Dr. Eric “VJ” Rubenstein.

Table of Contents

List of Tables	4
List of Figures	5
List of Abbreviations	6
Abstract.....	7
Specific Aims	8
Introduction	9
Research Methods	17
Plasmid Miniprep	17
Yeast Transformation	17
Cycloheximide Chase and Yeast Cell Harvest	18
Cell Lysis	19
Endoglycosidase H Treatment	19
SDS-PAGE Gel Electrophoresis and Transfer	20
Western Blotting	20
Statistical Analysis	20
Results.....	21
<i>OPI3</i> knockout stabilizes <i>Deg1</i> *-Sec62	21
<i>INO4</i> knockout does not cause a global translocation defect	21
<i>INO4</i> knockout does not destabilize Cue1 and Sbh1	22
<i>INO4</i> knockout does not stabilize a soluble substrate of a soluble ubiquitin ligase.....	22
Discussion	23
Summary of research findings	23
Future direction: Validating impact of mutations on lipid composition.....	24
Future direction: How does altered lipid homeostasis impact ER protein degradation?	25
Future direction: Uncoupling the impact of altered PE and PC synthesis on ER PQC	26
Future direction: Evaluating the extent of the impact of lipid synthesis on PQC.....	27
Future direction: How does impaired lipid synthesis slow <i>Deg1</i> *-Sec62 glycosylation?	27
Significance of research findings.....	28
Concluding remarks	29
References Cited	30
Tables	34
Figures.....	35

Figures 36

List of Tables

1. Table 1: Yeast strains used in these experiments
2. Table 2: Plasmids used in these experiments

List of Figures

1. Figure 1: Co-translational and post-translational translocation at the ER membrane
2. Figure 2: ER protein quality control mechanisms
3. Figure 3: *INO2* and *INO4* deletion stabilizes *Deg1**-Sec62
4. Figure 4: *Deg1**-Sec62 degradation is sensitive to perturbed phosphatidylcholine biosynthesis
5. Figure 5: *INO4* deletion does not broadly impair translocation
6. Figure 6: *INO4* deletion does not destabilize Cue1 and Sbh1
7. Figure 7: *INO4* deletion does not broadly impair PQC
8. Figure 8: Phospholipid synthesis pathway from phosphatidylserine to phosphatidylcholine

List of Abbreviations

1. ALS – amyotrophic lateral sclerosis
2. AP-MS – affinity purification-mass spectrometry
3. CPY – carboxypeptidase Y
4. CTT – co-translational translocation
5. E1 – ubiquitin activating enzyme
6. E2 – ubiquitin conjugating enzyme
7. E3 – ubiquitin ligase
8. Endo H – endoglycosidase H
9. ER – endoplasmic reticulum
10. ERAD – endoplasmic reticulum-associated degradation
11. ERAD-L – ERAD of proteins with luminal degrons
12. ERAD-M – ERAD of proteins with transmembrane degrons
13. ERAD-RA – ERAD of ribosome-associated proteins
14. ERAD-T – ERAD of translocon-clogging proteins
15. INM – inner nuclear membrane
16. INMAD – inner nuclear membrane-associated degradation
17. LB – Luria broth
18. MS – mass spectrometry
19. OPY – CPY with an Ost1 SS
20. PC – phosphatidylcholine
21. PE – phosphatidylethanolamine
22. PI – phosphatidylinositol
23. PQC – protein quality control
24. PS – phosphatidylserine
25. PTT – post-translational translocation
26. PVDF – polyvinylidene difluoride
27. RT – room temperature
28. SRP – signal recognition particle
29. SS – signal sequence
30. UPR – unfolded protein response
31. UPS – ubiquitin proteasome system

Abstract

Protein quality control (PQC) is an essential function for all living organisms, ensuring proper protein synthesis, folding, and clearance of potentially toxic, aberrant proteins in the cell. Impaired PQC is linked to human diseases such as Alzheimer's disease, amyotrophic lateral sclerosis (ALS), and type 2 diabetes, underscoring its importance. The role of phospholipid synthesis in maintaining efficient PQC is incompletely characterized. Previous work shows that impaired phospholipid synthesis via *INO4* deletion stabilizes the translocon-clogging Hrd1 substrate *Deg1**-Sec62 in *Saccharomyces cerevisiae*. *INO4* is required for the synthesis of several phospholipids. It is unknown if selective disruption of phosphatidylcholine (PC) synthesis (whose synthesis is regulated by the Ino2/Ino4 master transcriptional regulator) results in similar stabilization of model ER-associated degradation (ERAD) substrates. I showed that impaired PC synthesis partially stabilizes *Deg1**-Sec62. The mechanism by which impaired phospholipid synthesis impairs ERAD is unknown. Prior research suggests that impaired phospholipid synthesis might globally impair translocation into the ER. I determined that *INO4* deletion does not globally impair translocation, but partially impairs *Deg1**-Sec62 glycosylation. The breadth of the impact of impaired phospholipid synthesis on protein degradation is unclear. I showed that *INO4* deletion does not stabilize the soluble nucleoplasmic degradation substrate, $\alpha 2$ *-UH. I also showed that *INO4* deletion does not destabilize ER proteins Cue1 and Sbh1. Together, these results enhance our understanding of the impact phospholipid synthesis has on cellular physiology, potentially identifying unaccounted for features of human disorders associated with disrupted phospholipid synthesis.

Specific Aims

1. Characterize the impact of reduced phosphatidylcholine synthesis on *Deg1**-Sec62 degradation.
2. Determine if phospholipid synthesis is required for efficient ER protein translocation.
3. Characterize the broader impact of phospholipid biosynthesis on protein degradation.

Introduction

The endoplasmic reticulum (ER) in eukaryotes is required for the transport, secretion, and modification of proteins. Approximately 30% of all proteins produced in eukaryotic cells must first enter the ER for modification or transport [1]. These modifications include N-linked glycosylation, disulfide bond formation, protein folding, and cleavage. Transport includes the delivery to other areas of the cell such as the Golgi apparatus, plasma membrane, and lysosomes as well as secretion from the cell via exocytosis [2]. Proper folding is required for proteins to function. Misfolding can result in impaired cell function, contributing to the development of diseases such as Alzheimer's, Parkinson's, and Type II Diabetes [3]. Accumulation of misfolded proteins and an impaired ability to restore protein homeostasis (proteostasis) is a hallmark of aging [3]. The endomembrane system, cumulatively considered to be the nuclear envelope, lysosomes, the ER, Golgi apparatus, plasma membrane, and the vesicles of the cell, is a system conserved across eukaryotes including yeast and mammals, such as humans [4].

To enter the ER, proteins must move through the translocon ("translocate"). The translocon is a heterotrimeric protein complex that includes the universally conserved Sec61 channel protein alpha subunit along with the conserved beta and gamma subunits (Sbh1 and Sss1 in *S. cerevisiae*) [5]. There are two forms of protein translocation – co-translational translocation (CTT) and post-translational translocation (PTT). CTT occurs when the protein is translocated into the ER as it is translated by the ribosome complex (Figure 1). PTT occurs when the protein is translocated after translation is completed. CTT is facilitated by the interaction of the signal recognition particle (SRP) with the CTT signal sequence (SS) of a nascent chain protein emerging from the ribosome [6]. The SRP escorts the complex to the SRP receptor complex (SRP101 and SRP102) [7, 8]. Here, the SRP is released and the ribosome-nascent chain-complex docks to the Sec61 channel, where

translation proceeds as the protein is translocated into the ER [8]. Proteins possessing a PTT SS undergo translocation in an SRP-independent manner that is facilitated by the Sec62 and Sec63 proteins as part of a larger heptameric Sec61 complex [9, 10]. Molecular chaperones maintain the translocation-competent (i.e., unfolded) state of nascent proteins destined for PTT [11]. Efficient translocation is required to maintain proteostasis.

Protein synthesis is prone to errors including misfolding, incomplete translation, stalled translocation, and incorrect localization in the cell. These aberrant proteins account for up to 30% of all synthesized proteins in eukaryotic cells [12]. The relative abundance of these proteins suggests that protein misfolding is inevitable. ER protein misfolding results in protein dysfunction and potentially protein aggregation, which can impair ER function. The increased load of misfolded or aberrant proteins causes an increased burden on the molecules responsible for protein folding (e.g., molecular chaperones) in the ER [13]. Consequently, the cell has in place the unfolded protein response (UPR), a signaling pathway that results in increased synthesis of molecular chaperones and protein degradation machinery, as a mechanism to alleviate the excess accumulation of misfolded proteins in the ER [14]. These ER PQC mechanisms are essential to the health of the cell. Moreover, as cells age, they become less capable of ameliorating the accumulation of these misfolded proteins, contributing to the development of diseases such as Alzheimer's, Parkinson's [15] as well as Type 2 Diabetes [3]. Therefore, cells require a robust PQC system to attenuate the toxic effects of accumulated misfolded protein and aggregates.

ER PQC mechanisms are required to degrade misfolded proteins, proteins aberrantly engaged with the translocon, those with impaired function as well as proteins that are no longer needed. Many aberrant proteins are degraded by the ubiquitin proteasome system (UPS). In the UPS, proteins destined for degradation by the cytosolic proteasome are covalently modified by the

protein ubiquitin. The UPS involves the coordination of ubiquitin-activating enzymes (E1s) that activate ubiquitin molecules so they can be covalently linked to ubiquitin-conjugating enzymes (E2s). E2s associate with ubiquitin ligases (E3s) that then transfer these ubiquitin molecules to proteins targeted for degradation [16]. The accumulation of these ubiquitin proteins in the form of a polyubiquitin chain facilitates the ubiquitin-dependent degradation of proteins by the proteasome.

There are several E3s which function to target diverse proteins in a range of subcellular locations for proteasomal degradation. For example, multiple conserved ER-associated degradation (ERAD) pathways promote degradation of misfolded and otherwise aberrant ER proteins. These ERAD pathways are differentiated based on the location of the degradation signal found within the substrate protein. Hrd1 targets proteins clogged in the translocon (ERAD-T) (Figure 2A) and proteins with degradation signals in the lumen of the ER (ERAD-L) or in ER transmembrane segments (ERAD-M) [17]. Hrd1 functions with the ubiquitin-conjugating enzyme Ubc7, which is tethered to the plasma membrane by the transmembrane protein Cue1 [17]. This Hrd1 pathway is conserved in humans, with the SYVN1 and HRD1 enzymes corresponding to Hrd1 and the UBE2G2 enzyme corresponding to the Ubc7 protein [18]. In complex with the ubiquitin-conjugating enzyme Ubc6 and the Cue1-Ubc7 dimer, Doa10 is required for ERAD-C, or ERAD of proteins with cytosolic degradation signals, and inner nuclear membrane (INM)-associated degradation (INMAD) (Figure 2B) [19]. The *DOA10* gene is conserved in humans as the *MARCHF6* gene [20]. The Asi ubiquitin ligase complex functions alongside the Doa10 complex to facilitate INMAD [21]. Although not strictly an ERAD component, the transmembrane zinc metalloprotease Ste24 also promotes ER quality control. The yeast *STE24* has a human homolog, *ZMPSTE24* [22]. Ste24 cleaves proteins clogged at the translocon in a degradative

mechanism that is partially redundant with Hrd1 (Figure 2C) [23]. Clearance of translocon-clogged proteins is essential to the maintenance of proteostasis. Impaired translocation causes accumulation of unfolded proteins in the cytosol and the initiation of the UPR [24].

ERAD-T, or ER-associated degradation of translocon-associated proteins, is required for the clearance of translocon-clogging proteins at the ER. Translocon clogging prevents translocation of other proteins, contributing to the accumulation of proteins in the cytoplasm, which can lead to protein misfolding as the nascent proteins are stalled in the cytosol. While the genetic requirements for ERAD-L, -M, and -C have been extensively characterized, little is known about the genes required for ERAD-T. Therefore, the Rubenstein lab performed a genetic screen in yeast to identify genes required for ERAD-T of the model engineered translocon-clogging protein *Deg1**-Sec62, a substrate of the Hrd1 pathway [25]. A yeast strain expressing a variant of *Deg1**-Sec62 fused with the His3 protein was crossed with deletion or hypomorphic yeast strains to generate a library of approximately 6000 novel strains. These yeast were then queried for enhanced growth rate in media lacking histidine. Yeast strains with impaired ERAD-T would exhibit stabilized *Deg1**-Sec62-His3 and would thus be able to synthesize histidine in media lacking histidine, contributing to increased growth observable using optical densitometry. This screen revealed *INO2* and *INO4* as candidate genes required for efficient degradation of *Deg1**-Sec62, which was later confirmed using cycloheximide chase and western blot analysis (Figure 3).

The decision to use *S. cerevisiae*, or budding yeast, as our model organism to study PQC is grounded in its physiological relevance to human systems. The Hrd1, Doa10, and Ste24 PQC pathways are conserved in humans, making the translation of my research findings to human ER PQC pathways and disease pathology likely. In addition, yeast cells are amenable to genetic modification and plasmid insertion, allowing efficient knockout or insertion of genes to examine

their molecular roles [26]. The yeast genome is also fully sequenced and well annotated, allowing efficient retrieval of information from the *Saccharomyces* Genome Database for analysis [27]. Thus, *S. cerevisiae* serves as a valuable and efficient organism for the study of these pathways.

The *INO2* and *INO4* genes encode subunits of the heterodimeric transcription factor Ino2/Ino4. This complex derepresses genes involved in phospholipid biosynthesis [28]. The Ino2/Ino4 complex regulates genes, including *INO1* [29], *CHO1*, *CHO2* [30, 31], and *OPI3* [31], genes involved in the biosynthesis of various lipid molecules. *INO1* is required for phosphatidylinositol (PI) synthesis. *CHO1* is required for phosphatidylserine (PS) and phosphatidylethanolamine (PE) synthesis [32], while *CHO2* and *OPI3* are both required for phosphatidylcholine (PC) synthesis. Therefore, the Ino2/Ino4 complex regulates PI, PS, PE, and PC synthesis.

Phospholipids are essential components of cell membranes. These amphipathic molecules allow the plasma membrane to separate the cell from its external environment. Other cellular membranes also allow for the specialization of tasks. For example, the ER membrane allows for separation of the ER lumen from the cytosol, generating a specialized environment in the lumen required for the modification of nascent proteins. Further, organelles such as the mitochondria, Golgi apparatus, and lysosomes require a phospholipid membrane for the separation of tasks unique to these organelles. Disruption of phospholipid metabolism and synthesis in mammalian cells is associated with a range of metabolic disorders including atherosclerosis, insulin resistance, diabetes, liver disease (e.g., non-alcoholic fatty liver disease), and neurodegenerative diseases [33].

For this project, I addressed three questions related to the relationship between lipid biosynthesis and PQC. Research in the Rubenstein lab showed *INO4* and *INO2* deletion results in stabilization of *Deg1**-Sec62 [25]. Further, deletion of *INO1* results in similar stabilization,

indicating the importance of PI synthesis in the function of ERAD. The requirement of *INO2/INO4* for the synthesis of phospholipids other than PI suggests the potential requirements(s) of other lipids in maintaining the efficiency of ERAD. **The first question was, to what extent does the loss of PC synthesis impair ERAD?** To address this question, degradation of *Deg1**-Sec62 was assessed in yeast with deficient PC synthesis, such as those lacking the *CHO2* or *OPI3* genes. These genes encode enzymes that catalyze different steps in PC synthesis. Preliminary data indicated the loss of *CHO2* modestly stabilizes *Deg1**-Sec62 [25], supporting the role of PC in the maintenance of ERAD. To further investigate the requirement of PC in ERAD, I assessed the impact of loss of *OPI3* on *Deg1**-Sec62 degradation. *OPI3* has a human homolog (PEMT) and is required for the methylation of phosphatidyl-*N*-methylethanolamine, an intermediate of PC synthesis [34]. I found *OPI3* deletion modestly stabilizes *Deg1**-Sec62, to a degree that is intermediate to the impacts of *INO4* or *HRD1* deletion. Establishing the role of *OPI3* in ERAD efficiency identifies a potential contributor to pathology in human diseases with impaired PC synthesis.

The second question was, what is the impact of impaired lipid synthesis on protein translocation? In addition to stabilizing *Deg1**-Sec62, *INO4* deletion delayed post-translational modification (N-linked glycosylation) of the protein, which only occurs after translocon engagement. This delayed N-linked glycosylation suggested that *Deg1**-Sec62 stabilization could be the result of broadly impaired translocation in cells with disturbed phospholipid synthesis. To test this hypothesis, I inserted a plasmid expressing either CPY or OPY, model proteins undergoing PTT and CTT respectively, into WT, *ino4*Δ, and *sec61-13Myc* (a strain of yeast with established impaired PTT) and assayed CPY and OPY for changes in translocation efficiency in *ino4*Δ compared to WT yeast. I found that *INO4* deletion did not contribute to impaired translocation of

CPY or OPY, suggesting that disrupted phospholipid synthesis-mediated stabilization of ERAD-T substrates is not attributable to a broad translocation defect alone.

The third question was, what is the extent of the impact of disrupted lipid homeostasis on protein degradation? Stabilization of ERAD substrates in yeast with impaired phospholipid synthesis is not universal. In fact, impaired PC synthesis has been reported to increase the degradation rate of ER-resident proteins, Cue1 and Sbh1, which is counter to what is observed with the canonical ERAD substrate, *Deg1**-Sec62 [35]. Cue1 and Sbh1 are not typically targeted by the ERAD pathways. Cue1 anchors Ubc7 to the ER, as part of the Hrd1 and Doa10 ubiquitin-ligase complexes to facilitate ERAD [36]. Sbh1 is a subunit of the translocon complex [37]. Destabilization of Cue1 and Sbh1 in cells with impaired phospholipid synthesis is a potential explanation for the stabilization of canonical ERAD substrates such as *Deg1**-Sec62. Reduced abundance of Cue1 can impair Hrd1-mediated proteolysis. Reduced abundance of Sbh1 has the potential to impair translocation of ERAD substrates, resulting in reduced function of ERAD pathways because of reduced engagement of ERAD enzymes with the translocon (and therefore reduced integration into the ER membrane where they execute their functions). To determine if deletion of *INO4*, which stabilizes *Deg1**-Sec62, destabilizes Cue1 and Sbh1, I assessed the stability of an HA-tagged form of Cue1 or Sbh1 in WT, *opi3*Δ, and *ino4*Δ yeast. I found that *INO4* deletion does not destabilize these proteins, suggesting that the effect of *INO4* deletion on PQC efficiency is not due to destabilized Sbh1-mediated impaired translocation or destabilized Cue1-mediated impaired Hrd1 ubiquitination. The mechanism of *INO4* deletion-mediated stabilization of ERAD substrates remains unclear.

INO4 deletion stabilizes the model ERAD substrate *Deg1**-Sec62. Data from the Rubenstein lab indicate *INO4* deletion impairs degradation of ERAD-L, ERAD-M, and ERAD-C

substrates. It is unknown, however, if *INO4* deletion similarly stabilizes non-ER-associated substrates, such as soluble substrates of soluble ubiquitin ligases. Considering the uncharacterized mechanism for how *INO4* deletion stabilizes ERAD substrates, it is important to investigate the impact of *INO4* deletion on non-ERAD substrates, such as $\alpha 2^*$ -UH, a soluble protein targeted by the soluble ubiquitin ligase complex, Slx5/Slx8 [38, 39]. This pathway is independent of ERAD, so stabilization of $\alpha 2^*$ -UH would support the hypothesis that disturbed phospholipid synthesis broadly inhibits UPS function. To determine whether *INO4* deletion stabilizes non-ER substrates, I assessed the degradation of $\alpha 2^*$ -UH in WT, *ino4* Δ , and *slx8* Δ . I found that *INO4* deletion does not stabilize $\alpha 2^*$ -UH, indicating that *INO4* deletion does not broadly impair PQC, outside of the ER.

Taken together, my results address three questions about the impact impaired phospholipid synthesis has on PQC. I found that targeted disruption of PC synthesis stabilizes the ERAD substrate, *Deg1*^{*}-Sec62. *INO4* deletion does not broadly impair translocation. Finally, *INO4* deletion does not destabilize the ER-resident proteins, Cue1 and Sbh1, nor does it stabilize the non-ER resident substrate $\alpha 2^*$ -UH. This work answers important questions about the mechanisms and extent of impact of disrupted lipid homeostasis on PQC, with broad relevance to human diseases characterized by disruptions in lipid homeostasis, proteostasis, or both.

Research Methods

Plasmid Miniprep

Plasmids were stored in *Escherichia coli* as 25% glycerol permanents at -70 °C. Bacteria were streaked from the permanents onto Luria Broth (LB) plates containing ampicillin (LB+amp; 100 µg/mL ampicillin; 0.5% yeast extract, 1% tryptone, 0.5% NaCl). These plates were incubated overnight at 37 °C. The following day, individual colonies were inoculated into liquid LB media containing ampicillin (LB+amp; 100 µg/mL ampicillin) and incubated at 37 °C overnight with rotation. The following day, a plasmid miniprep was performed using the protocol from the QIAprep Miniprep Handbook (QIAGEN) for low throughput plasmid harvests.

Yeast Transformation

S. cerevisiae were streaked from glycerol permanents stored at -70 °C onto yeast extract-peptone-dextrose (YPD; 1% Bacto yeast extract, 2% Bacto peptone, 2% glucose, 0.2% adenine, 2% agar) plates. Samples were incubated at 30 °C for 2-3 days until sufficient growth for inoculation into liquid media (pinhead size colonies). Individual colonies were inoculated into liquid YPD (5 mL) and cultured overnight at 30 °C with rotation.

The following day, 500 µL aliquots of each strain were transferred into 1.5 mL microcentrifuge tubes and centrifuged at 5000 rpm for 2 minutes at room temperature (RT). Supernatant was removed, and yeast pellets were resuspended in 1000 µL sterile water. Samples were centrifuged at 5000 rpm for 2 minutes at RT. The supernatant was removed, and the cells were resuspended in 50 µL of sterile lithium acetate-Tris-EDTA (LTE; 0.1 M lithium acetate, 10 mM tris-CL pH 7.5, 1 mM EDTA). 20 µg of carrier DNA were added to the media along with 1 µL of the plasmid miniprep. A no-plasmid negative control was included for each strain.

Samples were vortexed and incubated at RT for 30 minutes. Following the incubation, 400 μ L of 40% polyethylene glycol (PEG) in LTE was added to each sample. Tubes were vortexed and incubated at RT overnight. The following day, samples were centrifuged at 5000 rpm for 2 minutes. The supernatant was removed, and the cell pellets were resuspended in 50 μ L of 1 M sorbitol. The suspension was plated onto plates corresponding to the selective yeast growth marker (SD Media; 0.67% yeast nitrogen base without amino acids, 2% glucose, 0.002% arginine, 0.001% histidine, 0.006% isoleucine, 0.006% leucine, 0.004% lysine, 0.001% methionine, 0.006% phenylalanine, 0.005% threonine, 0.004% tryptophan, 0.002% adenine, 0.004% uracil). Plates were incubated at 30 °C until colonies were present on positive control plates.

Cycloheximide Chase and Yeast Cell Harvest

Each transformed yeast strain was individually inoculated into 5 mL of selective media and incubated at 30 °C overnight with rotation. The following day, OD₆₀₀ (optical density at 600 nm) values were obtained for each sample. Yeast were diluted to an OD₆₀₀ value of 0.2. The samples were returned to the 30 °C incubator with rotation until the yeast suspensions were at an OD₆₀₀ value ranging from 0.8 to 1.2, corresponding to midlogarithmic (mid-log) growth phase for *S. cerevisiae*. For cell harvest, 2.5 OD₆₀₀ units (1 OD₆₀₀ unit is 1 mL of yeast at an OD₆₀₀ of 1.0) of yeast cells were collected by centrifugation at 5000 rpm for 2 minutes. Supernatants were removed, and samples were lysed or stored in the -20 °C freezer until cell lysis.

For cycloheximide chase analysis, 2.5 OD₆₀₀ units were harvested for each time point for the chase (typically 0, 30, and 60 minutes). Samples were centrifuged at 5000 rpm for 2 minutes and resuspended in 1 mL of selective media warmed to 30 °C for every 2.5 OD₆₀₀ units of sample harvested. Samples were incubated in pre-warmed media for 5 minutes. At time 0, cycloheximide (20 mg/mL) was administered to each suspension to a final concentration of 250 μ g/mL, followed

by vortexing. 950 μ L of sample were immediately harvested from the suspension and added to a 50 μ L solution of 20X ice-cold stop mix (200 mM sodium azide, 5 mg/mL bovine serum albumin) for 0-min time points. The sample was vortexed and placed on ice. Administration of cycloheximide to each sample of yeast was performed in 30-second increments. Additional 950 μ L harvests were performed at indicated times following cycloheximide addition (typically 30 and 60 minutes) for each sample. Samples were vortexed and combined with the 20X stop mix. All samples were centrifuged for 30 seconds at 7000 rpm at RT. The supernatants were removed, and the pellets stored at -20 $^{\circ}$ C until cell lysis.

Cell Lysis

Cell lysis was performed according to the Kushnirov protocol (2000). Cells were allowed to thaw at RT. Cell pellets were resuspended in 100 μ L of RT sterile water. 100 μ L of 0.2 M NaOH at RT was added to the samples, and the suspensions were vortexed. Samples incubated at RT for 5 minutes. After incubation, samples were centrifuged at 13,000 rpm for 30 seconds. The supernatants were removed. The pellets were resuspended in 100 μ L SDS sample buffer (0.06 M Tris-HCL, pH 6.8, 5% glycerol, 2% SDS, 4% β -mercaptoethanol, a pinch of bromophenol blue) and boiled for 5 minutes to lyse cells and denature proteins. Samples were stored in the -20 $^{\circ}$ C freezer or used immediately for SDS-PAGE gel electrophoresis or for endoglycosidase H treatment.

Endoglycosidase H Treatment

20 μ L aliquots of cell lysate (2 for each sample) were transferred to new tubes and treated with 2.2 μ L of 0.83 M potassium acetate. To 1 aliquot, 0.5 μ L of endoglycosidase H (Endo H; 250 units) was added, and, to the other, 0.5 μ L of sterile water (negative control) was added. Samples were incubated at 37 $^{\circ}$ C for 2.5 hours. Samples were boiled for 5 minutes to deactivate the enzyme.

Samples were stored in the -20 °C freezer or used immediately for SDS-PAGE gel electrophoresis.

SDS-PAGE Gel Electrophoresis and Transfer

Samples were run on 8%, 10%, or 12% SDS-PAGE gels to separate the proteins. Proteins were transferred from gels onto polyvinylidene difluoride (PVDF) membranes at 20 V for 1 hour at 4 °C. Membranes were blocked in a 5% milk protein (w/v) solution in Tris-buffered saline (TBS; 50 mM Tris, 150 mM NaCl) at RT for 1 hour, with rocking, or at 4 °C overnight.

Western Blotting

Antibodies were diluted in a 1% milk protein (w/v) solution in TBS with 1% Tween 20 (TBS/T) for all probes. Membranes were incubated in the presence of antibodies for 1 hour at RT, with rocking. Samples were washed between incubations 3 times for 5 minutes each, while rocking. The concentrations of antibodies were as follows: mouse anti-HA.11 (Clone 16B12; BioLegend) at 1:1000 and mouse anti-Pgk1 (Clone 22C5D8; LifeTechnologies) at 1:20,000. Rabbit anti-mouse secondary antibody was used to probe the mouse antibodies (AlexaFluor-680-conjugated rabbit anti-mouse (LifeTechnologies) at a concentration ranging between 1:20,000 to 1:40,000 for 1 hour at RT with rocking in 1% milk protein in TBS/T solution. Membranes were imaged using the Odyssey CLx IR Imaging System (Li-Cor).

Statistical Analysis

ImageStudio software was used to quantify the protein abundance. The abundance of protein was calculated using Microsoft Excel using the fluorescence intensity of the protein of interest for each strain, normalized to the intensity of the loading control (Pgk1). Experiments were repeated three times. Means of resultant values were then analyzed using RStudio using the appropriate statistical analyses one-way ANOVA to determine significance.

Results

***OPI3* knockout stabilizes *Deg1**-Sec62**

We established that broad disruption of PS, PI, PE and PC synthesis results in stabilization of several ERAD substrates. Moving forward, I sought to investigate the effects of selective disruption of PC synthesis on the ERAD-T substrate, *Deg1**-Sec62 using *OPI3* knockout yeast. Opi3 is a phospholipid methyltransferase required for the two-step methylation of PE into PC [40]. Knockout of *OPI3* allowed me to observe the effects of selective disruption of PC biosynthesis. Deletion of *OPI3* slowed degradation of *Deg1**-Sec62 (Figure 4), indicating that PC biosynthesis is required for efficient degradation of *Deg1**-Sec62.

***INO4* knockout does not cause a global translocation defect**

*Deg1**-Sec62 is glycosylated upon translocation into the ER lumen [41]. Deletion of *INO4* results in glycosylation impairment of *Deg1**-Sec62 as evidenced by increased mobility of the protein, which may reflect impaired translocon engagement (Figure 5A) [25]. I hypothesized that impaired translocation contributes to *Deg1**-Sec62 stabilization in *INO4* knockout yeast due to reduced engagement and recruitment of ERAD-T machinery. CPY (carboxypeptidase Y) and OPY (modified CPY with an Ost1 signal sequence) are model post-translationally translocated and co-translationally translocated proteins, respectively. Further, CPY and OPY become glycosylated upon entry into the ER lumen [42]. Analysis of CPY and OPY in *ino4Δ* yeast showed that *INO4* deletion does not broadly impair PTT and CTT (Figure 5B). It was previously shown that the addition of a 13Myc epitope to Sec61 results in impaired PTT of *Deg1**-Sec62 and CPY [43], so I expected a faster-migrating unglycosylated, untranslocated band to mirror that of the *sec61-13Myc* strain if translocation and subsequently glycosylation were impaired in *INO4* knockout yeast. This was not observed for CPY. To further validate that the slower-migrating bands in *ino4Δ*

yeast represent glycosylated proteins, I treated these lysates with endoglycosidase H (Endo H), which cleaves N-glycans. I observed a mobility shift in *Deg1**-Sec62, CPY, and OPY, confirming N-glycosylation and therefore translocation (Figure 5C).

***INO4* knockout does not destabilize Cue1 and Sbh1**

Recent research has shown *OPI3* deletion destabilizes Cue1, an ER membrane-bound anchor for Ubc7 and Sbh1, which is part of the heterotrimeric translocon complex [35]. Ubc7 is required for ERAD mediated by both Hrd1 and Doa10 [36]. Since *INO4* deletion disrupts *OPI3* transcription, I performed cycloheximide chase experiments to investigate the impact *INO4* deletion has on the stability of Sbh1 and Cue1. Destabilized Ubc7 or Sbh1 by *INO4* deletion would suggest a potential mechanism through which impaired phospholipid synthesis might stabilize ERAD substrates. However, in contrast to reported findings for *OPI3* deletion, I found that *INO4* deletion did not destabilize Cue1 or Sbh1 (Figure 6A and 6B.) While I reproduced the observation that loss of *OPI3* destabilizes Sbh1-HA, I did not observe significant stabilization of Cue1-HA in *opi3Δ* yeast. This suggests that *INO4* deletion-mediated stabilization of ERAD substrates is not attributable to Cue1 or Sbh1 destabilization.

***INO4* knockout does not stabilize a soluble substrate of a soluble ubiquitin ligase**

INO4 deletion has been shown to impair ERAD of cytosolic, membrane, and luminal substrates of the Doa10 and Hrd1 ERAD pathways [25]. Doa10 and Hrd1 are both ER-resident ubiquitin ligases. I tested whether *INO4* deletion broadly impairs the function of ubiquitin ligases, including those outside of the ER. Using a cycloheximide chase, I show that *INO4* deletion does not, however, impair the degradation of $\alpha 2^*$ -UH, a soluble, non-ER-associated substrate of the soluble ubiquitin ligase Slx5/Slx8 (Figure 7). I therefore conclude that *INO4* deletion does not broadly impair the ubiquitin-proteasome system outside of ERAD.

Discussion

Through a gene knockout screen, my lab identified genes involved in phospholipid synthesis as having a significant role in the degradation of the model engineered translocon-clogging substrate, *Deg1**-Sec62. This finding was further validated by cycloheximide chase and western blot analysis using *INO4* knockout yeast. The breadth of impact impaired phospholipid synthesis has on PQC remained unclear. Taking these findings into consideration, the objectives of my research were to determine if *OPI3* is required for the degradation of *Deg1**-Sec62 and determining if *INO4* deletion causes translocation defects. Further, I sought to determine if loss of *INO4* destabilizes the Ubc7-anchoring membrane protein, Cue1, providing a potential mechanism for the impact of loss of *INO4* on ERAD. Another means of ERAD impairment might be achieved via destabilization of the translocon subunit, Sbh1. Therefore, I sought to determine if *INO4* deletion destabilizes Sbh1. Finally, not knowing if disrupted phospholipid-mediated stabilization was limited to ER proteins, I sought to determine if *INO4* deletion stabilizes the model unstable soluble nucleoplasmic substrate, $\alpha 2$ *-UH, which is targeted by the soluble Slx5/Slx8 ubiquitin ligase.

Summary of research findings

Our data show that *OPI3* deletion significantly stabilizes *Deg1**-Sec62, further indicating disruption of phospholipid synthesis, in this case PC synthesis, disrupts *Deg1**-Sec62 degradation. I also show that *INO4* deletion does not cause global translocation impairment. This is evidenced by the observation that two model post-translationally and co-translationally translocated proteins (CPY and OPY, respectively) do not exhibit translocation impairment in *ino4* Δ yeast. This indicates broad translocation impairment is not the cause of impaired degradation of ERAD substrates in *ino4* Δ yeast. Rather, stabilization of translocon-clogging substrates mediated by *INO4*

deletion is achieved via another means. Further, *INO4* deletion does not destabilize the Ubc7-tethering protein Cue1 nor the translocon subunit Sbh1. I reason that *INO4* deletion-mediated stabilization of ERAD substrates is not attributable to disruption of the Hrd1 pathway mediated by Cue1 destabilization or to disruption of translocon function mediated by Sbh1 destabilization. Finally, $\alpha 2^*$ -UH degradation was not impaired in *ino4* Δ yeast, indicating that *INO4* deletion does not universally disrupt proteasome function. Alternative hypotheses for these results will be elaborated on below.

In recent years, there has been an increase in literature highlighting a relationship between altered lipid homeostasis and PQC efficiency. Studies published concurrently with this work showed that Hrd1 ERAD function is impeded by changes in the phospholipid membrane, specifically by the increase in the abundance of very-long-chain ceramides and disruption of sphingolipid biosynthesis [44]. Further, studies have shown that altered lipid composition in mammalian cells contributes to impaired glycan trimming and dislocation of ERAD substrates from the ER to the cytosol, ultimately disrupting proteostasis [45]. Yet another study shows that disrupted lipid droplet formation contributes to impaired proteostasis [46]. Consistent with these results, I demonstrate that disrupted PC biosynthesis similarly disrupts ERAD function.

Future direction: Validating impact of mutations on lipid composition

While it is established that Ino4 is part of a heterodimeric transcription factor that drives the expression of genes required for the synthesis of PE, PS, PI, and PC, the effect of *INO4* deletion on the composition of cell membranes is incompletely characterized. PC/PE ratios influence the function, fluidity, and integrity of biological membranes [47] due to the fact that PC and PE comprise approximately 50 and 20% of the total phospholipid content, respectively [48]. To confirm altered lipid profiles in *opi3* Δ and *ino4* Δ yeast, thin-layer chromatography could be used

to separate and identify the lipids present in the cell [49]. A higher-resolution alternative to thin-layer chromatography would be mass spectrometry (MS) analysis of yeast membrane preparations. This would allow for accurate determination of the PC/PE ratios, along with the abundance of all phospholipids, sphingolipids, ergosterols and their precursors and subtypes [50]. These analyses would validate the lipid-disrupting effects of my gene knockout approaches as well as elucidate the effects of those gene knockouts on the abundance of other phospholipids, as the synthesis of PC is downstream of PE and PS synthesis, and alteration of PC may homeostatically impact abundance of other lipids.

Future direction: How does altered lipid homeostasis impact ER protein degradation?

Our research shows that deletion of *INO4* does not cause broad translocation impairment. I also show that *INO4* deletion does not destabilize Cue1 or Sbh1, eliminating one potential mechanism for *INO4* deletion-mediated stabilization of ERAD substrates. Therefore, it is necessary to explore other potential mechanisms of impaired phospholipid synthesis-mediated disruption of ER protein degradation. It is possible that *INO4* or *OPI3* deletion changes membrane fluidity [51], contributing to a change in membrane-bound protein function. To assay for changes in membrane fluidity, fluorescence recovery after photobleaching (FRAP) can be used to measure the movement of fluorescently labeled membrane lipid into a photobleached region of the membrane [52].

It is possible that disrupted phospholipid synthesis alters the structure, and therefore, function of key proteins involved in ER PQC. X-ray crystallography or cryo-electron microscopy [17, 53] could be used to determine if lipid composition affects the structure of proteins like Hrd1, Cue1, and other cofactors purified and reconstituted into nanostructures containing different

distributions of lipid molecules. Further, I could use these techniques to assess the structure of the translocon complex in different lipid contexts.

Membrane dynamics influence protein complex formation. Complex formation in the ER of proteins involved in the Hrd1 pathway (e.g., Hrd1, Ubc7, and Cue1) could be analyzed to determine if changes in Hrd1 function are attributable to changes in complex formation. Affinity purification-mass spectrometry (AP-MS) could be used to determine if there is altered abundance of proteins purified with Hrd1 in yeast with disrupted phospholipid synthesis compared to WT. This approach can also be used to determine if translocon complex formation is compromised. Overall, notable differences in complex formation using AP-MS have the potential to highlight mechanisms for how disrupted lipid homeostasis contributes to impaired PQC.

To determine if lipid composition alters function of the Hrd1 ubiquitin ligase, the ability of Hrd1 to ubiquitinate a substrate (e.g., *Deg1**-Sec62) in WT, *ino4*Δ, or *opi3*Δ yeast could be performed. Immunoprecipitation of the substrate followed by a western blot probing for ubiquitin would allow me to determine if there is a change in the level of ubiquitination of these Hrd1 substrates.

Future direction: Uncoupling the impact of altered PE and PC synthesis on ER PQC

My lab has shown that PI, PC and ergosterol synthesis disruption impairs PQC efficiency (this study and [25]). In contrast, studying the effects of disrupted PE synthesis on PQC efficiency (without disrupting the synthesis of other phospholipids) has proven difficult given that PC synthesis is downstream of PE synthesis (Figure 8). Impaired PE synthesis inevitably affects downstream PC synthesis. To focus specifically on the role of PE in protein degradation, I propose to supplement yeast harboring knockouts of the PS decarboxylases *PSD1* and *PSD2* with choline. This will genetically disrupt PE synthesis while retaining PC synthesis via the alternative CDP-

choline synthesis pathway, which can utilize exogenous choline. Double knockouts of *PSD1* and *PSD2* are viable [54], and exhibit a significant reduction of PE abundance (12% PE content in *psd1Δ psd2Δ* yeast) relative to 25% in WT yeast [55]. This would allow me to assay the effects of disrupted PE synthesis while retaining PC synthesis.

Future direction: Evaluating the extent of the impact of lipid synthesis on PQC

In the work presented here and in Turk et al., 2023, we have shown that disrupted lipid homeostasis impairs multiple ER PQC pathways, including ERAD-C, ERAD-M, ERAD-L, and ERAD-T. However, PQC is not limited to the ER, so it is worthwhile to investigate the effect disrupted lipid homeostasis has on other PQC pathways. I demonstrate that *INO4* deletion does not stabilize the soluble nucleoplasmic substrate $\alpha 2^*$ -UH, establishing that disrupted lipid homeostasis does not universally impair PQC or proteasome function. Future work should be conducted to determine if impaired lipid synthesis disrupts INMAD, endosome and Golgi-associated degradation (EGAD), ERAD of ribosome-associated proteins (ERAD-RA), or vacuolar proteolysis. To assay INMAD, EGAD, or ERAD-RA efficiency, cycloheximide chase analysis of model substrates of these pathways would be performed in WT cells and cells with impaired phospholipid synthesis. To assay vacuolar proteolysis, cleavage of Pho8, which occurs in the vacuole to generate an active form with alkaline phosphatase activity can be assessed in wild type and lipid biosynthetic mutants. Treatment with a fluorometric substrate of Pho8, 4-Methylumbelliferyl Phosphate (4-MUP), allows vacuolar degradation efficiency quantification [56] using a fluorescence plate reader.

Future direction: How does impaired lipid synthesis slow *Deg1-Sec62 glycosylation?**

We hypothesized that delayed N-linked glycosylation of *Deg1**-Sec62 in *ino4Δ* yeast is due to impaired translocation. However, my results suggest this is not the case. It is possible the

observation of delayed glycosylation of *Deg1**-Sec62 is not due to an intrinsic defect in translocation but rather to prolonged and increased translocon engagement – without degradation – of other *Deg1**-Sec62 molecules. This prolonged engagement could cause an accumulation of pre-clogged *Deg1**-Sec62 due to the reduced availability of unoccupied translocons. To test for this, the model CTT and PTT proteins, OPY and CPY, respectively, would be useful. I have already shown that translocation efficiency in these proteins is not impaired in *ino4*Δ yeast. However, if translocation efficiency of these model proteins decreases in *ino4*Δ yeast expressing *Deg1**-Sec62, this would support a model whereby impaired degradation of abundant clogging-prone, translocon-associated proteins could impede translocation of other proteins.

Significance of research findings

The significance of these findings lies in the implication of impaired PQC in human diseases including Alzheimer's, Parkinson's and type II diabetes [3]. Further, disrupted lipid homeostasis and synthesis are observed in individuals with a range of metabolic disorders including atherosclerosis, insulin resistance, diabetes, liver disease (e.g., non-alcoholic fatty liver disease), and neurodegenerative diseases [57], so there is overlap in conditions marked by impaired PQC and lipid homeostasis. Observations that impaired phospholipid synthesis impairs PQC highlight an underappreciated factor potentially contributing to these conditions. It is possible that unexplained disruption of PQC is attributable to unnoticed disrupted lipid homeostasis. Further, this work identifies potential targets for treating conditions exacerbated by impaired PQC. For example, impaired PQC initiated, exacerbated, or maintained by altered lipid composition might be ameliorated using treatments directed at restoring lipid homeostasis [58]. Diseases marked by impaired lipid homeostasis might also be exacerbated by impaired ER PQC, highlighting a key area of future investigation into the cellular pathogenesis of these diseases.

Our findings further establish an influence of disrupted lipid homeostasis on PQC, but the mechanism for how this occurs remains unknown. My experiments eliminate a few potential explanations for how lipid homeostasis impacts ER protein degradation. For example, broad translocation impairment is not likely a major contributing factor to impaired PQC. Further, I show that that slowed degradation is not attributable to changes in the abundance of Cue1 and Sbh1. Stabilization of PQC substrates is not universal. The soluble, unstable substrate, $\alpha 2^*$ -UH, is not stabilized in *ino4* Δ yeast, indicating impaired lipid synthesis does not inhibit proteasome function.

Concluding remarks

To summarize, I have addressed several unique but related questions about the connection between lipid biosynthesis and ER protein degradation. I found that targeted disruption of PC synthesis modestly impairs degradation of the model translocon-clogging protein *Deg1*^{*}-Sec62. *INO4* deletion does not broadly impair translocation efficiency, nor does it destabilize ER-resident proteins Cue1 and Sbh1. Further, *INO4* deletion does not stabilize the soluble nucleoplasmic substrate $\alpha 2^*$ -UH. These findings provide an improved understanding about the relationship between conserved mechanisms of lipid homeostasis and PQC. Taken together with the observation that there is overlap between conditions in humans marked by impaired PQC and lipid synthesis, these findings highlight a potential relationship PQC and lipid synthesis in which disrupted lipid synthesis might contribute to impaired PQC. Further exploration into this relationship in humans has the potential to enhance our approach to treating conditions with established impaired PQC, focusing on lipid disruption as a target in therapy.

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Tables

Table 1: Yeast strains used in these experiments

Yeast Strain	Genotype
VJY18	<i>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 sec61-13myc:HIS3MX6</i>
VJY474	<i>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 ino4Δ::kanMX4</i>
VJY476	<i>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0</i>
VJY511	<i>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 hrd1Δ::kanMX4</i>
VJY643	<i>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 slx5Δ::kanMX4</i>
VJY659	<i>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 slx8Δ::kanMX4</i>
VJY1071	<i>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 opi3Δ::kanMX4</i>

All strains used in this study are of the BY4741 genetic background.

Table 2: Plasmids used in these experiments

Plasmid number	Plasmid name	Description	Yeast selection marker	Bacterial selection marker	Yeast plasmid type
pVJ27	pRS316	Empty vector	<i>URA3</i>	<i>AMP^R</i>	CEN
pVJ40	pRS315	Empty vector	<i>LEU2</i>	<i>AMP^R</i>	CEN
pVJ317	P416MET25- <i>Deg1*</i> -Sec62-ProtA	<i>Deg1*</i> -Sec62-ProtA driven by the <i>Met25</i> promoter. <i>Deg1*</i> = F18S, I22T	<i>URA3</i>	<i>AMP^R</i>	CEN
pVJ576	pRS316-GPD-CPY-2xProtA	CPY-2xProtA driven by GPD promoter	<i>URA3</i>	<i>AMP^R</i>	CEN
pVJ578	pRS316-GPD-OPY-2xProtA	OPY-2xProtA driven by the <i>GPD</i> promoter. OPY is CPY with the CPY signal sequence replaced with the Ost1 signal sequence.	<i>URA3</i>	<i>AMP^R</i>	CEN
pVJ667	pRS315-Cue1-HA	HA-tagged Cue1	<i>LEU2</i>	<i>AMP^R</i>	CEN
pVJ668	pRS315-Sbh1-HA	HA-tagged Sbh1	<i>LEU2</i>	<i>AMP^R</i>	CEN

Figures

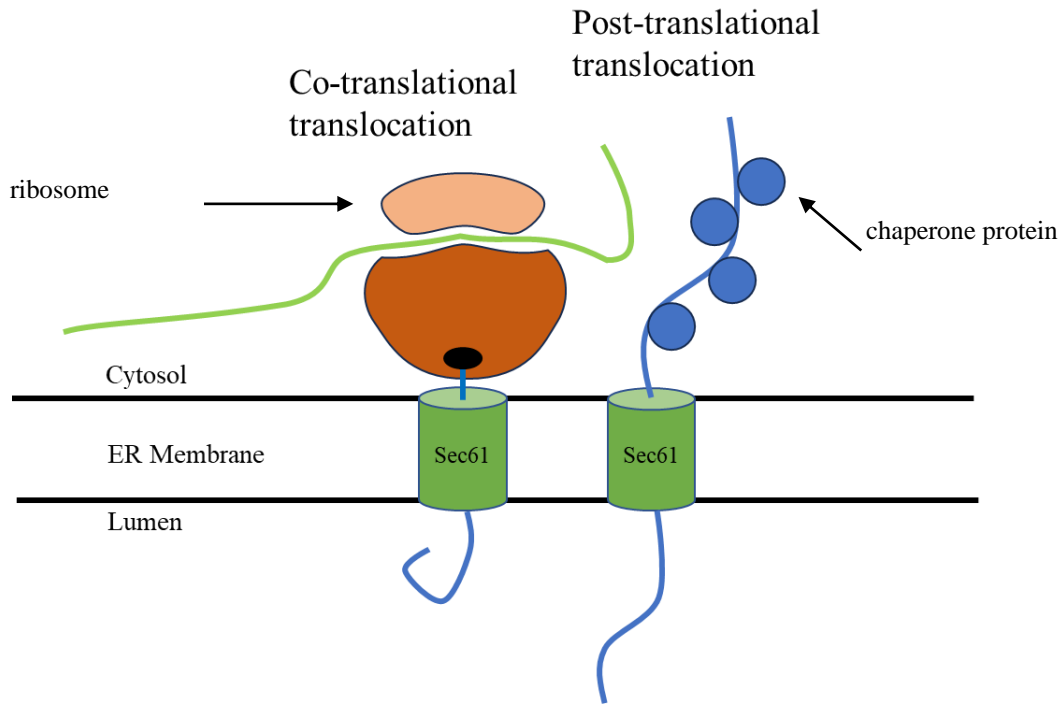


Figure 1: Co-translational and post-translational translocation at the ER membrane.

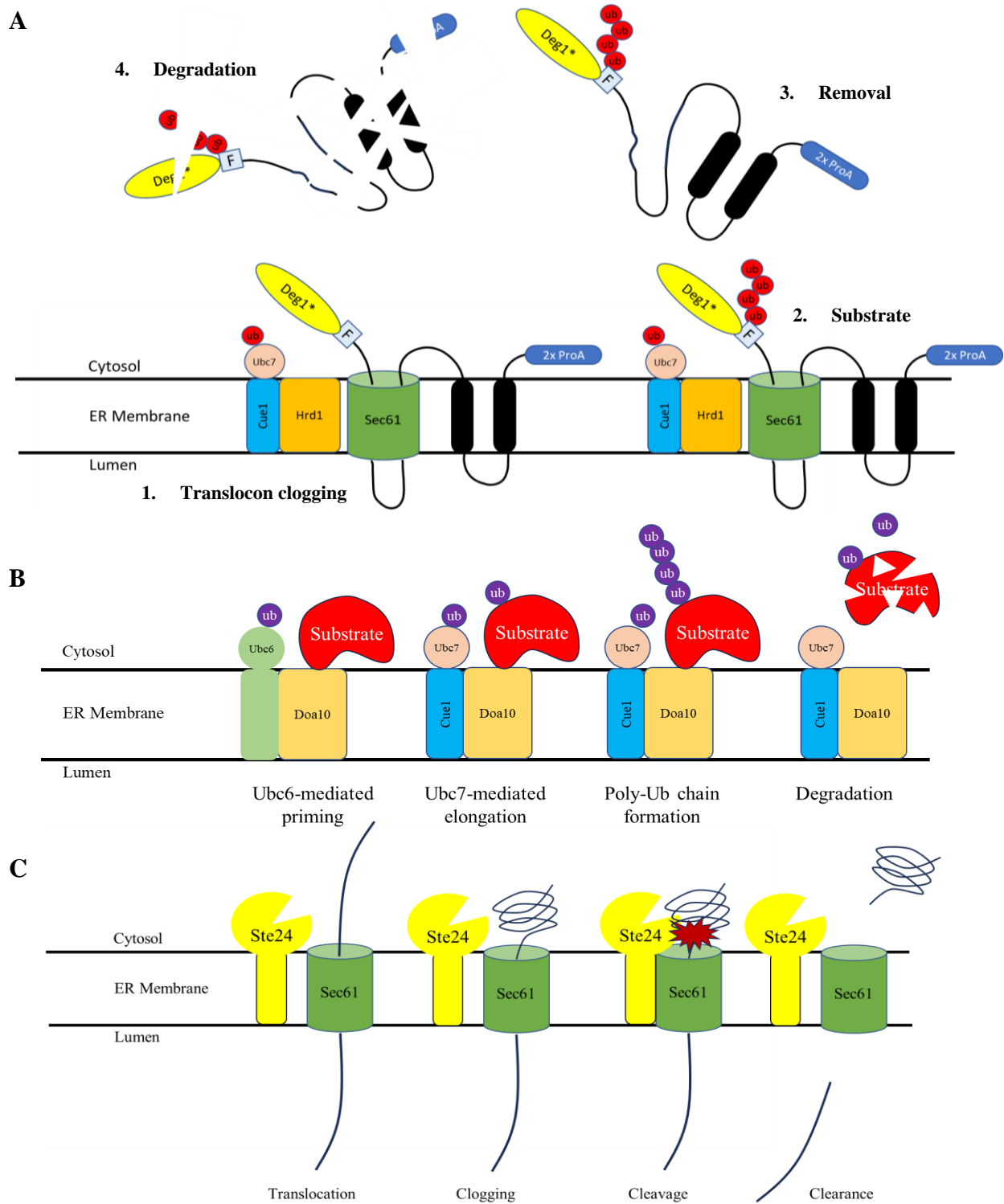


Figure 2: ER protein quality control mechanisms. A) Hrd1-mediated ERAD-T mechanism. B) Doa10 mediated ERAD-C mechanism. C) Ste24-mediated translocon quality control mechanism.

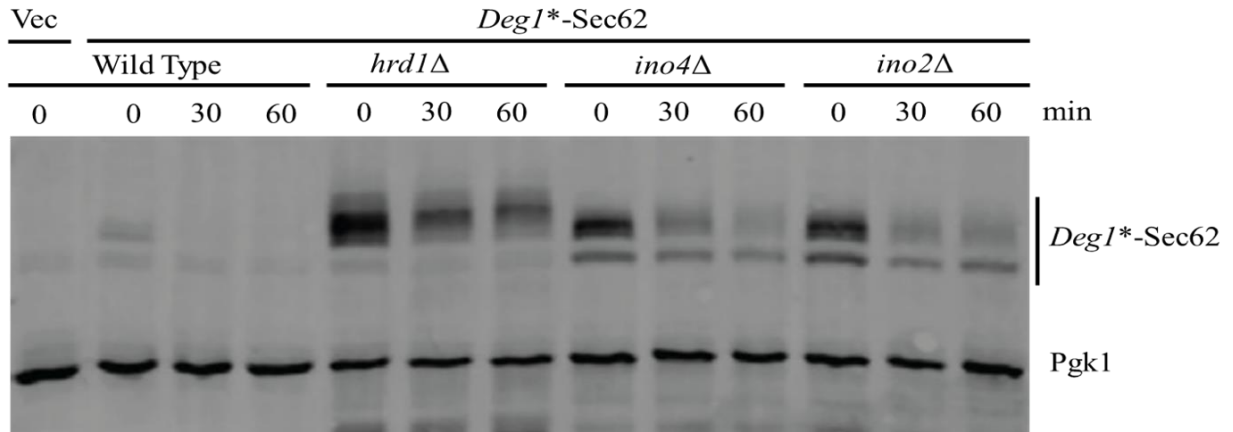


Figure 3: *INO2* and *INO4* deletion stabilizes *Deg1*-Sec62*. WT yeast and yeast lacking either *INO2* or *INO4* were transformed using a plasmid encoding *Deg1*-Sec62* or an empty vector and then treated with cycloheximide. Lysates of yeast collected at the indicated times following cycloheximide administration underwent SDS-PAGE gel electrophoresis and were probed for *Deg1*-Sec62* and Pgk1 by western blotting. Adapted from [Turk et al., 2023].

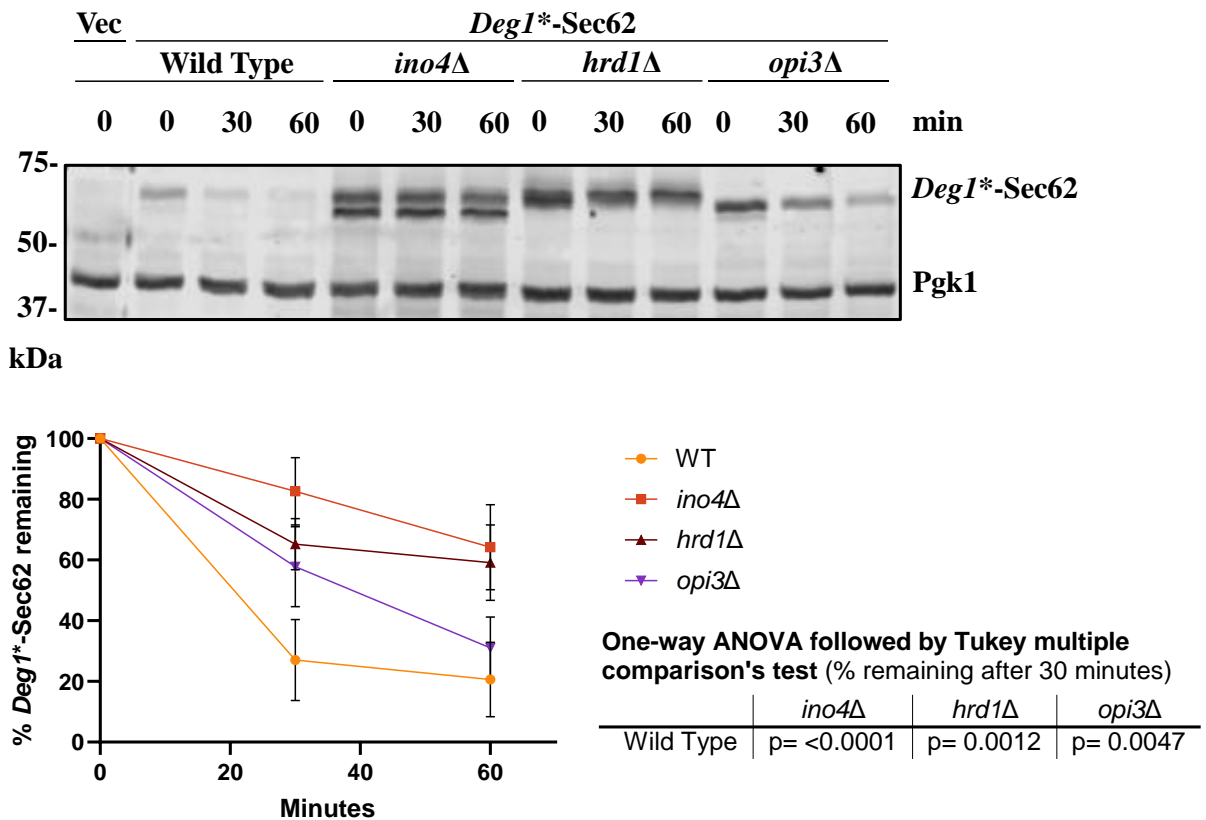


Figure 4: *Deg1*-Sec62* degradation is sensitive to perturbed phosphatidylcholine biosynthesis. Yeast were transformed with a plasmid expressing *Deg1*-Sec62* or vector with an auxotrophic *URA3* marker. Transformed yeast were subjected to cycloheximide chase followed by western blot analysis to detect *Deg1*-Sec62* and Pgk1 (loading control). 3-5 biological replicates were performed for each indicated strain. The average percent remaining at 30 and 60 minutes are plotted. Error bars represent the standard error of the mean. Means of percent of *Deg1*-Sec62* remaining at 30 minutes were evaluated using a one-way ANOVA followed by Tukey's multiple comparison test. All data were normal with equal variance.

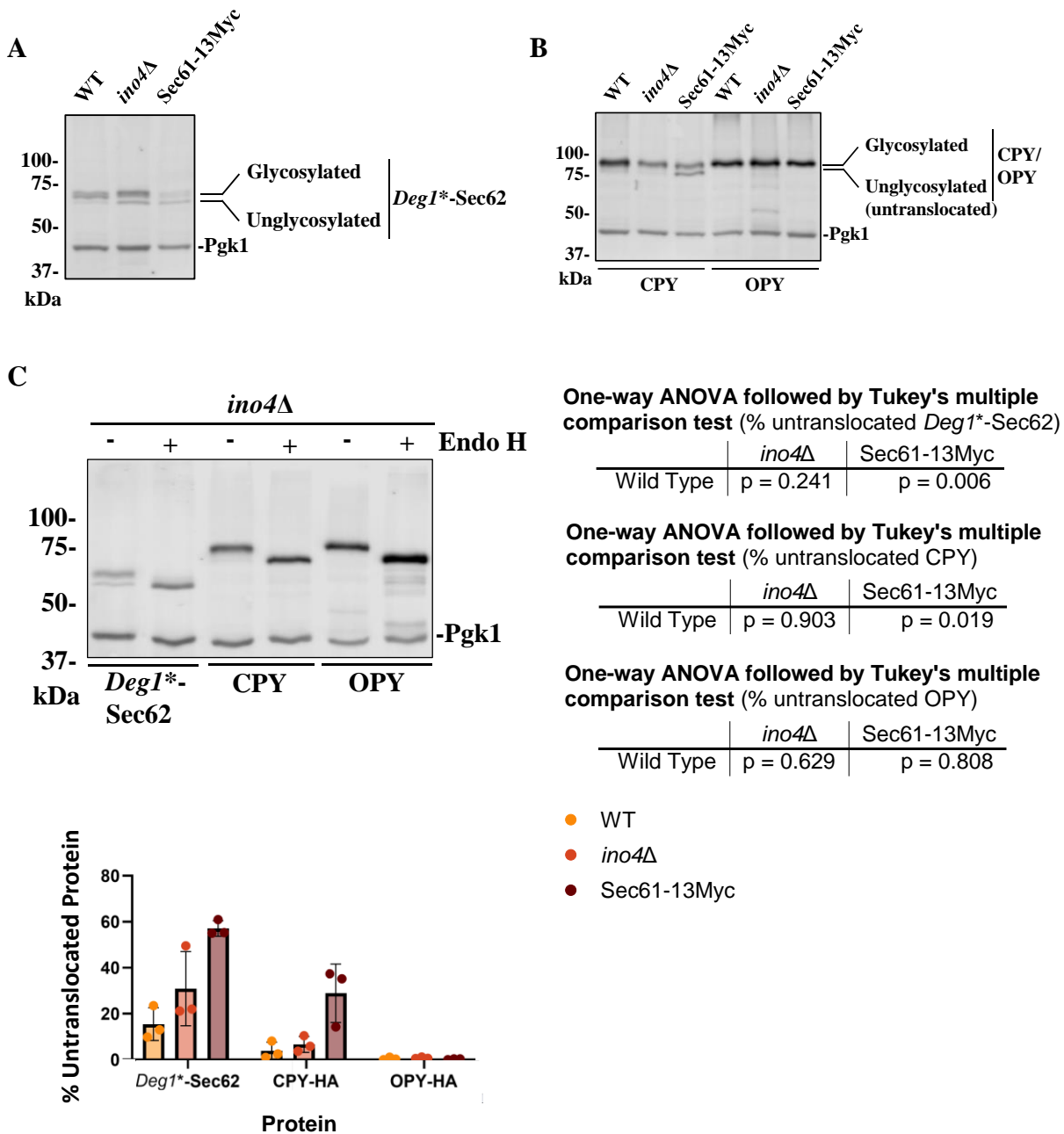


Figure 5: *INO4* deletion does not broadly impair translocation. A, B) Yeast were transformed with a plasmid expressing *Deg1*-Sec62*, CPY-HA, or OPY-HA or a vector with an auxotrophic *URA3* marker. 2.5 OD₆₀₀ units of yeast were harvested and analyzed by western blot analysis to detect *Deg1*-Sec62*, CPY-HA, or OPY-HA followed by Pgk1 detection (loading control). 3 biological replicates were performed. The lower, unglycosylated, untranslocated band quantification was divided by the total protein quantification to generate a % untranslocated value. Error bars represent the standard error of the mean percent untranslocated. Percent untranslocated values of *Deg1*-Sec62*, CPY-HA, or OPY-HA were evaluated using a one-way ANOVA followed by Tukey's multiple comparison test. All data were normal with equal variance. C) Lysates were treated with Endo H, resulting in a downward mobility shift representative of deglycosylation, indicating efficient glycosylation of proteins.

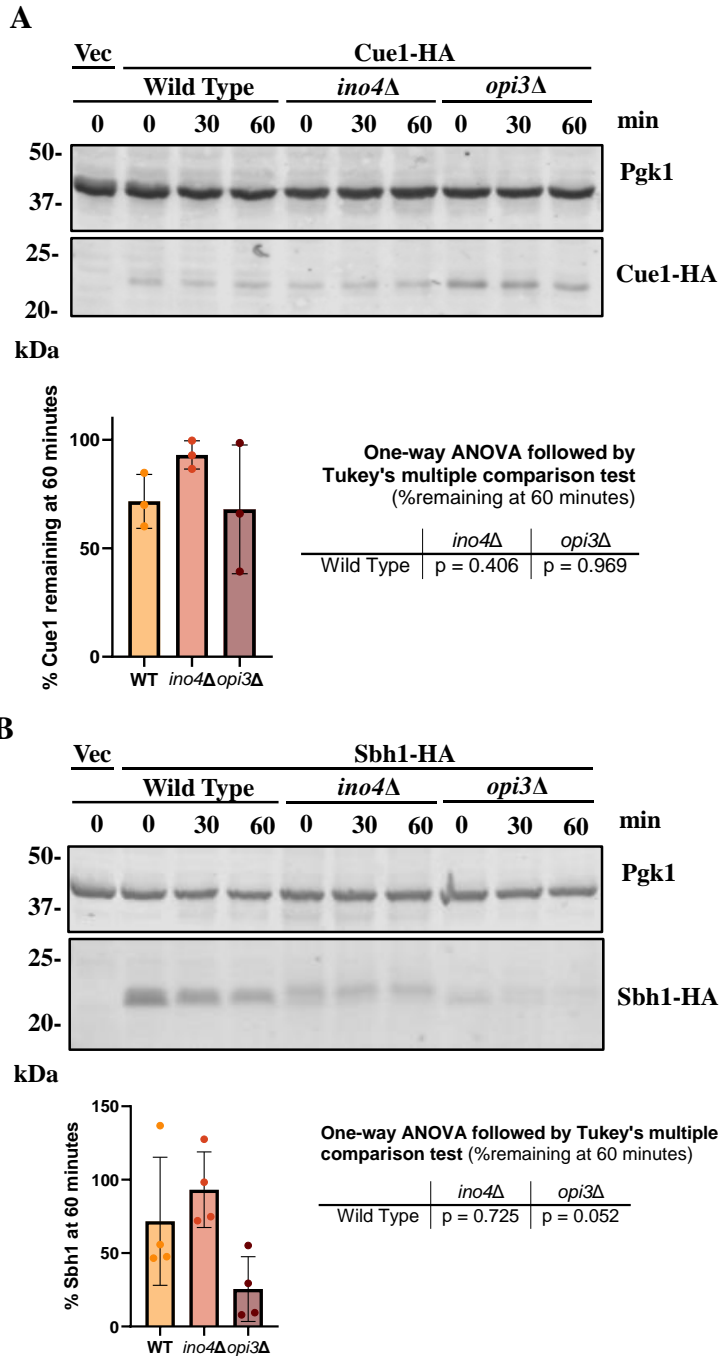


Figure 6: *INO4* deletion does not destabilize Cue1 and Sbh1. Yeast were transformed with a plasmid expressing Cue1-HA or Sbh1-HA or a vector with an auxotrophic *URA3* marker. Transformed yeast were subjected to a cycloheximide chase followed by western blot analysis to detect A) Cue1-HA or B) Sbh1-HA and Pgk1 (loading control). 3-4 biological replicates were performed and analyzed. The average percent remaining at 60 minutes is plotted. Error bars represent the standard error of the mean. Means of percent of Cue1-HA or Sbh1-HA remaining at 60 minutes were evaluated using a one-way ANOVA followed by Tukey's multiple comparison test. All data were normal with equal variance.

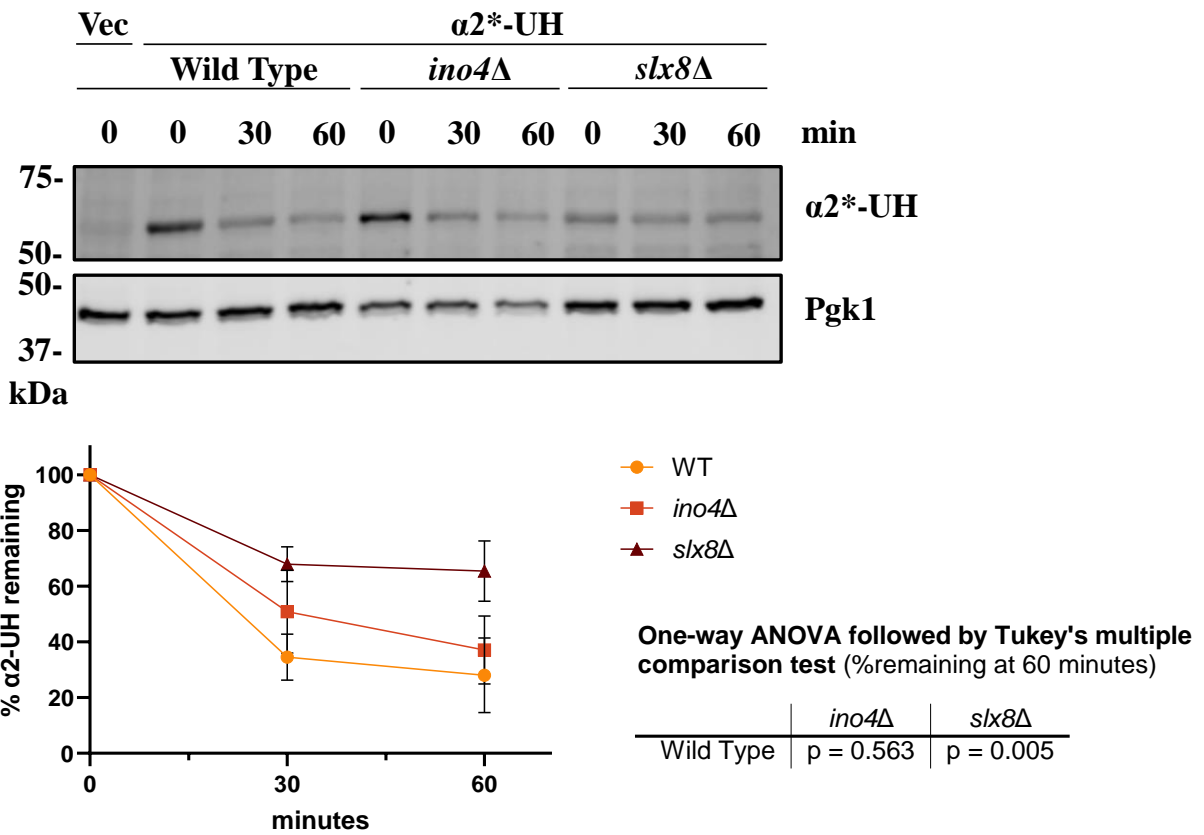


Figure 7: *INO4* deletion does not broadly impair PQC. Yeast were transformed with a plasmid expressing $\alpha 2^*$ -UH or vector with an auxotrophic *URA3* marker. Transformed yeast were subjected to cycloheximide chase followed by western blot analysis to detect $\alpha 2^*$ -UH and Pgk1 (loading control). 4 biological replicates were performed and analyzed. The average percent remaining at 30 and 60 minutes are plotted. Error bars represent the standard error of the mean. Means of percent of *Deg1**-Sec62 remaining at 60 minutes were evaluated using a one-way ANOVA followed by Tukey's multiple comparison test. All data were normal with equal variance.

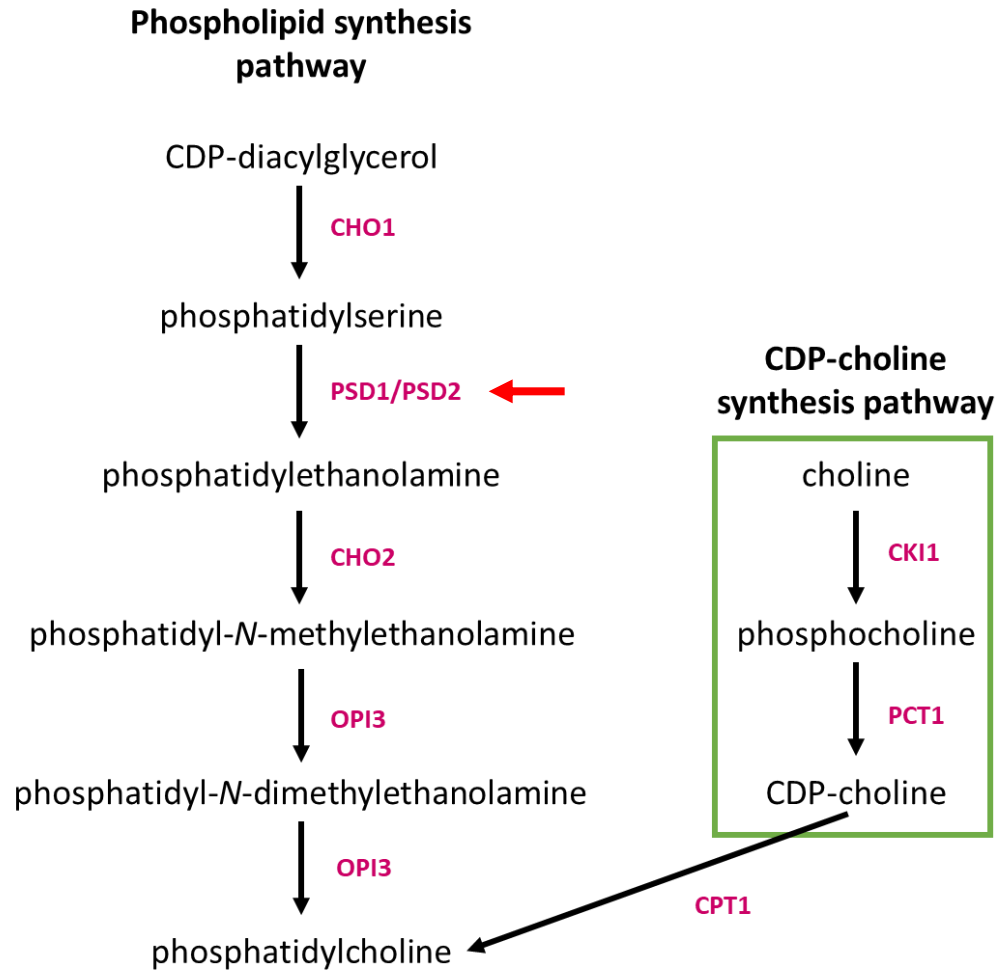


Figure 8: Phospholipid synthesis pathway from phosphatidylserine to phosphatidylcholine. CDP-choline synthesis pathway is also depicted. Red arrow shows where gene knockout would occur, in conjunction with choline supplementation, to allow for PC synthesis.