# THE RELATIONSHIP BETWEEN TRANSLOCON MODIFICATION AND TRANSLOCON QUALITY CONTROL

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**LIST OF ABBREVIATIONS**

**ApoB:** apolipoprotein b

**CPY:** carboxypeptidase Y

**CTT:** co-translational translocation

**DHFR:** dihydrofolate reductase

**E1:** ubiquitin-activating enzyme

**E2:** ubiquitin-conjugating enzyme

**E3:** ubiquitin ligase

**ER:** Endoplasmic Reticulum

**GFP:** green fluorescent protein

**LDL:** low-density lipoprotein

**LTE:** lithium acetate-tris-EDTA

**OD:** optical density

**OPY:** a hybrid protein of CPY with the signal sequence of the protein Ost1

**PCR:** polymerase chain reaction

**PEG:** polyethylene glycol

**PTT:** post-translational translocation

**PVDF:** polyvinylidene fluoride

**SD:** synthetic dextrose media

**SDS-PAGE:** sodium dodecyl sulfate-polyacrylamide

**SRP:** signal recognition particle

**TBS/T**: tris-buffered saline with tween 20

**TBS:** tris-buffered saline

**UPS:** ubiquitin proteasome system

**YPD:** yeast extract-peptone-dextrose media

**Background and Significance**

*Endoplasmic Reticulum translocation is essential to eukaryotic function*

While most protein synthesis is isolated to the cytosol of the eukaryotic cell, proteins are needed throughout the cell, as well as exported outside the cell. Proteins bound for the cellular membrane or exported outside the cell are trafficked through a series of membranes, organelles, and vesicles. These structures are collectively known as the endomembrane system (Park and Rapoport 2012). The endoplasmic reticulum (ER) is an entry point for a variety of proteins bound for the endomembrane system (Aviram and Schuldiner 2017). Over 30% of proteins synthesized by the cell use the ER to travel to their place of effect (Chen et al. 2005). The ER is also responsible for post-translational processing, containing many enzymes responsible for modifying proteins once they have entered the ER (Schwarz and Blower 2016).

The foremost entrance into the ER is the translocon. The translocon is a protein complex centered around Sec61 (Plath et al. 1998). Sec61, along with Sss1 and Sbh1 create a pore allowing the passage of polypeptides into the ER, where they fold and are modified into their functional configurations (Deshaies and Schekman 1987).

Translocation of polypeptides may occur by one of two primary mechanisms: co-translational translocation (CTT) or post-translational translocation (PTT) (Figure 1). CTT involves recruitment of an actively translating ribosome to the translocon (Ast, Cohen, and Schuldiner 2013). This is done through a signal recognition particle (SRP) that binds to the newly synthesized protein. Through interactions with the SRP receptor, the translating ribosome is lead to the Sec61 pore, and the protein is translocated as the ribosome continues to synthesize it (Linxweiler, Schick, and Zimmermann 2017).

PTT occurs when a protein is completely translated in the cytosol and then translocated into the ER lumen. This requires a set of accessory proteins that are not required for CTT. Cytosolic Hsp40 and

Hsp70 proteins escort newly synthesized proteins to the translocon complex. This stabilizes proteins and prevents folding that would hinder movement through the translocon (Kimura, Yahara, and Lindquist 1995; Cyr and Douglas 1994). In addition to the Sec61/Sss1/Sbh1 heterotrimer, the membrane-bound tetramer composed of Sec62, Sec63, Sec71, and Sec72 are also essential for PTT substrates to bind the translocon complex and travel into the lumen of the ER. Recent data suggest Sec62 and Sec63 may also be involved in CTT (Linxweiler, Schick, and Zimmermann 2017).

Dysfunction of the translocon occurs when proteins are unable to pass through the Sec61 channel and enter the ER. This dysfunction has extensive effects for human health, including links to kidney disease, liver disease, type 1 diabetes, and cancer (Lang et al. 2017). For example, mutations in Sec63 cause dysfunction in CTT that limits the movement of polycystins into the ER, which in turn leads to autosomal-dominant polycystic liver disease (Drenth et al. 2005), and translocon clogging has been proposed as a mechanism that contributes to diabetes (Kayatekin et al. 2018). The relationship between translocon dysfunction and disease is not entirely understood.

*Yeast is a useful model organism to study translocons*

This work utilizes the model organism *Saccharomyces cerevisiae* (budding yeast) to study protein quality control at the translocon. Yeast contain several traits that can be advantageous for experiments performed to understand fundamental eukaryotic biology. Yeast grow at fast rates and are cost-effective compared to mammalian cell culture (Duina, Miller, and Keeney 2014). Translocation and translocon quality control are conserved in yeast and in humans (Lang et al. 2017; Kaneko et al. 2016; Joshi et al. 2017; Fisher, Khanna, and McLeod 2011; Ast, Michaelis, and Schuldiner 2016; Crowder et al. 2015; Vibhuti Joshi 2017). Discoveries in yeast are therefore likely to be relevant to human physiology.

*Cells can remove proteins that aberrantly interact with the translocon*

One cause of translocon dysfunction is when proteins aberrantly interact with the translocon and stall in the pore of Sec61 (Rubenstein et al. 2012). This stalling blocks translocation of other proteins through the translocon pore until the offending protein is removed (Ast, Michaelis, and Schuldiner 2016; Toshiaki Izawa 2012). This clogging dysfunction can occur during either PTT (Rubenstein et al. 2012) or CTT (Crowder et al. 2015).

Cells have multiple mechanisms for removing proteins that aberrantly stall in the translocon. One of these mechanisms centers around homologs of the enzyme Hrd1 (Deak and Wolf 2001). Hrd1 is a ubiquitin ligase (E3) that acts as part of the ubiquitin-proteasome system (UPS; Figure 2) (Nandi et al. 2006). In the UPS, a ubiquitin-activating enzyme (E1) transfers the small signaling molecule ubiquitin to a ubiquitin-conjugating enzyme (E2) (Mayor 2014). The ubiquitin-conjugating enzyme is then recruited to the targeted substrate by an E3. The E3 is responsible for facilitating the transfer of the ubiquitin from the E2 to the substrate. E3 ligases may transfer ubiquitin molecules to another ubiquitin molecule, creating chains of ubiquitin molecules on a targeted substrate. For a protein to be targeted by the UPS, multiple ubiquitin molecules must be added to the substrate. Once the protein has been polyubiquitylated, it is degraded by the proteasome (Tommer Ravid 2008). After the protein has been degraded, the amino acid components can be recycled for new protein synthesis, and the free ubiquitin molecules can be reused in the UPS (Zhangyuan Yin 2020). In the case of removing clogged proteins from the translocon, members of the Hrd1 family of ubiquitin ligases transfer ubiquitin molecules to aberrant proteins in the translocon channel (Figure 3) (Rubenstein et al. 2012). This mechanism is conserved from yeast to mammals, with the mammalian Hrd1 homolog Gp78 targeting the translocon-clogging protein apolipoprotein B (apoB, discussed below).

Another major mechanism for removing stalled proteins from the translocon is Ste24 (Figure 4). Ste24 is a metalloprotease which cleaves stalled proteins in the translocon (Ast, Michaelis, and Schuldiner 2016). Once cleaved, the cytosolic portion becomes unbound from the translocon and is targeted by the

proteasome for degradation. The luminal section of the stalled protein, now also free from the translocon, is believed to continue into the lumen of the ER, where it may be eventually signaled for degradation by the proteasome (Ast, Michaelis, and Schuldiner 2016). This mechanism is conserved between yeast and humans (Ast, Michaelis, and Schuldiner 2016).

Hrd1 and Ste24 are both important in the removal of aberrant post-translationally translocated proteins. Proteins may also stall through aberrant interaction with the ribosome during CTT. For example, if the stop codon signal in an mRNA molecule is missed, translation will continue into the 3' untranslated region and, unless a stop codon is fortuitously detected, into the poly-A tail (Bennett 2014). A ribosome that aberrantly skips the stop codon will begin to translate poly-A tail. This creates a polylysine chain at the end of the normal amino acid sequence. These lysine residues aberrantly interact with the internal ribosomal structure, causing the protein to stall in not only the ribosomal machinery, but in the translocon as well, if the protein is ER-targeted (Crowder et al. 2015; Malsburg, Shao, and Hegde 2015). The E3 ligase responsible for ubiquitylation and removal of these ribosomal stalled proteins is Rkr1/Ltn1 (Figure 5). Rkr1/Ltn1 function in targeting ER-targeted CTT substrates is conserved between yeast and mammals (Karina von der Malsburg 2015).

*Model proteins engineered to stall in the translocon are used due to absence of characterized natural proteins that stall in the translocon*

There are a limited number of characterized natural proteins known to stall in the translocon. In humans, the low-density lipoprotein (LDL) precursor apolipoprotein B (apoB) is known to stall in the translocon as it waits for available lipids. If no lipids are available, the enzyme Gp78 (a mammalian Hrd1 homolog (Vibhuti Joshi 2017)) targets translocon-associated apoB for degradation, allowing for normal translocon function to resume (Yeung, Chen, and Chan 1996). Thus, removal of aberrant proteins from the translocon can also serve a regulatory function, as well as a quality control function.

ApoB is toxic to yeast, making it a poor model for studies of translocon quality control in a yeast model system (Eric Rubenstein, unpublished data). Two model substrates are used for studying protein quality control at the yeast translocon: *Deg1*-Sec62 and Clogger (Figure 6). *Deg1*-Sec62 is an engineered protein with the two-transmembrane protein Sec62 fused to the N-terminal 67 residues of the protein Matα2 (*Deg1*) (Rubenstein et al. 2012). When *Deg1*-Sec62 is first translated, the two transmembrane segments of the protein enter the translocon and are integrated into the ER membrane. Once the protein is fully integrated, persistent interaction with the translocon allows for a portion of the N-terminal tail to post-translationally translocate into the ER lumen. This interaction is stabilized by a disulfide bond between *Deg1*-Sec62 and the interior of the translocon. Only a portion of *Deg1*-Sec62 is able to translocate into the ER lumen, causing the protein to aberrantly stall in the translocon (Rubenstein et al. 2012). Hrd1 plays the major role in targeting *Deg1*-Sec62 for degradation (Rubenstein et al. 2012). However, Ste24 has also been shown to promote degradation of this protein, although to a smaller extent (Runnebohm et al. 2020). In this study, we also use a variant of *Deg1*-Sec62 that contains point mutations. This version of *Deg1*-Sec62 (denoted as *Deg1**-Sec62) has been shown to behave the same as "normal" *Deg1*-Sec62 during clogging experiments (Rubenstein et al. 2012). The point mutations in *Deg1**-Sec62 have the added advantage of preventing clogging protein from being degraded by other quality control mechanisms independent of translocon clogging.

Clogger is also an engineered protein, created by fusing the PTT substrate Pdi1, to the *E. coli* enzyme dihydrofolate reductase (DHFR). The DHFR portion of the protein creates a rapid and stable fold that cannot pass through the translocon. The protein is engineered to possess multiple N-linked glycosylation sites. If these sites are exposed to the ER lumen, they are modified, allowing discernment of cytosolic, clogged, and fully translocated protein via mobility differences as observed in sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) and western blot experiments. Clogger was initially believed to be

specifically degraded by Ste24. However, Hrd1 has also been shown to promote Clogger degradation (Runnebohm et al. 2020).

*The relationship between protein quality control mechanisms at the ER is unclear*

Unexpectedly, deleting either *HRD1 or STE24* does not cause a growth defect (Crowder et al. 2015; Ast, Michaelis, and Schuldiner 2016). If translocon quality control is critical to cellular homeostasis, then we would expect to observe negative phenotypes associated with impaired mechanisms. Our hypothesis was this observation is due to redundancy between the two quality control mechanisms. This was supported by the observation that *hrd1Δ ste24Δ* double mutant cells exhibit a synthetic slow-growth phenotype (Figure 7A) (Runnebohm et al. 2020).

*The addition of a 13myc epitope tag to Sec61 reduces translocon clogging by a model substrate*

The engineered proteins *Deg1**-Sec62 and Clogger have played a large role in the research of translocon quality control. However, knowledge of natural proteins that stall in the translocon would allow for a deeper understanding of the mechanisms that control translocon quality control, and the protein features that lead to clogging. One way to identify novel translocon-clogging substrates would be to purify translocons from cells with dysfunctional quality control mechanisms. From these purified translocons, it may be possible to identify associated translocon-clogging proteins.

To purify translocons, a 13myc tag was added to Sec61 in *hrd1Δ ste24Δ* yeast. Epitope tags such as 13myc are useful tools in studying protein abundance and interactions, particularly when antibodies that bind directly to a protein of interest are not commercially available. This modification had two unexpected effects. *hrd1Δ ste24Δ* cells expressing 13myc-tagged Sec61 grew better than the double knockout cells that did not contain the tag, effectively rescuing cells lacking these translocon quality control mechanisms (Figure 7A). Biochemically, the addition of the tag also impaired clogging by *Deg1**-

Sec62, which occurs by PTT (Figure 7B). This was surprising because a previous report indicated the 13myc tag does not impair translocon function (Carvalho, Stanley, and Rapoport 2010).

I hypothesized that selective impairment of translocation seen in *sec61-13myc hrd1Δ ste24Δ* cells was due to dysfunction of PTT. It is possible that weaker interactions between translocating proteins and the translocon caused by this tag also increased viability of cells lacking translocon quality control mechanisms, since Hrd1 and Ste24 target model translocon-clogging proteins that engage the translocon post-translationally. Reduced protein clogging at the translocon would reduce cells' dependence on unclogging mechanisms such as Hrd1 and Ste24. If this hypothesis is correct, then cells with PTT impaired in other ways should also exhibit similar phenotypes to cells containing *sec61-13myc.*

I also hypothesized that tagging the translocon with a different epitope tag such as GFP and moving the tag farther from the central channel may improve translocon function. This approach may allow for isolation of proteins that naturally interact with the translocon without impairing translocon function. Identification of novel clogging proteins could reveal features of proteins that render them more likely to clog the translocon and enable elucidation of mechanisms in translocon dysfunction and repair. This in turn may lead to new discoveries about protein quality control at the ER and the implications these mechanisms have on cellular health.

In this study, I investigated the relationship between translocon function and translocon quality control mechanisms. I hypothesized that the 13myc on the Sec61 subunit of the translocon rescues the phenotype associated with loss of *HRD1* and *STE24* by impairing PTT. I tested this by causing PTT dysfunction through elimination of the Sec72 subunit of the translocon and measuring the effect this has on ER import of various translocation substrates and on the *hrd1*Δ *ste24*Δ synthetic phenotype. I also attempted to tag the translocon in a way that perturbs function less than the 13myc tag on Sec61. By testing the effects of a different epitope tag, GFP, on different translocon subunits, I hoped to

identify a tagged translocon with wild type function, which would be useful for future experiments to identify natural translocon-clogging proteins. This study shows deletion of *SEC*72 causes impairment of model PTT substrates similar to impairment seen in sec61-13myc cells. Additionally, deletion of *SEC*72 paired with deletion of *HRD1* and *STE24* rescued the negative growth phenotype seen in *hrd1Δ ste24Δ* cells. This shows a novel relationship between the translocon and translocon quality control machinery, in which impairment of translocon interactions reduces cells' reliance on pathways that relieve translocon clogging. Translocon modifications involving GFP resulted in a variety of translocation phenotypes. While no translocon modification tested impaired translocation of a model CTT substrate, only two of the six tested GFP-tagged translocon subunits, Sec71-GFP and Sec72-GFP, showed no impairment of model PTT substrates. These two modifications may be useful in future experiments to isolate natural translocon-clogging substrates. Discovery of novel translocon-clogging proteins can lead to a better understanding of the foundational mechanisms behind diseases such as liver disease, diabetes, and cancer.

**Specific Aims**

**Aim 1:** To determine the effect of compromised post-translational translocation on cells with impaired translocon quality control.

> **Hypothesis:** Impaired post-translational translocation will lead to fewer clogging-prone proteins interacting with the translocon, which will in turn reduce reliance on protein quality control at the translocon.

**Aim 2:** To determine the impact of epitope tags on various translocon subunits on co-translational and post-translational translocation.

> **Goal:** Identify an epitope tag that, when attached to the translocon, causes the least impairment of translocon function.

**Methods**

*Plasmid DNA Isolation*

A QIAprep (Qiagen) spin miniprep kit was used to isolate plasmid DNA from bacterial cells. Manufacturer instructions were used for this protocol. Plasmids used in this study are presented in Table 1.

*Bacterial culture*

Bacterial strains were stored at -70°C when not in use. Cells were cultured at 37°C in Luria broth (0.5% Bacto yeast extract, 1% tryptone 0.5% sodium chloride) plates containing ampicillin (100 µg/ml) for plasmid selection.

*Yeast culture*

Yeast strains were stored at -70°C when not in use. Cells were cultured on plates containing yeast peptone dextrose (YPD) media (1% Bacto yeast extract, 2% Bacto peptone, 2% glucose, 0.002% adenine; 2% agar) at 30°C. Yeast strains used in this study are presented in Table 2. To select by auxotrophic marker, cells were grown on plates containing synthetic dextrose (SD) media (0.67% yeast extract without amino acids, 2% glucose, 0.2% arginine, 0.6% isoleucine, 0.1% histidine, 0.6% leucine, 0.4% lysine, 0.1% methionine, 0.6% phenylalanine, 0.5% threonine, 0.4% tryptophan, 0.002% adenine, 0.002% uracil; 2% agar) lacking the appropriate metabolite at 30°C.

*Lithium Acetate Transformation of Yeast Strains with Plasmids*

To transform plasmid DNA into a yeast strain, yeast cells were cultured in liquid YPD media overnight at 30°C. 500 µl of cell culture were pelleted by centrifugation at 5000 rpm for 2 minutes. Cells were washed with 1 ml of sterile water and pelleted again under the same conditions. Cells were resuspended in LTE (10 mM Tris-Cl pH 7.5, 1 mM EDTA, 0.1 M lithium acetate). 20 µl of salmon sperm DNA (100 µg/ml) and 1 µl of plasmid DNA was added to the cell suspension. This mixture was incubated at room

temperature for 30 minutes. 400 µl of 40% polyethylene glycol (PEG) in LTE was added, and the mixture incubated again at room temperature overnight. Cells were pelleted by centrifugation at 5000 rpm for 2 minutes. Cell were resuspended in 50 µl of 1 M sorbitol and plated onto selective SD media.

*Yeast DNA Preparation*

A Yeast DNA Purification Kit (Masterpure) was used to isolate DNA from yeast cells. Manufacturer instructions were used for this protocol.

*PCR*

For preparative PCR for gene replacement, the following were combined in a PCR tube: Thermopol buffer, deoxynucleotide triphosphates (500 nM each), *Taq* polymerase (0.04 units/ml), 2 µl of template yeast DNA, and primers (1 µM each) engineered to match the DNA product. Template DNA was amplified using a thermocycler using the following conditions: 1 cycle at 95$^{o}$C for 5 minutes; 40 cycles of 95$^{o}$C for 30 seconds, 55$^{o}$C for 30 seconds, 72$^{o}$C for 1 minute; and 1 cycle at 72$^{o}$C for 10 minutes.

For PCR genotyping, the following reagents was combined in a PCR tube: Thermopol buffer, deoxynucleotide triphosphates (500 nM each), *Taq* polymerase (0.04 units/ml), 2.5 µl of template yeast DNA, primers (1 µM each) engineered amplify either the wild type or mutant alleles. Template DNA was amplified in a thermocycler using the following conditions: 1 cycle 1 at 95$^{o}$C for 5 minutes; 40 cycles of 95$^{o}$C for 30 seconds, 55$^{o}$C for 30 seconds, 72$^{o}$C for 1 minute; and 1 cycle at 72$^{o}$C for 10 minutes.

All PCR products were verified using 1% agarose gel electrophoresis.

*DNA purification*

A QIAquick Gel Extraction Kit (Qiagen) was used to purify DNA products created via PCR. The manufacturer's instructions were used for this protocol.

*Strain Generation via High-Efficiency Transformation and Homologous Recombination*

Yeast cells were cultured in liquid YPD media overnight at 30°C. Cells were diluted to $OD_{600}$ of 0.2 and incubated at 30°C until mid-logarithmic growth. Cells were pelleted via centrifugation at 3500 rpm for 7 minutes. The supernatant was removed, and cells washed with 25 ml of sterile water and pelleted again. The supernatant was removed again, and cells resuspended in 1 ml of 0.1 M lithium acetate. Cells were pelleted and the supernatant removed and replaced by 400 µl of 0.1 M lithium acetate. 100 µl of this cell suspension was aliquoted to a microcentrifuge tube and the following added sequentially: 240 µl 50% (w/v) PEG, 36 µl 1.0 M lithium acetate, 20 µl fresh salmon sperm DNA (100 µg/ml), and PCR product containing an antibiotic-resistance gene flanked with sequences homologous to the target gene. This mixture was subjected to heat shock at 42°C for 40 minutes. Cells were pelleted at 10,000 rpm for 30 seconds and the supernatant removed. Cells were resuspended in sterile water and plated onto YPD. Plates were incubated at 30°C. Plates were replica plated onto selective media and incubated until colonies formed. Gene replacement was confirmed by PCR.

*Strain Generation via Mating and Sporulation*

Novel strains were created by mixing two strains, each containing different desired characteristics, onto a YPD plate. This plate was incubated overnight at 30°C. These strains have opposite mating types, allowing them to form a diploid containing all desired traits. Selection was performed by plating on media that only allowed the diploid strain (but not either haploid strain) to grow. Sporulation was induced by first replica plating the diploid colonies onto a GNA plate (5% glucose 3% Difco nutrient broth, 1% yeast extract, 2% bacto agar) and incubating overnight at 30°C. Cells were replica plated onto a second GNA plate and incubation was repeated. Diploid cells were replica plated onto a sporulation plate (1% potassium acetate, 0.004% w/v uracil, 10 µl/L histidine, 10 µl/L leucine, 2% bacto agar). Cells were incubated at room temperature until tetrads form, as judged by microscopic investigation. Tetrads

were dissected on a YPD plate and incubated at 30°C until colonies form. Relevant genotypes were determined by growth on selective medium and PCR.

*Yeast Cell Lysis*

Strains were incubated in selective liquid media overnight at 30°C. Strains were diluted to an $OD_{600}$ of 0.2 and incubated again at 30°C until cultures reached mid-logarithmic phase. 2.5 $OD_{600}$ units were collected and pelleted in a centrifuge at 7000 rpm for 30 seconds. The supernatant was removed, and cells resuspended in 200 μl of 0.1 M sodium hydroxide. After 5 minutes incubation at room temperature, samples were centrifuged at 13,000 rpm for 30 seconds. The supernatant was removed, and samples resuspended in 100 μl Laemmli loading buffer, which lyses cells. Lysates were heated at 95°C for five minutes and then centrifuged at 13,000 rpm for 30 seconds to pellet insoluble material.

*Endoglycosidase H (Endo H) Assay*

80 μl of yeast lysates lysate were combined with 8.4 μl 0.83 M potassium acetate (pH 5.6) and 1 μl Endo H (0.5 units/ml) (New England BioLabs). This mixture was incubated at 37°C for 1-3 hours.

*Western Blot*

Lysates were loaded onto an 8% SDS-PAGE gel. Samples were run at 200 V. Proteins were transferred from the polyacrylamide gel to a polyvinylidene fluoride (PVDF) membrane that had been activated with methanol and submersed in transfer buffer (2.5 mM tris acetate, 1.4% w/v glycine, 0.1% w/v SDS, 20% methanol) at 20V for 60 min at 4°C. After proteins were transferred to the PVDF membrane, the membrane was soaked in blocking solution (5% powdered milk in 5 mM Tris-buffered saline (TBS; 0.5 M Tris-Base, 1.5 M NaCl) overnight.

AlexaFluor-680-conjugated rabbit anti-mouse antibody (Life Technologies Inc; 1:40,000) was used to detect *Deg1**-Sec62 (which possesses two copies of *Staphylococcus aureus* Protein A, which binds non-

specifically to mammalian antibodies (Hjelm 1972). Pgk1 was detected with mouse anti-phosphoglycerate kinase 1 (Pgk1; clone 22C5D8; Life Technologies, Inc; 1:20,000) followed by AlexaFluor-680-conjugated rabbit anti-mouse antibody (1:40,000). Clogger was detected with mouse anti-HA (Life Technologies Inc.; 1:5,000) followed by AlexaFluor-680-conjugated rabbit anti-mouse antibody (1:40,000). GFP was detected with mouse anti-GFP (Life Technologies Inc.; 1:5,000) followed by AlexaFluor-680-conjugated rabbit anti-mouse antibody (1:40,000). Membranes were imaged using Li-Cor Odyssey CLx imaging instrumentation and analyzed using Image Studio Lite imaging software.

**Results**

*sec61-13myc impairs Clogger translocation*

The attachment of the 13myc epitope tag to Sec61 caused impairment in translocation of the model translocon-clogging substrate *Deg1**-Sec62. This was inferred based on reduced extent of glycosylation of *Deg1**-Sec62 when the epitope tag is present (Figure 7B). In sec61-13myc cells, the appearance of a faster migrating band visible via SDS-PAGE and western blot represents protein that has not engaged the translocon and become glycosylated (which occurs in the ER lumen). To test the impact of sec61-13myc on Clogger interaction with the translocon, I analyzed the migration of Clogger in both wild type and *sec61-13myc* cells. I also observed differing glycosylation of Clogger when the 13myc tag is present. In wild type cells, the majority of Clogger migrates as a fully glycosylated species (Figure 8). This represents Clogger that has fully translocated into the ER. There is also a minor pool of Clogger that has not interacted with the translocon, and therefore remains unglycosylated. Very little partially glycosylated, translocon-clogged Clogger is present.

In cells containing sec61-13myc, a greater proportion of Clogger is not fully glycosylated, indicating reduced flux through the translocon. In contrast to wild type cells, a substantial proportion of Clogger remains partially glycosylated, indicating accumulation of translocon-clogged species. This is different than the observed impact of 13myc on *Deg1**-Sec62 translocation (Figure 7B). The presence of sec61-13myc reduced *Deg1**-Sec62 interaction with the translocon and clogging frequency, culminating in a substantial abundance of protein that that could not interact with the translocon and remained unglycosylated. However, in both cases, the addition of 13myc to Sec61 decreased movement of proteins through the translocon.

*sec61-13myc specifically impairs translocation of PTT substrates*

The sec61-13myc modification impairs translocation of two model translocon-clogging substrates, *Deg1**-Sec62 and Clogger. Both these model substrates translocate through PTT mechanisms. I also tested translocation of model PTT and CTT substrates that translocate normally (i.e. without clogging). CPY is a model PTT substrate, while OPY is a model CTT substrate (Willer, Forte, and Stirling 2008). In sec61-13myc cells, translocation of CPY is impaired (Figure 9). When the epitope tag is not present, CPY migrates as one glycosylated species on an SDS-PAGE gel. When the 13myc epitope tag is present, a proportion of CPY remains unglycosylated and migrates lower on the gel. This impairment is similar to the impaired translocation seen in the clogging PTT substrates, *Deg1**-Sec62 and Clogger. In contrast, there is no change in OPY glycosylation or migration when the epitope tag is present or absent. This suggests that the epitope tag modification sec61-13myc specifically impairs PTT pathways, while leaving CTT unperturbed.

*Generation of hrd1Δ ste24Δ sec72Δ yeast*

To determine if impaired PTT rescues the *hrd1*Δ *ste24*Δ growth defect, I generated a strain that lacked both translocon quality control enzymes (Hrd1 and Ste24) and the PTT subunit Sec72. The Rubenstein lab possessed a *hrd1Δ ste24Δ* yeast strain (VJY456; Table 2). *SEC72* was knocked out in this strain and replaced with a hygromycin B resistance gene (*hphMX4*). An *hphMX4* knockout cassette was amplified from plasmid pAG32 (pVJ134; Table 1 (Goldstein and McCusker 1999)) using PCR with flanking sequences homologous to sequences immediately upstream and downstream of the *SEC72* locus. Following yeast transformation, the *hphMX4* cassette replaced *SEC72* by homologous recombination. Stable transformants were selected by growth on media containing hygromycin B. Cells were also cultured on media containing G418 as well to confirm the pre-existing knockout of *HRD1* (which was replaced with the *kanMX4* cassette).

Insertion of the hygromycin cassette at *SEC72* was validated by three-primer PCR at both the 5' and 3' junctions of the *sec72Δ::hphMX4* locus (Figure 10). By performing PCR with primers that amplified products of different sizes for the wild type and mutant alleles, the *SEC72* genotype was validated at both ends of the locus, ensuring the entire gene was replaced during homologous recombination. A strain wild type at the *SEC72* locus was used as a negative control. Seven candidate *sec72Δ::hphMX4* clones created through homologous recombination were analyzed by PCR. Five clones yielded PCR products at both junctions consistent with *SEC72* knockout. Clone 1 (VJY841) was used for subsequent experiments.

*Mutation of SEC72 rescues slow growth of hrd1Δ ste24Δ cells.*

Previous experiments demonstrated that eliminating either Hrd1 or Ste24 individually yields no observable negative phenotype (Crowder et al. 2015; Ast, Michaelis, and Schuldiner 2016), while simultaneously mutating both genes causes a negative growth defect (Runnebohm et al. 2020). Modification to the central pore of the translocon via epitope tag (*sec61-13myc*) fully rescued the *hrd1Δ ste24Δ* growth defect (Runnebohm et al. 2020). I hypothesize that the *sec61-13myc* modification to the translocon reduces reliance on translocon quality control mechanisms due to impaired engagement between the translocon and translocating proteins. Cells lacking *SEC72* exhibited no discernable growth defects (Figure 11). When the same mutation was introduced in cells lacking Hrd1 and Ste24, cellular growth was again rescued, matching results in *hrd1Δ ste24 sec61-13myc* cells. Thus, two different mutations that impair PTT rescue the growth defect associated with impaired translocon quality control.

*sec72Δ reduces frequency of translocation of PTT substrates.*

I also observed translocation efficiency in *sec72Δ* cells. I analyzed translocation efficiency of three translocation substrates: *Deg1**-Sec62, CPY, and OPY. Entry of each substrate into the ER results in glycosylation, which causes a slower migration by SDS-PAGE (Rubenstein et al. 2012; Runnebohm et al.

2020). Mutations of *SEC72* mildly increased the proportion of both *Deg1**-Sec62 and CPY (which enter the translocon via PTT) that remained unglycosylated (Figure 12A and 12B). This indicates that a higher proportion of these substrates were unable to engage the translocon and enter the ER. This dysfunction is similar to dysfunction observed in *sec61-13myc* cells (Runnebohm et al. 2020). No dysfunctional translocation of OPY was observed in *sec72Δ* cells (Figure 12C). OPY is a CTT substrate, and Sec72 is only necessary for PTT function. Therefore, it is reasonable that OPY would be unaffected by *SEC72* mutation. Since *sec72Δ* only affects PTT, dysfunction of PTT is likely sufficient to rescue the negative phenotype associated with impaired translocon quality control. I note that the effect of *SEC72* deletion on CPY translocation is mild. However, this modest reduction in translocation efficiency is reproducible. A second experiment demonstrating impaired translocation of CPY in *sec72*$\Delta$ cells is illustrated in Figure 12D.

*GFP-tagged translocon subunits cause varying dysfunction of PTT*

Yeast possessing several different GFP-tagged translocon subunits were analyzed for translocation efficiency of three model translocating substrates: *Deg1**-Sec62, CPY, and OPY (Figure 13). Tagged translocons with wild type function may be used in translocon immunoprecipitation experiments to identify physiological translocon-clogging proteins. *sec61-13myc* cells were used as a positive control for dysfunctional PTT. sec63-GFP and sec61-GFP both caused accumulation of unglycosylated and likely untranslocated PTT substrates *Deg1**-Sec62 and CPY (Figure 13A, 13B), but did not alter modification of CTT substrate OPY (Figure 13C). The tagged translocon subunit sss1-GFP reduced the steady state abundance of all three substrates tested (Figure 13A, 13B, 13C). This would not be an ideal choice for use in immunoprecipitation experiments. Sec71-GFP and Sec72-GFP did not impact apparent PTT of *Deg1**-Sec62 and CPY or CTT of OPY. Either Sec71-GFP or Sec72-GFP would potentially be a good candidate for immunoprecipitation experiments based on unperturbed translocation of the three tested substrates.

I also tested sss1-3HA for translocation efficiency of *Deg1**-Sec62, CPY, and OPY (Figure 14). Sss1 is the smallest subunit of the translocon, is a component of the central heterotrimer, and the HA epitope tag is smaller than GFP or 13myc (Esnault et al. 1993; Zappelli et al. 2014). However, sss1-3HA caused worse translocation function than sss1-GFP, as demonstrated by increased abundance of unglycosylated and likely untranslocated species of PTT substrates *Deg1**-Sec62 and CPY. Translocation of CTT substrate OPY remained unaffected.

**Discussion**

Our experiments show that modification to the translocon can have significant effects on translocation into the ER and impact cellular health in the context of impaired translocon quality control. Previous experiments have shown that the translocon modification sec61-13myc both impairs translocon clogging by *Deg1**-Sec62 , which occurs by the PTT mechanism, and rescues cellular health in cells lacking translocon quality control machinery (Runnebohm et al. 2020). I observed that the sec61-13myc modification also impaired translocation of a second model clogging PTT substrate, Clogger, as well as the model PTT substrate CPY. sec61-13myc did not impair translocation of model CTT substrate OPY.

I was able to replicate these phenotypes in cells lacking Sec72. Sec72 is a translocon subunit that is required for efficient PTT. *sec72*Δ cells exhibit reduced clogging by *Deg1**-Sec62, impaired PTT, and robust cellular health when translocon quality control is impaired. I also show that other modifications to the translocon impair translocation of PTT substrates. C-terminal GFP epitope tags of translocon subunits Sec61, Sss1, and Sec63 impaired translocation of model clogging substrate *Deg1**-Sec62 and model PTT substrate CPY. The addition of the epitope tag HA to the Sss1 subunit also caused impairment of *Deg1**-Sec62 and CPY. However, GFP epitopes attached to Sec71 or Sec72 did not cause impairment of *Deg1**-Sec62 or CPY translocation. In addition, no tested epitope tag configurations caused translocation impairment of model CTT substrate OPY.

*Why does loss of Hrd1 and Ste24 cause a growth defect?*

In cells expressing either functional Hrd1 or Ste24, there is no discernable growth phenotype compared to wild type (Ast, Michaelis, and Schuldiner 2016; Crowder et al. 2015; Kaneko et al. 2016). However, when both proteins are missing, cells exhibit a strong negative growth phenotype. This strongly suggests an overlapping function between Hrd1 and Ste24 that is not performed when both proteins are missing. This is likely a function in ER translocon quality control, as this is a known overlapping function of both

proteins. A natural translocon-clogging protein, for instance, may be cleared by either Hrd1 or Ste24. In cells without either translocon quality control measure, translocon clogging is unable to be resolved, and cellular health decreases. This hypothesis is supported by observations of increased stabilization (*Deg1**-Sec62) or abundance (Clogger) of model translocon-clogging proteins in *hrd1Δ ste24Δ* cells than in single mutants (Runnebohm et al. 2020). The *hrd1Δ ste24Δ* double mutant is unable to completely stabilize either model protein, suggesting still other pathways may contribute to translocon quality control.

However, Hrd1 and Ste24 perform other functions in the cell beside their roles in translocon quality control. Hrd1 ubiquitylates several different categories of proteins in the ER membrane and lumen, including many that do not clog translocons. These functions are collectively known as ER-associated degradation (ERAD) (Rubenstein et al. 2012). Ste24 protease also cleaves other proteins that do not aberrantly engage the translocon. Along with translocon quality control, Ste24 cleaves proteins possessing CAAX boxes. This allows for maturation of proteins at the ER (Ast, Michaelis, and Schuldiner 2016). These other functions of Hrd1 and Ste24 have not been isolated from their translocon quality control function in these experiments. Therefore, further testing must be done to discern how much of the observed phenotypes are due to the roles Hrd1 and Ste24 play at the translocon versus other functions of these two proteins. This could be done through targeted knockout of accessory proteins that are involved in the different functions of these two proteins. If I see the same phenotypes both with and without these accessory proteins, the phenotypes are most likely due to action of Hrd1 and Ste24 at the translocon.

*Impaired post-translational translocation rescues the negative growth phenotype of hrd1Δ ste24Δ cells.*

To further characterize *hrd1Δ ste24Δ* cells, I introduced a 13myc epitope tag to the Sec61 subunit of the translocon. This had the unintended consequence of rescuing the negative growth phenotype observed in *hrd1Δ ste24Δ* cells. Further investigation showed that the addition of this tag to the translocon also caused a population of PTT substrates (but not of a model CTT substrate) to fail to efficiently engage the translocon, as indicated by a reduction of glycosylation of these proteins.

I hypothesized the reduced translocation in *sec61-13myc* cells led to more robust cellular health when translocon quality control machinery was absent. This was supported in similar experiments using *sec72Δ* cells. Western blot analysis showed that translocation of *Deg1**-Sec62, Clogger, and CPY (all PTT substrates) was impaired in *sec72Δ* cells. The impairment of translocation was smaller than that observed in Sec61-13myc cells. However, this phenotype was consistent and shows that both modifications to the translocon specifically impair translocation of PTT substrates.

*sec72Δ* cells exhibit similar growth phenotypes to cells expressing sec61-13myc in the context of impaired translocon quality control. In both cases, when translocation is impaired, cells become less reliant on translocon quality control machinery for optimal growth. It is possible that impaired PTT reduces the number of clogging events happening in the cell at any one time. For instance, a naturally clogging protein may only clog during a small number of individual translocation events. PTT impairment may lower the overall instances of translocon clogging of these proteins by reducing the number of total PTT translocation events. With fewer instances of translocon clogging, there would be reduced need for the translocon quality control proteins Hrd1 and Ste24. In turn, this would lead to increased cellular health in cells with impaired translocation in the context of *HRD1* and *STE24* deletion.

While modifications to the translocon caused impaired translocation of model PTT substrates, translocation of the model CTT substrate OPY was unaffected by any translocon modification tested. This indicates that these translocon modifications specifically inhibit translocation of PTT substrates.

From this, I can infer the likely translocon-clogging protein targets of Hrd1 and Ste24: proteins that enter the ER via PTT. I speculate that accumulation of PTT substrates clogging wild type translocons when translocon quality control is impaired leads to poor cellular health by impairing the movement of other proteins into the ER. This suggests that Hrd1- and Ste24-mediated degradation of translocon-clogging PTT substrates is vital to cellular health.

Consistent with a specific role in targeting PTT translocon-clogging proteins, Hrd1 and Ste24 do not contribute to the degradation of model translocon-clogging substrates that use the CTT pathway (Crowder et al. 2015)(Kennedy, Rubenstein unpublished data). In these studies, the engineered protein OPY-K12 was utilized to simulate a CTT clogging protein. OPY is a model CTT protein. By adding multiple positive lysine residues to the C-terminus of the protein, the amino acid sequence aberrantly interacts with the negatively charged exit channel of the ribosome, stalling the newly formed protein in the ribosome. As the protein simultaneously engages the ribosome and translocon, the translationally stalled protein also remains stalled in the translocon. These stalled proteins are degraded through the UPS and the E3 ligase Rkr1/Ltn1. However, Hrd1 and Ste24 do not participate in the degradation of this model CTT clogging protein, suggesting that Hrd1 and Ste24 may only be used in protein quality control of the PTT translocon.

*How do sec61-13myc and sec72Δ cause impairment of translocation?*

Both sec61-13myc and *sec72Δ* specifically impair PTT. This is most likely due to changes in interactions among specific subunits of the PTT translocon. PTT requires multiple subunit interactions to facilitate the transfer of nascent proteins to the Sec61 pore and further translocation. The proteins considered part of the PTT translocon are Sec61, Sss1, Sbh1, Sec62, Sec63, Sec71 and Sec72. Subtle perturbation of interactions between translocon subunits or between the translocon and the translocating protein may explain translocation impairment of PTT substrates.

In cells expressing sec61-13myc, the epitope tag is attached at the at the C-terminus of Sec61 (Carvalho, Stanley, and Rapoport 2010). Sec61 contains ten transmembrane segments with both the N- and C-termini on the cytosolic side of the ER membrane (Linxweiler, Schick, and Zimmermann 2017). While Sec62 binds to the N-terminus of Sec61, Sec63 binds to cytosolic loops 6 and 7 of Sec61. These loops are in close proximity to loops 8 and 9 of Sec61 (Wu 2019). Binding of Sec62 and Sec63 cause conformational changes to Sec61 that facilitate translocation of PTT substrates. Mutation of *SEC63* inhibits translocation of some model PTT substrates, while having no effect on others (Lang 2012; Gorlich 1993). The addition of a 13myc tag to the C-terminal end of Sec61 may sterically disrupt the interaction between Sec63 and Sec61. Since PTT of model substrates *Deg1**-Sec62, Clogger, and CPY is only partially impaired, the presence of 13myc may weaken, but not abolish, the Sec61-Sec63 interaction. Flexibility in the 13myc epitope may result in a transient translocation block, or inhibition of a subset of translocon complexes (Figure 15).

Sec72 is another component of the PTT translocon, with a primary function of recognizing proteins bound to cytosolic Hsp70 (Wu 2019). This cytosolic protein is recruited to the translocon by Sec63, along with the integral membrane protein Sec71. Sec72 and Sec71 (which are not present in higher organisms) are not essential for PTT (Wu 2019). However, deletion of *SEC72* modestly impairs translocation of PTT substrates *Deg1**-Sec62, CPY, and Clogger. The impairment is likely due to the translocon not being able to recognize PTT substrates as efficiently without Sec72 being able to recruit Hsp70-bound PTT substrates. The reproducible impairment is milder than the impairment in Sec61-13myc cells. The milder effect is likely due to the non-essential nature of Sec72 compared to the interaction between Sec61 and Sec63.

*Which tagged translocon subunits do not cause translocation impairment?*

To identify an epitope-tagged translocon with unimpaired translocation, I analyzed the function of cells expressing various translocon subunits with a GFP epitope. Along with GFP-tagged translocons, I also created one 3HA-tagged translocon to test for translocation efficiency. Similar to sec61-13myc and *SEC72* deletion, none of the epitope-tagged translocons tested affected translocation of model CTT substrate OPY. Translocation of model PTT substrates *Deg1**-Sec62 and CPY was differentially affected by epitopes linked to different translocon subunits. Modification of Sec61, Sec62, Sec63, and Sss1 subunits caused impairment of PTT, while modification to Sec71 and Sec72 caused little impairment.

Of all the subunit-epitope combinations tested, tags attached to the Sec61 and Sss1 subunits showed the most impairment of PTT. Both subunits contribute to the central pore of the translocon, suggesting that even slight perturbation to the central pore could impair function. Sec71 and Sec72 are the most peripheral subunits of the translocon, which may explain the minimal impact of tagging these proteins. However, an epitope on a subunit contributing to the central pore that mildly impairs PTT may still be useful in experiments designed to identify physiological translocon-clogging proteins. In the presence of all tested epitopes, a substantial amount of PTT substrate translocates into the ER and becomes glycosylated, and a substantial fraction of Clogger protein clogged the translocon in cells expressing sec61-13myc. This suggests that clogging proteins (which likely predominantly enter the translocon via PTT) may be isolated through co-immunoprecipitation experiments using tags close to the central pore. Attaching the tag to the central pore also has advantages compared to tagging more peripheral subunits, such as Sec71 or Sec72. By attaching the tag to a peripheral subunit of the translocon, the interaction between this subunit and the central pore must be maintained to precipitate clogging proteins. Attaching the epitope tag to the central pore of the translocon will bypass this issue, potentially allowing for more efficient isolation of physiological translocon-clogging proteins.

**Conclusions and future directions**

This research reveals modifications to the ER translocon that specifically impair PPT, and how this impairment leads cells to have less reliance on the translocon quality control pathways mediated by Hrd1 and Ste24. This could lead to new discoveries and therapies for diseases that have been correlated with clogged translocons, such as cholesterol and diabetes. Apolipoprotein B (ApoB) is a known translocon-clogging protein that is regulated by Gp78, the mammalian Hrd1 homolog (Yeung, Chen, and Chan 1996). ApoB interacts with and clogs the translocon, waiting for lipids in the ER lumen to bind and create LDL molecules. If lipids do not bind to translocon-engaged ApoB, Gp78 ubiquitylates ApoB, leading to its degradation, allowing the translocon to resume normal function. Dysfunction in this system can lead to problems in cholesterol metabolism and cardiovascular disease in mammals. Future experiments will be performed to determine if Ste24 (or mammalian homolog ZMPSte24) contributes to ApoB regulation and cholesterol metabolism. The overlapping function of Hrd1 and Ste24 suggests that targeting ZMPSte24 or Gp78 may represent viable strategies for treating cholesterol-related disease.

Diabetes also can be regulated through actions at the translocon. Type 2 diabetes is characterized by insulin resistance and β cell dysfunction. One of the causes of β cell dysfunction is misfolding and aggregation of a normally secreted protein, islet amyloid polypeptide (IAPP). Recent studies indicate that the pathologic impact of this protein may be mediated in part through translocon clogging by the aggregated form of IAPP. Toxicity associated with this protein is modulated by ZMPSte24, which may target the protein for degradation (Kayatekin 2018). Similar to ApoB in cholesterol function, it is unknown whether Gp78 also has an overlapping role in the regulation of IAPP. Ste24, and potentially Gp78, are potential targets for new strategies in diabetes therapy and prevention.

Many questions remain unanswered regarding the relationship between Hrd1 and Ste24 in translocon quality control. Both Hrd1 and Ste24 have many functions outside their roles as translocon quality control. It is possible that disruption of these other functions in *hrd1Δ ste24Δ* cells have some effect on the negative growth phenotype observed in these cells. Moreover, whether Hrd1 cofactors that

contribute to degradation of misfolded Hrd1 substrates are also required for its function in translocon quality control is not known. More research needs to be done to untangle the varying roles of these enzymes to further understand their roles in translocon quality control.

A better understanding of the features of translocon-clogging proteins recognized by Hrd1 and Ste24 homologs, facilitated by identification of physiological translocon-clogging proteins, may inform strategies to treat medical conditions associated with clogged translocons. The work presented here has relied on two model translocon-clogging proteins to study translocon quality control: *Deg1**-Sec62 and Clogger. While these engineered proteins have proven useful, they may not interact with the translocon or translocon quality control in the same way as natural clogging proteins. In the future, I would like to identify novel, natural translocon-clogging proteins through co-immunoprecipitation and proteomics-based identification. Based on the epitope-tagged translocons tested, Sec71-GFP and Sec72-GFP cause the least impaired translocation. However, sec61-13myc retains sufficient translocon function and would likely prove useful in co-immunoprecipitation experiments. Purifying a central pore subunit may prove more successful in enrichment of translocon-clogging proteins than precipitating a peripheral subunit of the translocon. I hope that the discovery of novel translocon-clogging proteins will lead to new insights about the nature of translocon-clogging proteins, potentially leading to new strategies for treating diseases associated with translocon clogging, such as cholesterol-related pathology and diabetes.

**Table 1. List of plasmids used in this study**

| Plasmid Number | Plasmid Name | Description | Yeast Selection Marker | Bacterial Selection Marker |
|---|---|---|---|---|
| pVJ27 | pRS316 | empty vector | *URA3* | AmpR |
| pVJ134 | pAG32 | HphMX4 deletion cassette | - | AmpR |
| pVJ317 | p416-MET25-*Deg1**-Sec62-ProtA | *Deg1**-Sec62 expression plasmid; expression driven by *MET25* promoter | *URA3* | AmpR |
| pVJ576 | pRS316-GPD-CPY-ProtA | CPY expression plasmid; expression driven by *GPD* promoter | *URA3* | AmpR |
| pVJ578 | pRS316-GPD-OPY-ProtA | OPY expression plasmid; expression driven by *GPD* promoter | *URA3* | AmpR |
| pVJ605 | p416-MET25-Clogger | Clogger-HA expression plasmid; expression driven by *MET25* promoter | *URA3* | AmpR |

**Table 2. List of yeast strains used in this study**

| Yeast Number | Alias | Genotype |
|---|---|---|
| VJY6 | MHY500 | *his3-Δ200, leu2-3-112, ura3-52, lys2-801, trp1-1, gal2* |
| VJY18 | MHY7659 | *his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, sec61-13myc::HISMX6* |
| VJY260 | MHY7582 (105-3-2) | *his3-Δ200, leu2-3-112, ura3-52, lys2-801, trp1-1, gal2, sec61-13myc-TRP1* |
| VJY456 | | *his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, hrd1Δ::kanMX, ste24Δ::natMX4* |
| VJY462 | | *his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, hrd1Δ::kanMX4, ste24Δ::HygroR, sec61-13myc::HISMX6* |
| VJY476 | YSC1048, BY4741 | *his3Δ1, leu2Δ0, met15Δ0, ura3Δ0* |
| VJY588 | YKM936 | *his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, sec63-EGFP-kanMX4* |
| VJY765 | | *his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, sec61-GFP::HIS5sp* |
| VJY766 | | *his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, sss1-GFP::HIS5sp* |
| VJY767 | | *his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, sec71-GFP::HIS5sp* |
| VJY783 | | *his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, sec72-GFP::HIS5sp* |
| VJY786 | | *his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, sec72Δ::hphMX4* |
| VJY803 | | *his3-Δ200, leu2-3-112, ura3-52, lys2-801, trp1-1, gal2, sss1-3HA::His3MX6* |
| VJY841 | | *his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, hrd1Δ::kanMX4, ste24Δ::natMX4, sec72Δ::hphMX4* |

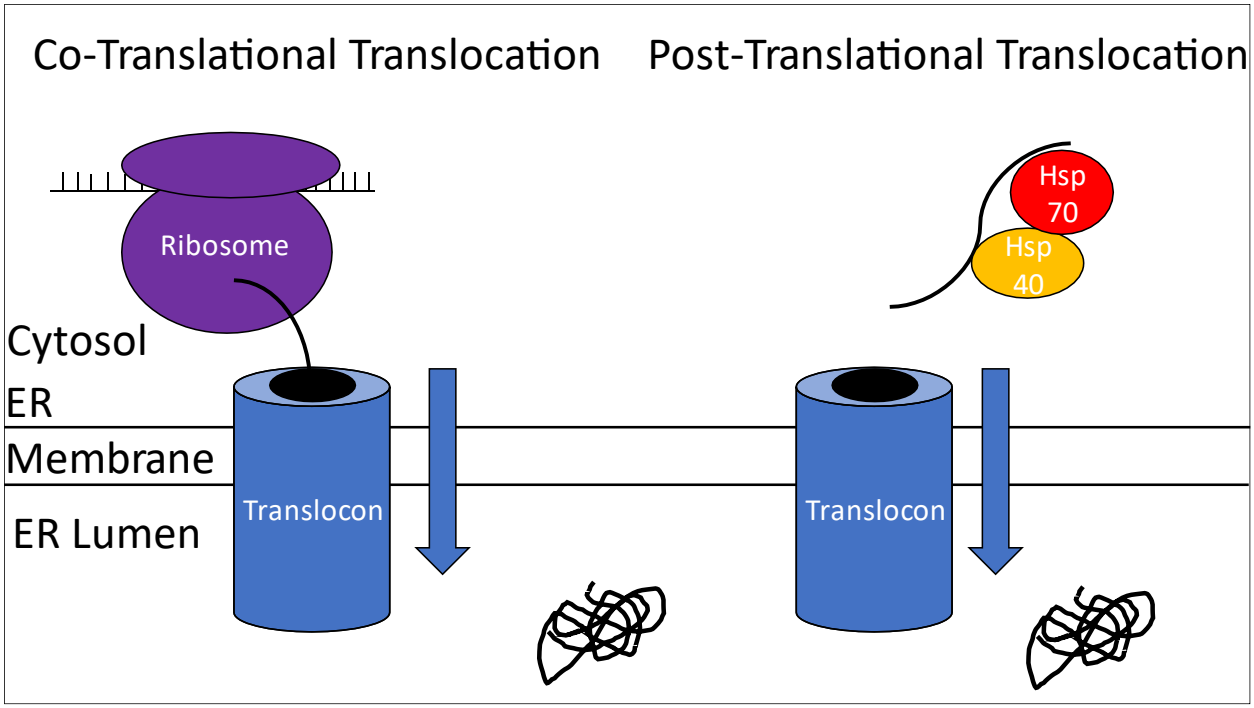



**Figure 1. Different mechanisms of translocation into the ER lumen**. During co-translational translocation (CTT), polypeptide chains are simultaneously translated by the ribosome and translocated into the ER. During post-translational translocation (PTT), translation occurs independently of translocation. After translation is completed in the cytosol, the newly formed polypeptide is transported to the translocon with the aid of Hsp70 and Hsp40 proteins.

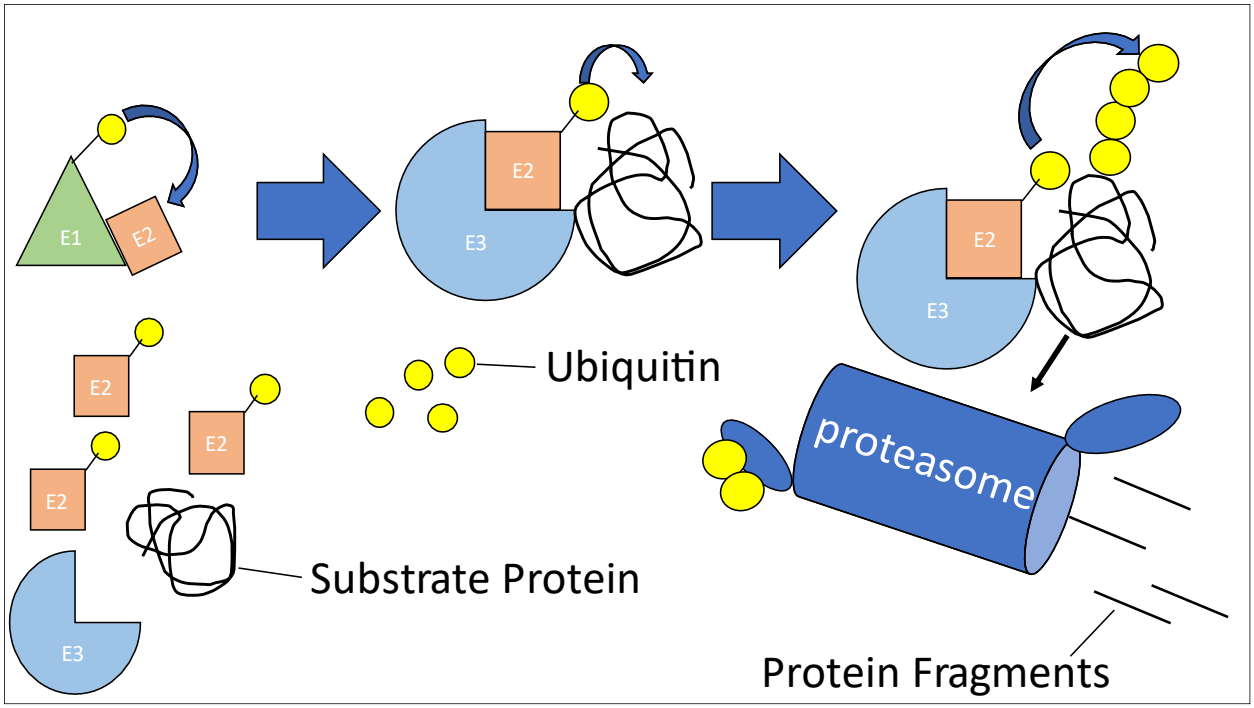



**Figure 2. The Ubiquitin-Proteasome System.** A ubiquitin-activating enzyme (E1) transfers a ubiquitin molecule to a ubiquitin-conjugating enzyme (E2). A ubiquitin ligase enzyme (E3) then recruits the charged E2 to a protein targeted for degradation. The E3 is also responsible for facilitating the transfer of the ubiquitin molecule from the E2 to the targeted protein. After consecutive ubiquitin molecules have been transferred to the targeted protein, the protein is then targeted by the proteasome for degradation, and its constituent parts are recycled.

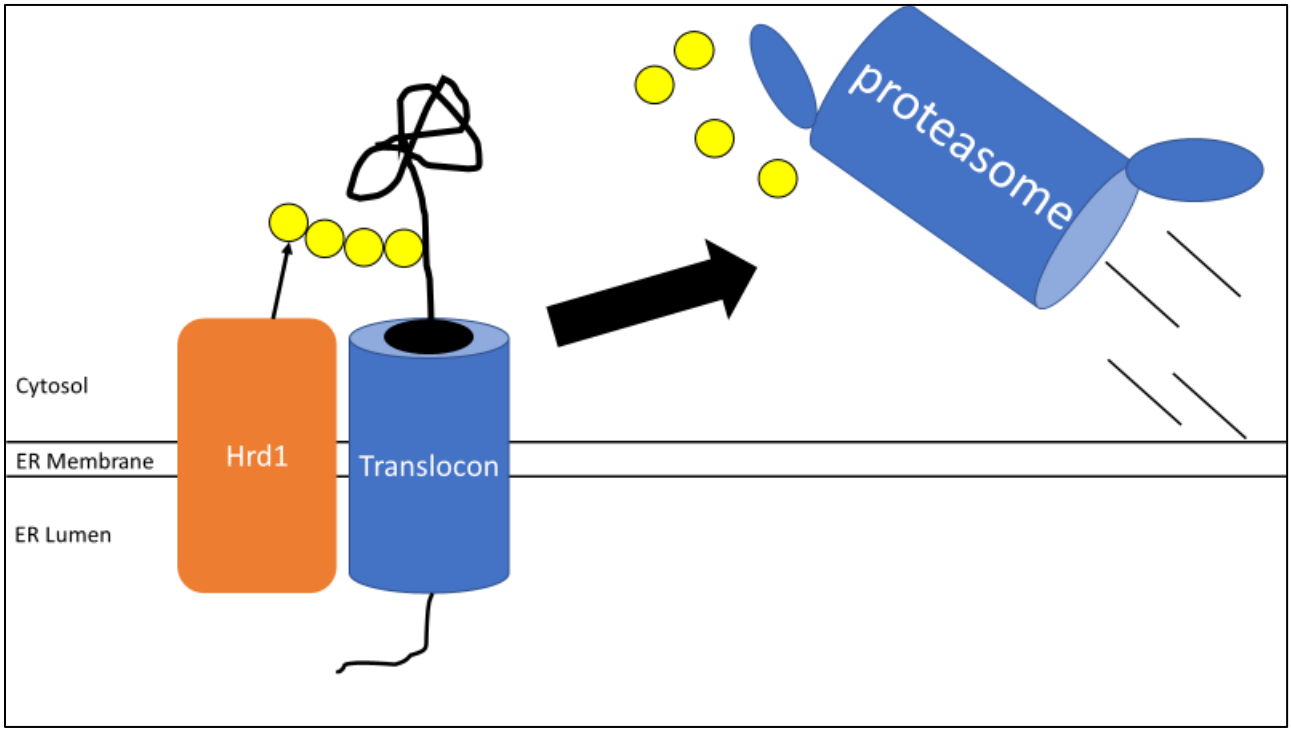



**Figure 3. ER-associated degradation of translocon-clogging proteins mediated by the E3 ubiquitin ligase Hrd1.** The E3 ligase Hrd1 attaches ubiquitin to protein molecules that clog the translocon. The clogging proteins are then removed from the translocon and recruited to the proteasome for degradation.

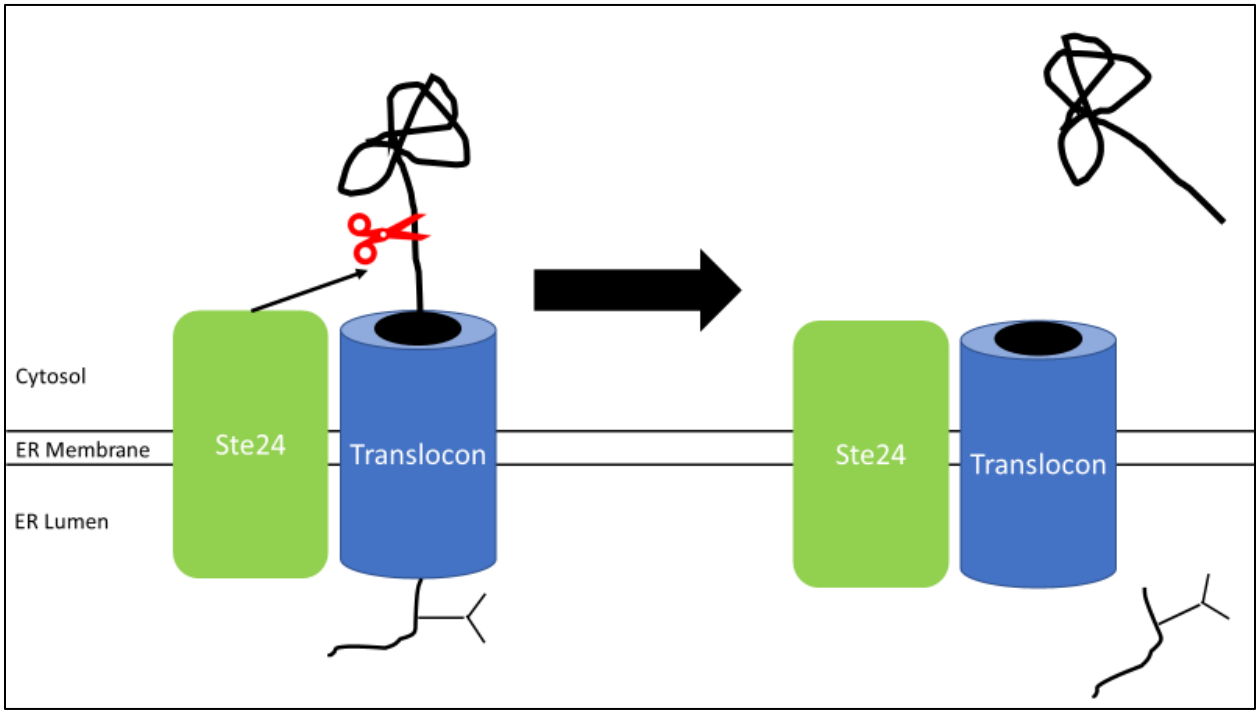



**Figure 4. Ste24 protease cleaving stalled proteins from the translocon**. Ste24 is a protease enzyme that, once recruited to the translocon, cleaves clogging proteins.

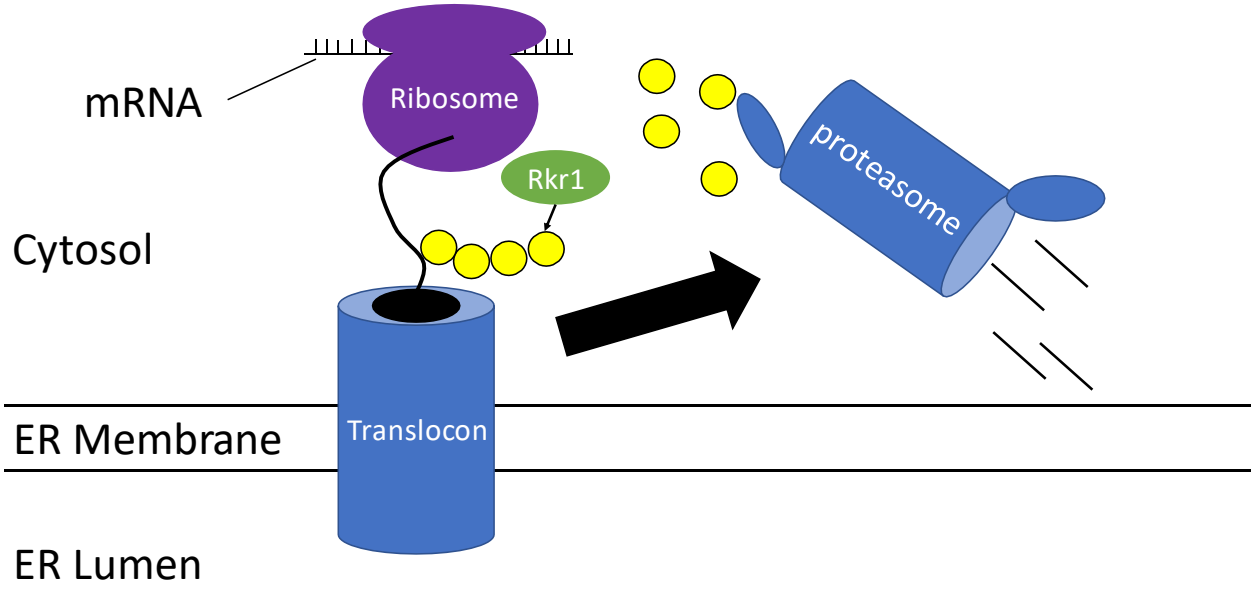



**Figure 5. Ribosome-associated degradation mediated by Rkr1/Ltn1.** As proteins stall in the translocon during translation, Rkr1/Ltn1 is recruited to the dysfunctional ribosome and ubiquitylates the offending protein. The ubiquitylated protein is then removed from the translocon and transported to the proteasome for degradation.

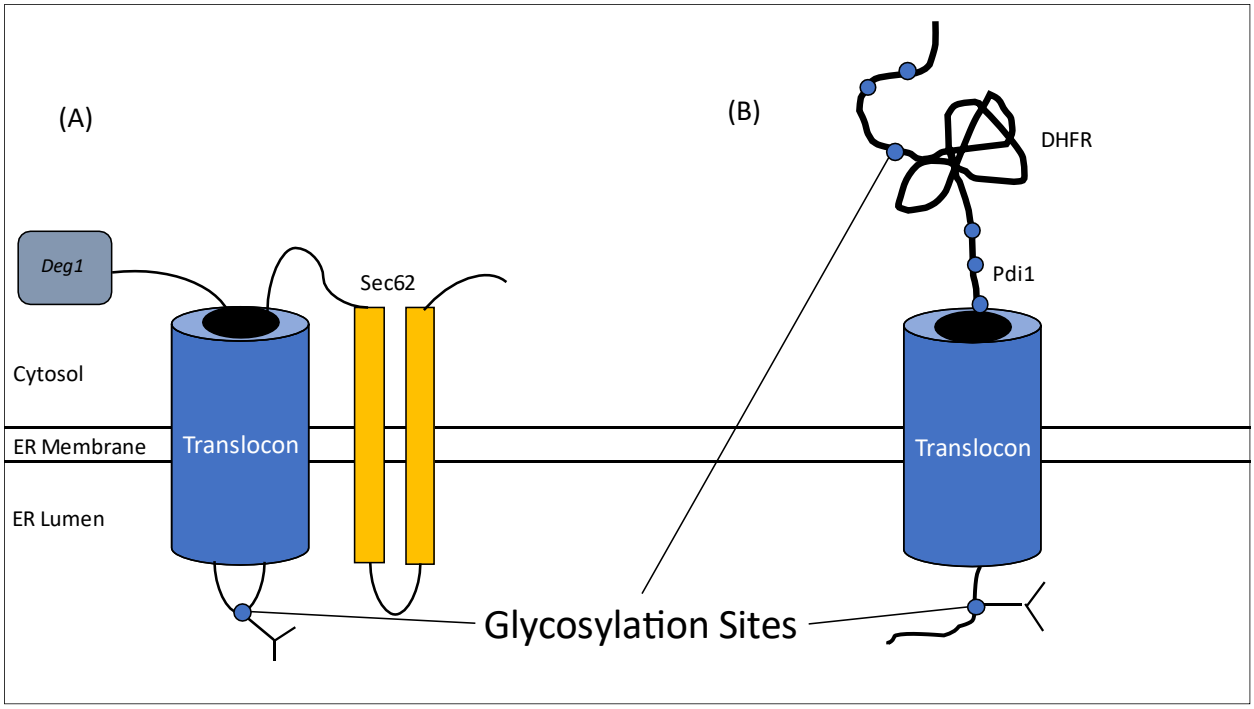



**Figure 6. Schematic of *Deg1*-Sec62 and Clogger interaction with the translocon.** (A) *Deg1*-Sec62 consists of the degron *Deg1* from Matα2 fused to the N-terminus of the translocon subunit Sec62. This fusion causes the cytosolic portion of the N-terminus to loop into the central pore of the ER and aberrantly interact with the translocon. There is one glycosylation site in the portion of *Deg1*-Sec62 that is exposed to the ER lumen. (B) Clogger is a fusion of the PTT substrate Pdi1, and the *E. coli* enzyme dihydrofolate reductase (DHFR). DHFR rapidly folds, causing steric hindrance at the translocon. Clogger contains several glycosylation sites throughout the protein. Glycosylation sites are indicated as blue circles.

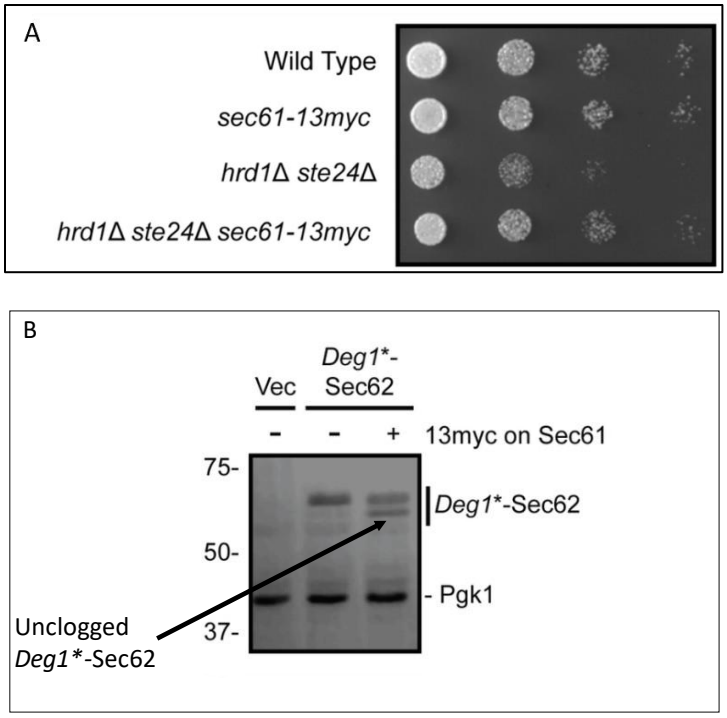



**Figure 7. sec61-13myc causes impairment of the translocon** (A) Six-fold serial dilutions of yeast of the indicated genotypes were spotted onto rich growth medium and incubated at 30°C. (B) Protein abundance and mobility of *Deg1**-Sec62 in wild type cells and *sec61-13myc* cells. As *Deg1**-Sec62 interacts with and clogs the translocon, it is modified via N-linked glycosylation. Impairment of the translocon leaves *Deg1**-Sec62 unmodified. Modifed (clogged) and unmodified (unclogged) *Deg1**-Sec62 travel as two discrete species. Pgk1 is used as a loading control.

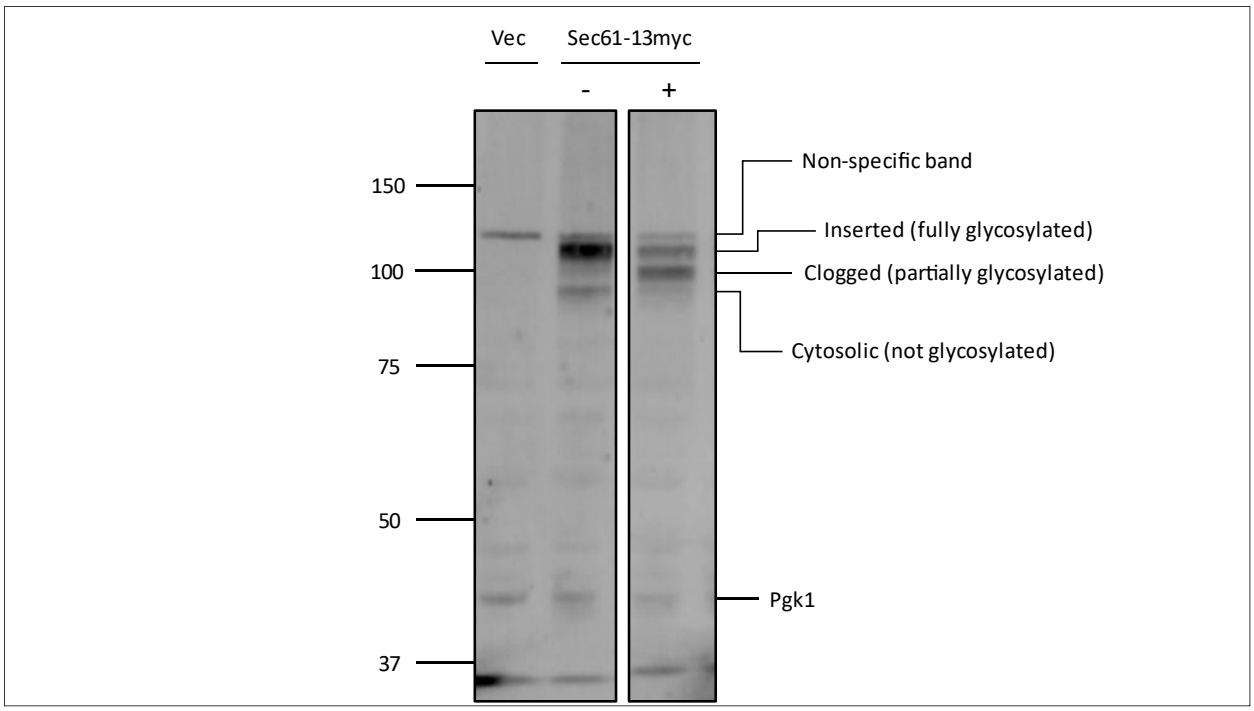



**Figure 8. sec61-13myc modification causes impairment of translocation of Clogger protein.** Protein abundance and migration of Clogger were analyzed in wild type cells and *sec61-13myc* cells. As translocating substrate interacts with the translocon, it is modified via N-linked glycosylation. Impaired translocation resuls is substrate molecules with fewer modifcations. Differences in translocation result in species that migrate as discrete bands. Clogger was detected by western blot with antibodies that bind to a C-terminal HA epitope tag. Pgk1 was used as a loading control.

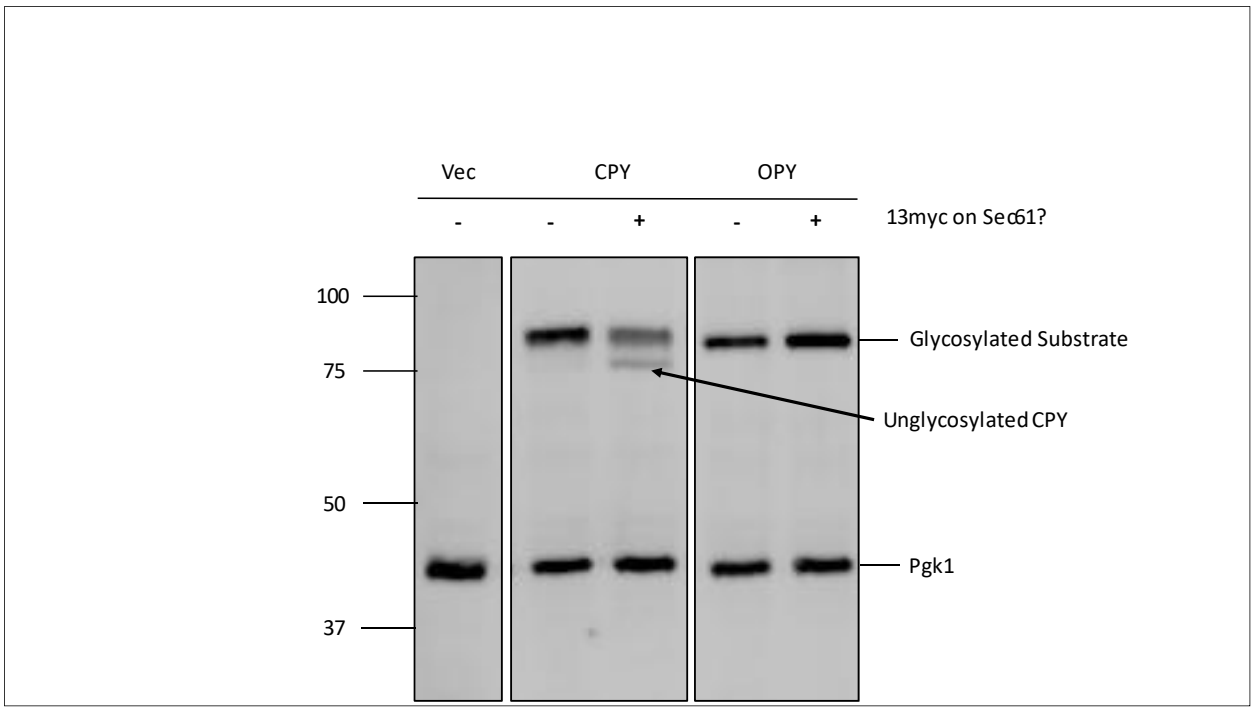



**Figure 9. sec61-13myc modification causes impairment of translocation of model PTT substrate.**

Protein abundance and migration of model PTT substrate CPY and model CTT substrate OPY were analyzed in wild type cells and *sec61-13myc* cells. As translocating substrate interacts with the translocon, it is modified via N-linked glycosylation. Impaired translocation resuls is substrate molecules with fewer modifcations. Substrates with varying modifications migrate as discrete bands. CPY and OPY was detected by western blot with antibodies that bind to a C-terminal ProtA epitope tag. Pgk1 was used as a loading control.

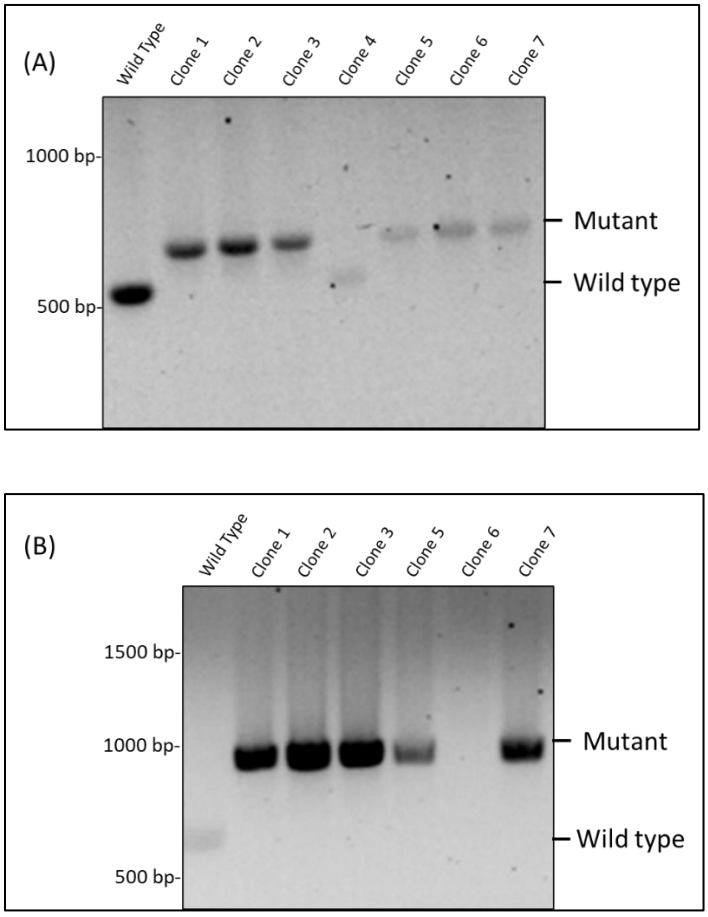



**Figure 10. Validation of *SEC72* genotype in *hrd1Δ ste24Δ sec72Δ* strains.** (A) Seven candidate *hrd1Δ ste24Δ sec72Δ* clones were genotyped at the 3' end of the *SEC72* locus. Wild type expected product size: 501 bp. *sec72Δ* expected product size: 615 bp. (B) Validated clones from the 3' end were then analyzed at the 5' end of the *SEC72* locus. Wild type expected product size: 601 bp. *sec72Δ* expected product size: 880 bp.

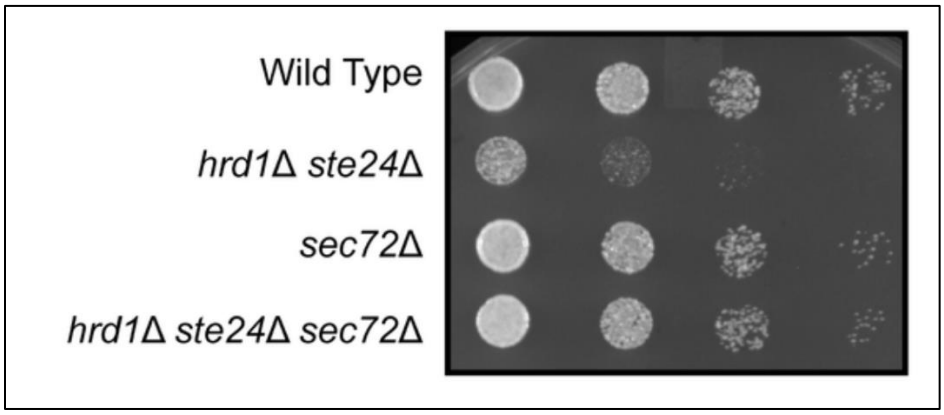



**Figure 11. *SEC72* deletion rescues negative growth phenotype of *hrd1Δ ste24Δ* cells.** Six-fold serial dilutions of yeast of the indicated genotypes were spotted onto rich growth medium and incubated at 30°C.

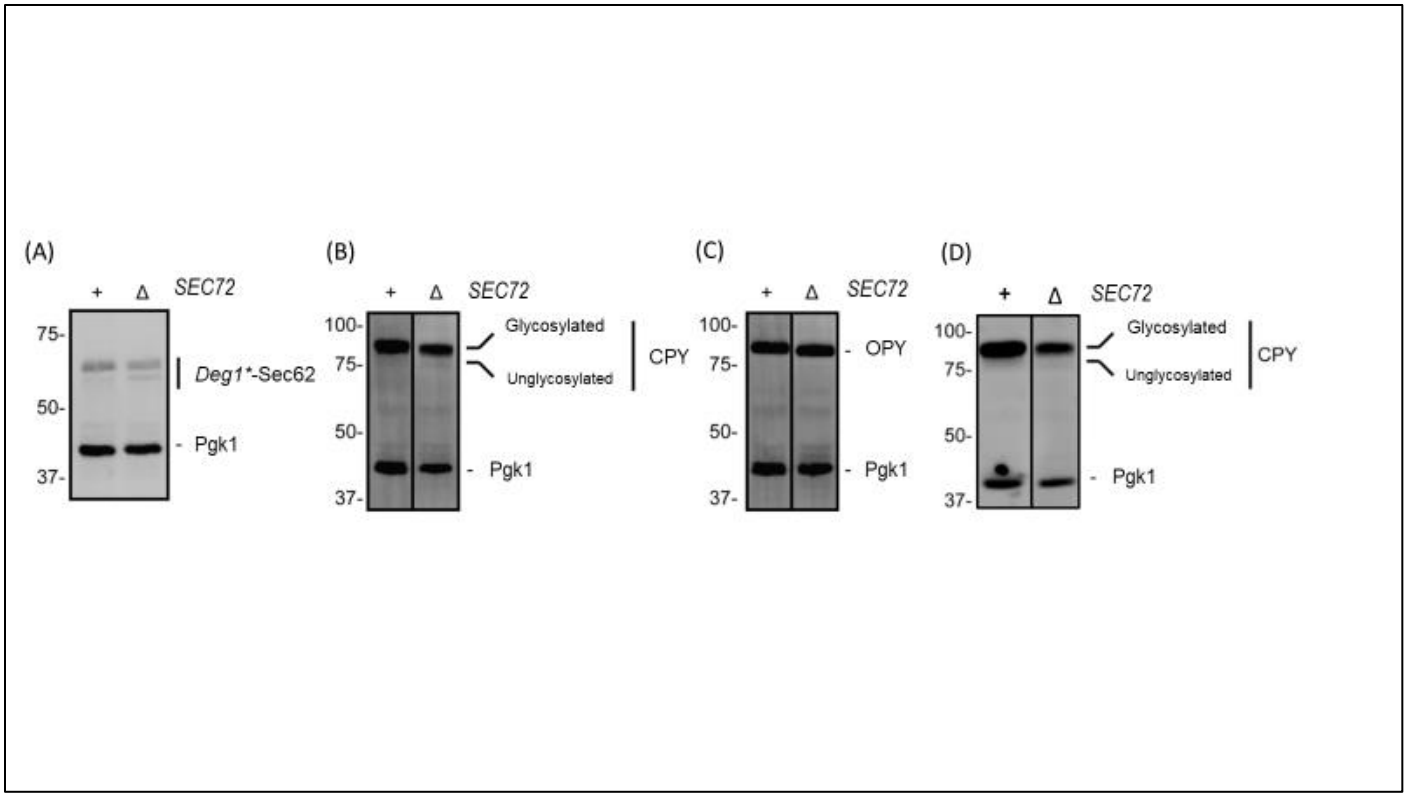
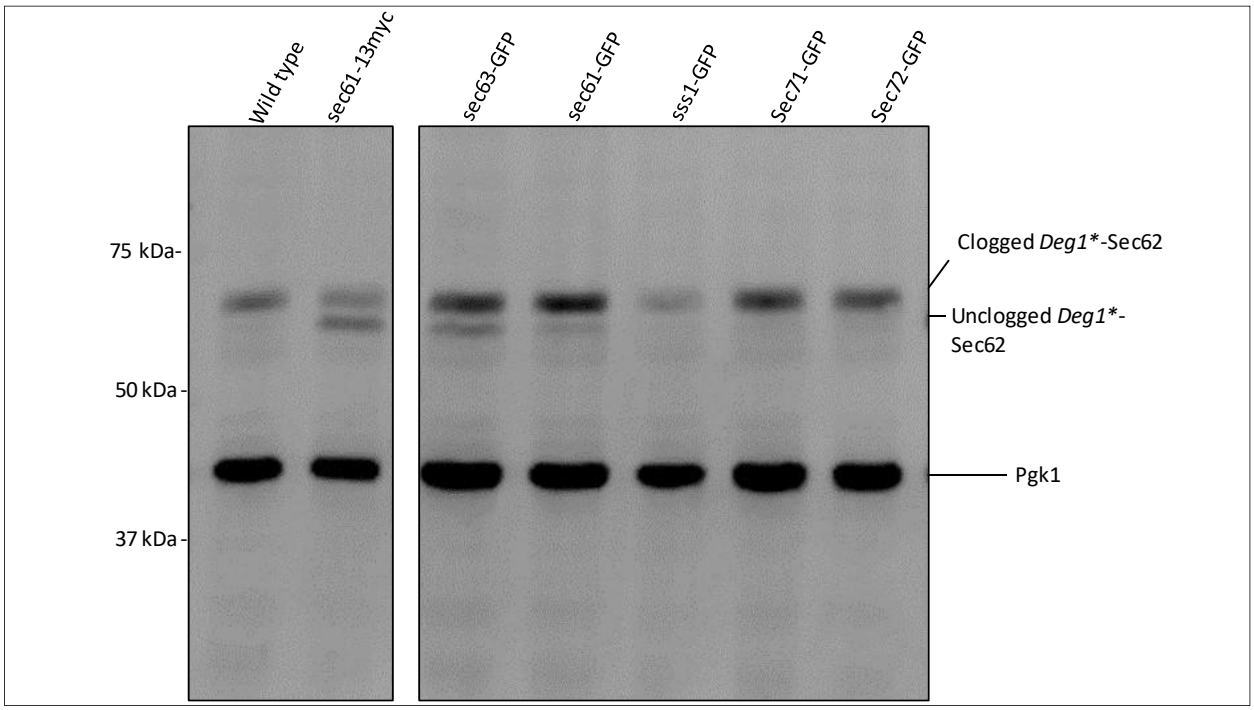
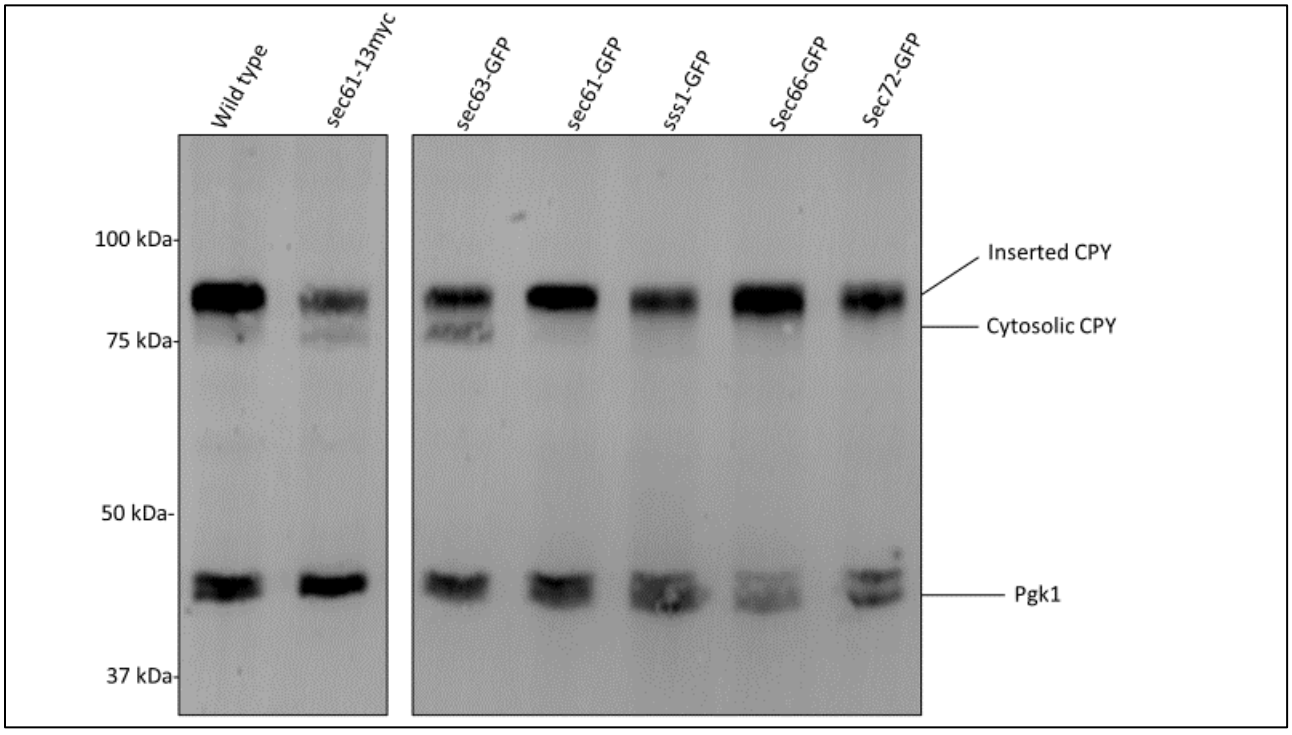



**Figure 12. *SEC72* deletion causes dysfunction of the translocon.** (A) Protein abundance and migration of of *Deg1**-Sec62 were analyzed in wild type cells and *sec72Δ* cells. (B, D) Protein abundance and migration of CPY were analayzed in wild type cells and *sec72Δ* cells. (C) Protein abundance and migration of OPY were analyzed in wild type cells and *sec72Δ* cells. (A-D) As translocating substrates interact with the translocon, they become modified via N-linked glycosylation. Impaired translocation results in hypomodified substrate. Modifed and unmodified substrate travel as discrete bands. DS, CPY, and OPY were detected by western blot with antibodies that bind to a C-terminal Protein A epitope tag. Pgk1 was used as a loading control.

(A)


Wild type
sec61-13myc
sec63-GFP
sec61-GFP
sss1-GFP
Sec71-GFP
Sec72-GFP
75 kDa-
50 kDa-
37 kDa-
Clogged *Deg1**-Sec62
Unclogged *Deg1**-Sec62
Pgk1

(B)


Wild type
sec61-13myc
sec63-GFP
sec61-GFP
sss1-GFP
Sec66-GFP
Sec72-GFP
100 kDa-
75 kDa-
50 kDa-
37 kDa-
Inserted CPY
Cytosolic CPY
Pgk1

(C)

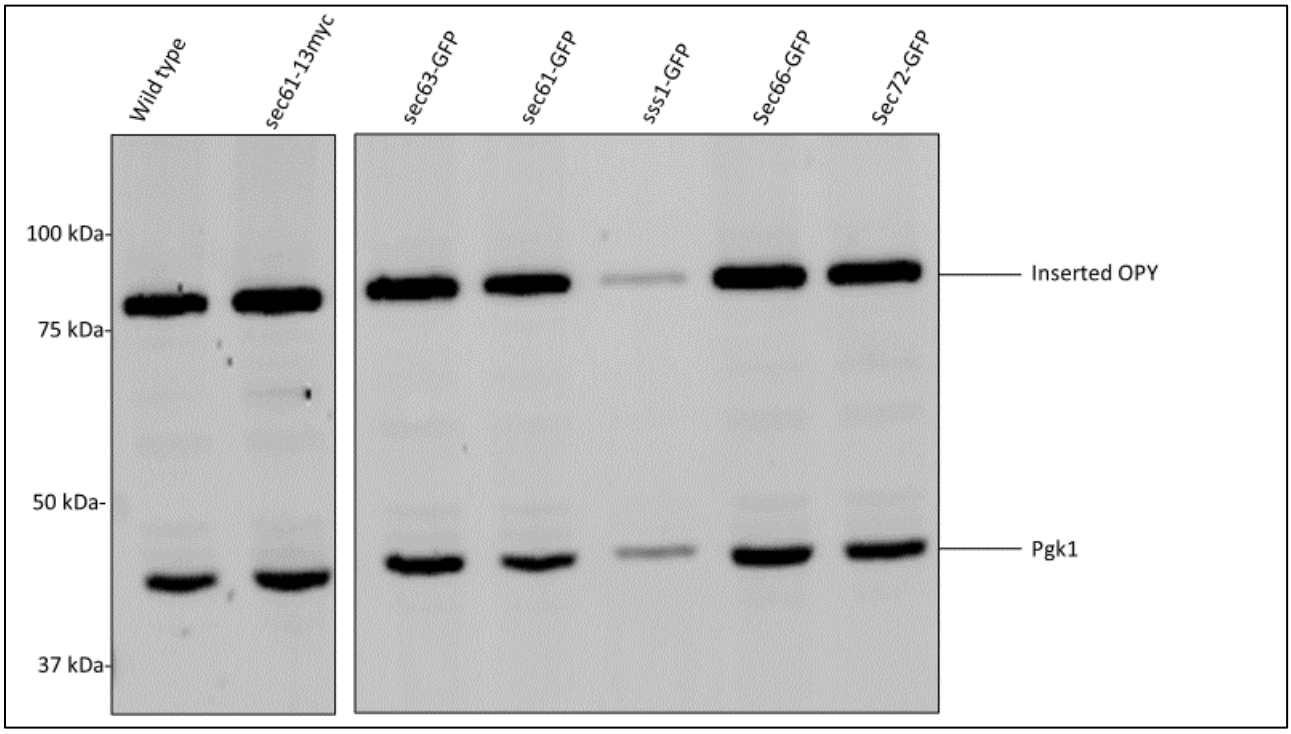



**Figure 13. Impact of the addition of C-terminal GFP tag on translocon subunits on translocon engagement.** (A) Protein abundance and migration of *Deg1**-Sec62 were analyzed in wild type cells, *sec61-13myc* cells, and cells containing indicated GFP-tagged translocon subunit. (B) Protein abundance and migration of CPY were anlyzed in wild type cells, *sec61-13myc* cells *sec72Δ* cells, and cells containing indicated GFP-tagged translocon subunit. (C) Protein abundance and migration of OPY were anlayzed in wild type cells , *sec72Δ* cells, and cells containing indicated GFP-tagged translocon subunit. *Deg1**-Sec62, CPY, and OPY were detected by western blot with antibodies that bind to a C-terminal Protein A epitope tag. Pgk1 was used as a loading control.

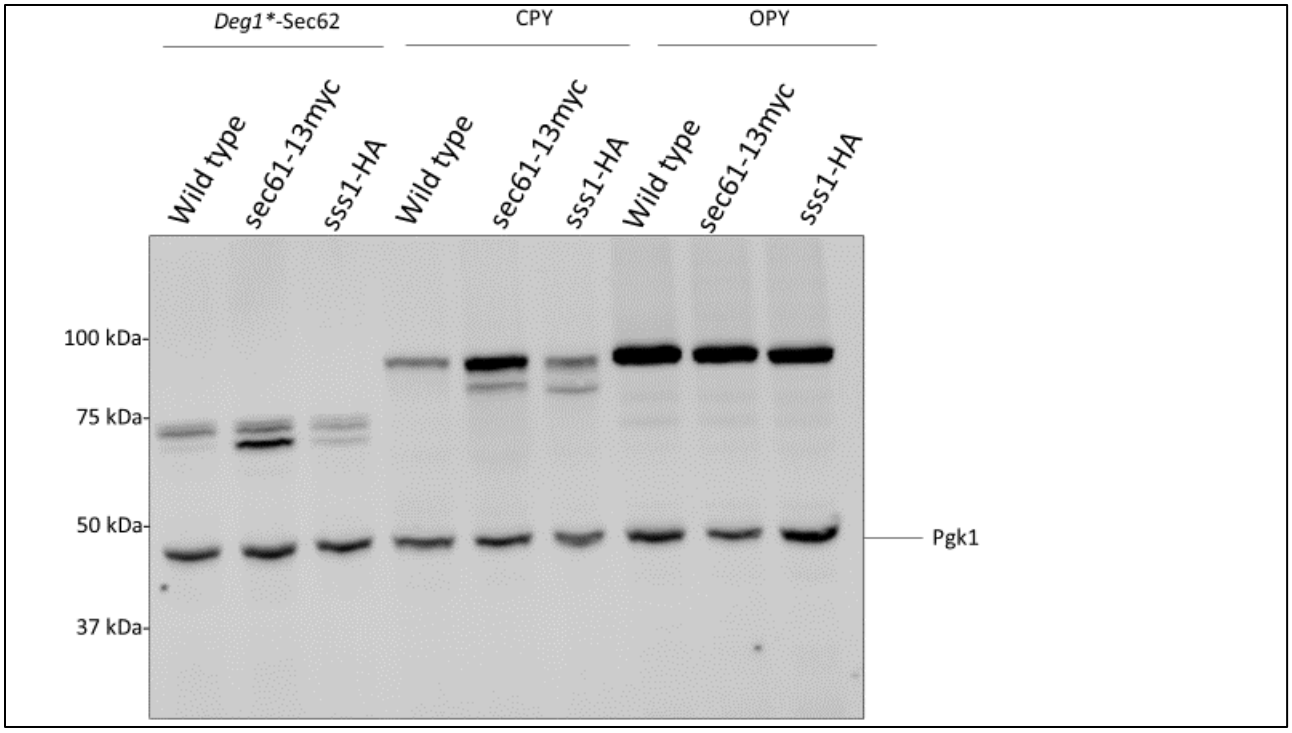



**Figure 14. sss1-3HA causes dysfunction of translocation.** Protein abundance and migation of *Deg1**-Sec62, CPY, and OPY were analyzed in wild type, *sec61-13myc*, and *sss1-3HA* cells. As translocating substrates interact with the translocon, they become modified via N-linked glycosylation. Impaired translocation results in hypomodified substrate. Modifed and unmodified substrate travel as discrete bands. *Deg1**-Sec62, CPY, and OPY were detected by western blot with antibodies that bind to a C-terminal Protein A epitope tag. Pgk1 was used as a loading control.

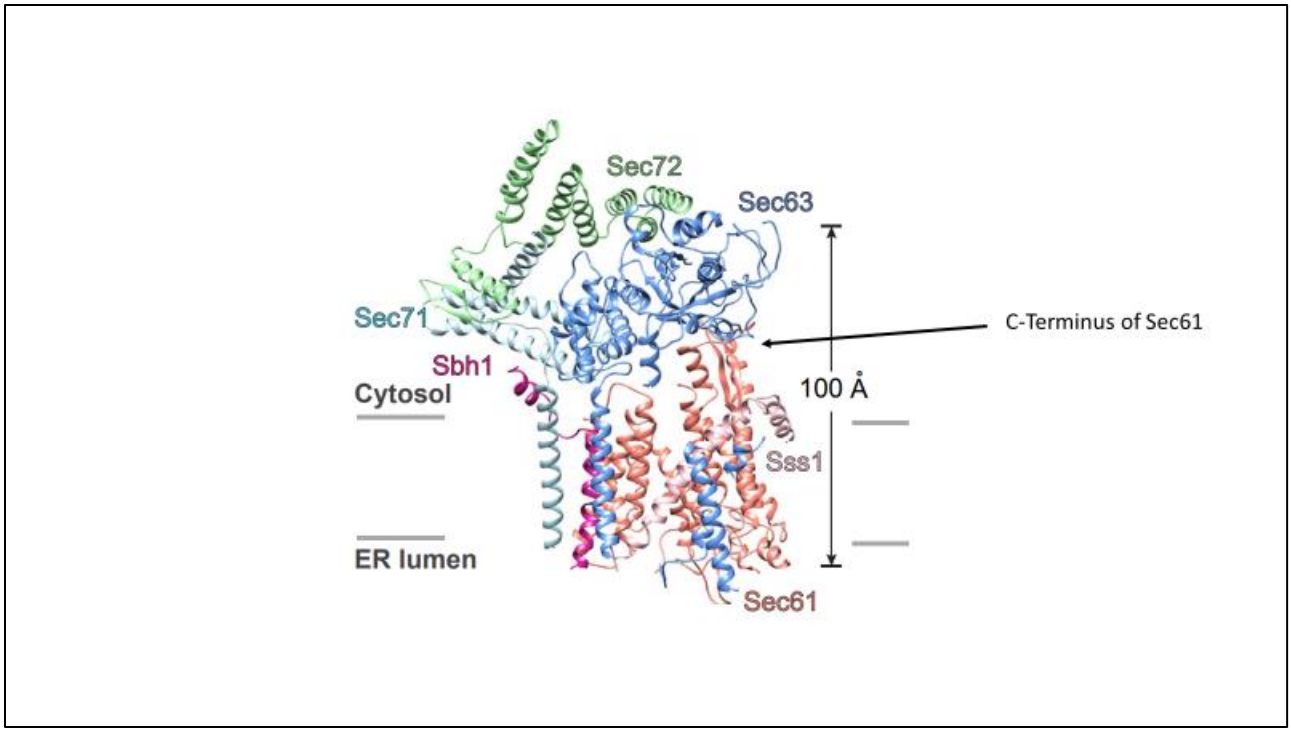



**Figure 15. The C-Terminus of Sec61 is important to efficient subunit interactions.** Ribbon diagram of the PTT translocon (reproduced from (Wu, Cabanos, and Rapoport 2019). The C-terminal end of Sec61 is located in close proximity to the domain that binds to Sec63.

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