THE RELATIONSHIP BETWEEN ER STRESS AND PROTEIN QUALITY
CONTROL AT THE TRANSLOCON

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LIST OF ABBREVIATIONS

apoB: apolipoprotein B

DTT: dithiothreitol

E1: ubiquitin-activating enzyme

E2: ubiquitin-conjugating enzyme

E3: ubiquitin ligase

EDTA: ethylenediaminetetraacetic acid

ER: endoplasmic reticulum

ERAD: endoplasmic reticulum-associated degradation

ERAD-C: ERAD of proteins with cytosolic degrons

ERAD-L: ERAD of proteins with luminal degrons

ERAD-M: ERAD of proteins with intramembrane degrons

ERAD-RA: ERAD of ribosome-associated proteins

ERAD-T: ERAD of translocon-associated proteins

ERpQC: ER stress-induced pre-emptive quality control

ERSU: ER surveillance

GFP: green fluorescent protein

LB: luria broth

LDL: low-density lipoprotein

LTE: lithium acetate-tris-EDTA

PEG: polyethylene glycol

PTMs: post-translational modifications

PVDF: polyvinylidene difluoride

RESET: rapid ER stress-induced export

SD: synthetic defined media

SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis

SHRED: stress-induced homeostatically regulated protein degradation

TBS: tris-buffered saline

TBS/T: tris-buffered saline with Tween-20

TE: tris-EDTA

Tm: tunicamycin

UPR: unfolded protein response

UPRE: unfolded protein response element

UPS: ubiquitin-proteasome system

YPD: yeast extract-peptone-dextrose media

INTRODUCTION

Proteins play an essential role in cellular function and homeostasis. For example, proteins can act as enzymes, messengers, and antibodies. To maintain this homeostasis, cells have developed a balanced relationship between protein synthesis and degradation. As proteins are synthesized, there is the potential for error, sometimes leading to misfolded, damaged, or otherwise aberrant proteins [1]. When aberrant proteins accumulate, they must be degraded to maintain cellular health [2]. Proteins are also degraded when they are no longer needed for regulatory purposes.

Saccharomyces cerevisiae and Protein Quality Control

Protein degradation is one branch of protein quality control [3]. Quality control can be studied in the model organism *Saccharomyces cerevisiae*, also known as budding yeast. Many genes and many biochemical pathways of budding yeast are highly conserved with other eukaryotes, including humans, making them a useful model organism to study eukaryotic molecular mechanisms [4]. Biological discoveries in yeast may potentially be applied to other eukaryotic organisms such as humans. The yeast genome is relatively easy to manipulate making it possible to introduce, edit, or delete genes as desired. In addition, yeast also have a relatively short doubling time, allowing for rapid cell culture.

The ubiquitin-proteasome system (UPS) is responsible for the majority of regulated degradation of misfolded proteins (**Figure 1**). Degradation through this pathway begins with an E1, or a ubiquitin-activating enzyme [5, 6]. This enzyme carries a small protein called ubiquitin. The E1 will then transfer the ubiquitin to an E2, or a

ubiquitin-conjugating enzyme. E2s associate with E3s, ubiquitin ligases, which then target a specific protein for degradation. The ubiquitin molecule is finally transferred to a protein marked for degradation. This series repeats until a poly-ubiquitin chain has been formed on the protein. A complex referred to as the proteasome can then recognize the ubiquitin chain and will degrade the protein. When this occurs, the ubiquitin molecules are released and reused.

A branch of the UPS, endoplasmic reticulum (ER)-associated degradation (ERAD), involves the degradation of proteins at the ER membrane [7, 8]. There are three characterized E3s (Hrd1, Doa10, and Rkr1) involved in multiple ERAD pathways [8, 9, 10]. These ligases target proteins that possess a degradation signal (or degron) or that exhibit specific types of abnormal behavior. A degron is a portion of a protein that is aberrant (e.g. misfolded); the degron for an ER-localized protein can be located in the cytosol, membrane, or lumen of the ER [11].

There are several subtypes of ERAD (**Figure 2**). In the ERAD-C (cytosol) pathway, proteins with degrons located in the cytosol are targeted for degradation [12]. These proteins can be integrated into the ER membrane or be soluble cytosolic proteins. The main E3 involved in ERAD-C is Doa10 [13]. The ERAD-M (membrane) pathway targets proteins with degrons located within transmembrane segments [14]. In ERAD-L (lumen), proteins with luminal degrons are degraded [15]. Similar to ERAD-C, this can involve soluble luminal proteins or proteins integrated in the ER membrane. ERAD-T targets proteins that persistently engage (i.e. clog) the translocon located in the ER membrane (as described below). The primary E3 that functions in ERAD-M, ERAD-L, and ERAD-T is Hrd1 [16, 17]. Lastly, the ERAD-RA (ribosome-associated) pathway

targets proteins that have stalled within the ribosome. After ribosomal stalling, these proteins also stall in the translocon. Rkr1 (also called Ltn1) is the main E3 functioning in ERAD-RA [9]. An additional protein quality control pathway is mediated by the metalloprotease Ste24, which proteolytically cleaves a subset of translocon-clogging proteins [18].

The Translocon

Because proteins are large and polar, they cannot simply diffuse across the ER membrane. Therefore, proteins synthesized by cytosolic ribosomes must pass through the translocon into the ER where they will be folded and modified. The translocon is a channel used by the majority of endomembrane system proteins to travel into the ER lumen or membrane [17]. Proteins can be co- or post-translationally translocated.

On occasion, the translocon can become clogged [9, 10, 18], which leads to a number of problems for the cell. The clogging protein will not be able to enter the ER and will not be properly folded, modified, or localized. This clogging also prevents other proteins from entering the ER and reaching other endomembrane system components where they are needed, leading to impaired cellular function [18]. Further, it is likely that protein quality control machinery (such as molecular chaperones and ubiquitin ligases) are not able to efficiently enter the ER, potentially resulting in the accumulation of misfolded proteins in the ER.

The causes and frequency of translocon clogging are not completely understood. Several factors may promote clogging. For instance, premature folding of the cytosolic portion of a protein [18], disulfide linkage between the translocon interior and a

translocating protein [10], and translational stalling [9] have all been found to cause translocon clogging.

Translocon-Clogging Proteins

One human protein known to clog translocons is apolipoprotein B (apoB) [19, 20, 21]. ApoB utilizes the translocon en route to becoming part of a low-density lipoprotein (LDL). ApoB enters the translocon co-translationally. Before translation and translocation have terminated, the lumenally exposed N-terminus of the protein binds to lipids. When this occurs, translation and translocation complete uninterrupted, and an LDL molecule is formed. If apoB is unable to bind to lipids, its translation pauses and it stalls within the translocon; eventually, it is marked for degradation by the mammalian Hrd1 homolog gp78 [22].

There are medications currently on the market for elevated cholesterol that function by reducing lipid or apoB protein abundance. Mipomersen, for example, has been introduced for reducing apoB synthesis in patients with familial hypercholesterolemia. Mipomersen is a modified antisense oligonucleotide that forms base pairs with apoB mRNA, preventing its translation [23]. Accelerating apoB degradation has been suggested as a complementary route to therapy. Therefore, a better understanding of how cells recognize and destroy translocon-clogging proteins could lead to new therapeutic strategies.

Other than apoB, the majority of characterized translocon-clogging proteins are engineered. *Deg1*-Sec62 is an engineered transmembrane protein that clogs the translocon [10]. It is composed of *Deg1* (a soluble degron derived from the Doa10

substrate MAT α 2), Sec62 (a component of the translocon complex with two transmembrane segments), and two copies of the Protein A epitope tag (derived from *Staphylococcus aureus*). *Deg1*-Sec62 is a fusion protein that was originally created to be a membrane-bound substrate of the ERAD-C pathway, which is mediated by Doa10 [24]. However, it was found that fusion of *Deg1*-Sec62 converted the protein to a substrate that aberrantly interacts with the translocon. Following co-translational translocation of the Sec62 transmembrane segments, a portion of the N-terminal tail loops into and stalls within the translocon (**Figure 3**). During this interaction a disulfide bond forms between *Deg1*-Sec62 and Sec61, the pore subunit of the translocon. This aberrant translocon engagement causes *Deg1*-Sec62 to become a Hrd1 ERAD-T target rather than a Doa10 ERAD-C target [10].

A variant of *Deg1*-Sec62, *Deg1**-Sec62, possesses point mutations in the *Deg1* degron. Like *Deg1*-Sec62, *Deg1**-Sec62 is degraded by Hrd1 following translocon clogging [10]. *Deg1*-Sec62 and *Deg1**-Sec62 have been used interchangeably to study ERAD-T [10, 25].

Clogger is also an engineered protein [18]. It is a post-translationally translocated protein in which a portion of the protein rapidly folds before translocation is complete and produces a plug that cannot fit through the translocon. Clogger consists of, in sequence, Pdi1 (a post-translationally translocated ER-targeted protein containing five glycosylation sites), an engineered rapidly folding version of DHFR (clogging element), three additional glycosylation sites, and an HA tag (useful for western blotting). Clogger is targeted for degradation by the metalloprotease Ste24. Hrd1 also contributes substantially to Clogger degradation, consistent with a broad role for Hrd1 in the

degradation of translocon-clogging proteins (Avery Kirschbaum and Eric Rubenstein, unpublished results).

Rkr1 is the E3 involved in degradation of ribosomally stalled proteins, which persistently engage the translocon [9]. *In vivo* substrates of Rkr1 include proteins that have been aberrantly translated beyond their stop codons to the polyA tail (resulting in a series of lysine residues, each encoded by the codon AAA). To engineer proteins to stall in the ribosome (and the translocon through which the protein is moving), a stretch of lysine residues can be hard-coded into the gene sequence, immediately upstream of the stop codon. The positively charged lysine residues may cause ribosomal stalling by forming electrostatic interactions with the negatively charged ribosome exit tunnel.

ER Stress

ER stress occurs when there is an increase in the number of misfolded proteins in the ER or during lipid perturbation [26, 27, 28, 29]. Professional secretory cells have great flux through the ER and are under elevated basal levels of ER stress [30]. Cells with such a high secretory burden include B cells (which secrete antibodies) and islet cells (which secrete insulin). ER stress is also characteristic in a number of conditions such as heart disease, neurodegeneration, and cancer [21, 22, 23, 24]. It is not clear if stress is a contributing cause or consequence of these diseases. However, it is known that prolonged stress leads to cell death [31].

To study ER stress in yeast, ER stress can be introduced pharmacologically by the addition of compounds that cause proteins to misfold in the ER. Dithiothreitol (DTT) is a drug that inhibits disulfide bonds, which are formed in the ER [32]. An additional

chemical, tunicamycin (Tm), inhibits N-linked glycosylation, a post-translational modification specific to the ER [32]. Both compounds lead to soluble and integral membrane protein misfolding, resulting in ER stress. Perturbing lipid homeostasis or altering the lipid composition of the ER membrane can also cause ER stress. This can be accomplished through inositol depletion [32]. This treatment is expected to lead to integral membrane protein misfolding or misassembly. DTT, Tm, and inositol depletion all induce the unfolded protein response (UPR), described below. Translocation slows during ER stress, presumably to reduce the burden of unfolded proteins in the ER [33]. Knowing the consequences of ER stress can lead to an improved understanding of diseases associated with increased levels of ER stress.

ER Stress-Sensing Pathways

Several pathways exist that sense and reduce levels of ER stress, including the UPR, ER Surveillance (ERSU), Rapid ER Stress-Induced Export (RESET), and Stress-Induced Homeostatically Regulated Protein Degradation (SHRED). The UPR is the best-characterized pathway that is triggered during ER stress [8, 14, 32]. A bifunctional transmembrane enzyme in the ER, Ire1, can sense the accumulation of misfolded proteins in the ER. During non-stress conditions, Kar2 (a chaperone protein) is bound to the luminal portion of Ire1, keeping Ire1 inactive. When misfolded proteins accumulate, Kar2 dissociates from Ire1, and allows Ire1 to dimerize. Ire1 is a kinase that undergoes trans-autophosphorylation, where each Ire1 molecule phosphorylates the opposite Ire1 in the dimer. Phosphorylation of Ire1 activates its second enzymatic activity, in which it initiates splicing of *HAC1* mRNA. Spliced *HAC1* mRNA can be translated to the Hac1

transcription factor protein. Hac1 moves to the nucleus and associates with the unfolded protein response element (UPRE) in the promoters of genes that encode E3s and chaperone proteins to restore ER homeostasis. The green fluorescent protein (GFP) gene can be integrated downstream of the UPRE as a powerful tool to study the induction of the UPR [34]. During ER stress, the GFP gene will be transcribed and translated into a protein that fluoresces and can be detected through various lab techniques such as flow cytometry and western blotting.

The ERSU pathway, mediated by the Slt2 MAP kinase, prevents the inheritance of stressed ER containing misfolded proteins to daughter cells [35]. This is a signal transduction pathway initiated by the cell wall protein Wsc1 and culminating in activation of Slt2. The activating signal for Wsc1 and the substrate(s) for Slt2 are not known. In the SHRED pathway, the Ubr1 ubiquitin ligase is activated by a variety of conditions that cause cell stress (e.g. tunicamycin and DTT) [36]. Once activated, Ubr1 ubiquitylates misfolded proteins to target them for degradation. Lastly, in the RESET pathway, misfolded glycosylphosphatidylinositol (GPI)-anchored ER-localized proteins are trafficked to the lysosome via the secretory pathway [37]. Export from the ER occurs via the COPII pathway and requires a p24 export factor. The UPR, ERSU, and SHRED pathways have been characterized in yeast and are conserved in mammalian cells. The RESET pathway has been characterized in mammalian cells and is predicted to be mediated by the p24 protein Emp24 in yeast.

ER Stress and ERAD-T

The overall goal of this project is to study the relationship between ER stress and degradation of translocon-clogging proteins. Other work [45] has shown that ER stress preferentially impairs specific ERAD pathways. ER stress impairs Hrd1-mediated ERAD-T and ERAD-L. However, Hrd1-mediated ERAD-M is unaffected indicating that ER stress does not directly impair Hrd1 function. Impairment of ERAD-L by ER stress has been previously reported. ER stress also impairs Ste24-mediated cleavage of Clogger. Lastly, ER stress does not affect Doa10-dependent degradation (ERAD-C) or Rkr1-mediated degradation (ERAD-RA). It was unexpected that ER stress would inhibit Hrd1-mediated degradation of luminal and translocon-clogging proteins, since Hrd1 is induced by the UPR and Hrd1 catalytic function is not directly impaired by ER stress. Further, the observation that ER stress impairs turnover of translocon-clogging substrates of two different enzymes suggests ER stress may broadly impair degradation of translocon-clogging substrates regardless of enzyme specificity.

How does ER stress inhibit Hrd1-dependent degradation of translocon-clogging proteins? ER stress does not inhibit Hrd1 function directly, since degradation of some Hrd1-targeted proteins (such as ERAD-M substrates) is unaffected by ER stress. While ER stress impairs modification of translocon-clogging proteins [45] this is likely not the reason for impaired degradation, since it has been shown modifications are not required for degradation [10, 25]. Inhibition of ERAD-L by ER stress is likely to occur by saturation of the multifunctional ER chaperone Kar2 [7]; Kar2 overexpression partially rescues impaired ERAD-L during conditions associated with elevated ER stress [38]. However, Kar2 overexpression does not restore ERAD-T during ER stress [45]. **We**

hypothesize that one of several characterized ER stress response mechanisms (UPR, ERSU, SHRED, or RESET) specifically inhibits ERAD-T during ER stress.

Expression of Clogger is shown to induce ER stress. This stress may be due to reduced ER import of enzymes required for protein quality control when the translocon is clogged [18]. This suggests a reciprocal relationship between translocon clogging proteins and ER stress.

It is not known if ER stress induction is unique to Clogger. **We hypothesize that translocon-clogging proteins broadly induce ER stress.** This can be tested by determining if overexpressing other translocon-clogging proteins (such as *Degl-Sec62*) induces ER stress.

Other Forms of Cellular Stress and ERAD-T

Is ERAD-T inhibition unique to ER stress caused by misfolded proteins? Cells may be subjected to a range of different forms of stress that disrupt ER homeostasis or cellular proteostasis. This includes ER stress caused by membrane perturbation, oxidative stress, temperature stress, and nutrient limitation or overabundance. Membrane perturbation alters lipid composition and activates the UPR without misfolding proteins in the ER lumen [36]. Oxidative and temperature stress are predicated to broadly increase protein misfolding, not just in the ER [39]. Nutrient limitation or overabundance activates a diverse set of stress responses [40, 41]. **We hypothesize ERAD-T inhibition occurs in response to a range of cellular stresses.**

Cycloheximide Chase Analysis

Protein abundance is a reflection of the rates of protein synthesis and degradation. Therefore, analyzing the steady state abundance (i.e. abundance at a single time point) of a protein by western blot is not sufficient to study protein degradation, because the method cannot discriminate between altered synthesis and degradation. To monitor protein degradation over time, cycloheximide chase followed by western blot analysis may be used [42]. Cycloheximide inhibits eukaryotic cytosolic translation. Samples are harvested at specific times and abundance of a protein of interest may be monitored (e.g. by western blotting) following cycloheximide addition. Because cycloheximide is toxic to cells, this method is suitable for relatively short time periods (less than three hours).

Our objective was to contribute to the ER stress field by dissecting the relationship between ER stress and degradation of translocon-clogging proteins. To do so, we specifically investigated the roles of ER stress-sensing pathways in translocon-quality control, the effects of high-level expression of translocon-clogging substrates on ER stress, and the impacts of various forms of stress on translocon quality control. The goal of the first aim was to determine if characterized ER stress-sensing pathways are required for impairment of ERAD-T observed during ER stress. Degradation of an ERAD-T substrate was monitored in stressed and non-stressed conditions under the impairment of the four characterized pathways. The goal of the second aim was to determine if abundance of a second characterized translocon-clogging protein, *Deg1*-Sec62, induces the UPR similar to Clogger. This would be consistent with a model in which translocon clogging causes ER stress generally. We overexpressed the *Deg1*-Sec62 protein and analyzed the abundance of a UPR reporter by flow cytometry. The goal of the third aim was to explore the impact of different forms of stress including ER membrane

perturbation, oxidative stress, elevated temperature, glucose limitation, and glucose overload on ERAD-T. This showed if ERAD-T impairment is specific to ER stress caused by protein misfolding (e.g. following DTT or tunicamycin treatment) or if impairment occurs during a range of stress conditions. This work aimed to provide insights into how eukaryotic cells respond to ER stress, and how conserved translocon quality control pathways are affected by ER stress.

HYPOTHESES

1. One or more of the characterized ER stress-sensing pathways are required for impairment of ERAD-T during ER stress.
2. Elevated abundance of an ERAD-T substrate induces the unfolded protein response (UPR).
3. ER membrane perturbation, oxidative stress, elevated temperature, glucose limitation, and glucose overload impair ERAD-T.

SPECIFIC AIMS

1. Are characterized ER stress-sensing pathways (UPR, ERSU, RESET, or SHRED) required for impairment of ERAD-T during ER stress?
2. Does elevated abundance of an ERAD-T substrate induce the unfolded protein response (UPR)?
3. Do different forms of stress (ER membrane perturbation, oxidative stress, elevated temperature, glucose limitation or glucose overload) impair ERAD-T?

MATERIALS AND METHODS

Plasmid miniprep

The QIAprep Spin Miniprep kit was performed following manufacturer's instructions to isolate plasmid DNA from bacterial cells.

Yeast Growth Conditions

For non-selective growth of yeast cells, yeast extract-peptone-dextrose (YPD) medium was used (1% Bacto yeast extract, 2% Bacto peptone, 2% glucose, 0.002% adenine). For plasmid-selective growth of yeast cells, Synthetic Defined (SD) medium was used (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). To maintain plasmid selection, a nutrient (i.e. uracil, histidine, tryptophan, or leucine) that can only be produced if a plasmid-borne gene product is synthesized was omitted from the media. All experiments were conducted with mid-logarithmic culture yeast cells. For cells subjected to specific stress conditions, cells were cultured to mid-logarithmic growth prior to introduction of specified stress.

Lithium Acetate Yeast Transformation

Yeast were grown overnight in YPD media at 30°C. 500 µL of culture was centrifuged at 5,000 rpm for 2 minutes at room temperature. Pellets were resuspended in 1 mL of sterile water, and centrifugation was repeated. Pellets were then resuspended in 50 µL of sterile 1X LTE (0.1 M lithium acetate, 10 mM tris-Cl pH 7.5, 1 mM EDTA). 20 µg of carrier

DNA (denatured salmon sperm DNA) was added to each transformation with 1 μL of plasmid miniprep (or 1 μL of sterile water for negative controls). Transformations were mixed by vortexing and incubated at room temperature for 30 minutes. After incubation, 400 μL of 40% PEG in 1X LTE was added to the transformations, and the samples were mixed by vortexing. The transformations were then incubated at room temperature overnight. Transformations were centrifuged at 5,000 rpm for 2 minutes. Pellets were resuspended in 50 μL of 1 M sorbitol. Suspensions were spread onto selective plates and incubated for 3-5 days at 30°C.

Cycloheximide Chase, Cell Harvest, and Lysis

Transformed strains were inoculated into appropriate SD selective media and incubated at 30°C rotating overnight. The optical density was measured, and the overnight cultures were diluted to an OD_{600} of 0.2 in fresh media. The samples were incubated rotating at 30°C. When cells reached mid-logarithmic growth (i.e. when OD_{600} reached ~ 0.8 -1.2), 2.5 OD_{600} units per time point to be assayed were harvested. Cells were centrifuged at room temperature at 3,000 x g for 2 minutes. Pellets were resuspended in 1 mL pre-warmed media per 2.5 OD_{600} units. Samples were incubated in a 30°C heat block for 5 minutes (and the duration of the experiment). Cycloheximide was added to each sample to a final concentration of 250 $\mu\text{g/mL}$. At each time point (0, 30, 60 minutes), 950 μL was harvested and added to a tube containing 50 μL of 20X stop mix (1X stop mix contains 200 mM sodium azide and 5 mg/mL BSA). Samples in stop mix were kept on ice until the end of the chase. Once all samples were collected, tubes were centrifuged at 6,500 x g at room temperature for 30 seconds and supernatant was aspirated. Cells were lysed using

the post-alkaline lysis method [36].

For cycloheximide chase experiments in which samples were subjected to the presence or absence of stress-inducing conditions, overnight cultures were diluted to an OD₆₀₀ value of 0.2 in fresh appropriate media and incubated at 30°C shaking until mid-logarithmic growth was reached. Cultures were split into appropriate treatment groups. Cultures were incubated in the presence or absence of treatments at 30°C for specified treatment time. At the end of treatment, 2.5 OD₆₀₀ units per time point to be assayed were collected for each culture, and the cycloheximide chase was performed as above. The presence or absence of treatment was maintained until the point of transferring cells to 20X stop mix.

Western Blot Analysis

Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a 10% polyacrylamide gel and transferred to polyvinylidene difluoride (PVDF) membrane by wet transfer at 20 V for 60 minutes at 4°C. *DegI*-Sec62 possesses two tandem C-terminal Protein A tags (derived from *Staphylococcus aureus*), which bind non-specifically to antibodies. Membranes were blocked by incubation in 5% skim milk in tris-buffered saline (TBS; 0.05 M Tris-base, 0.15 M NaCl) with 0.1% (v/v) Tween 20 (TBS/T) for one 1 hour. To detect *DegI*-Sec62, membranes were probed with polyclonal secondary (Rabbit anti-Mouse IgG (H+L) Cross-Absorbed, Alexa Fluro 680) antibody (Fischer Scientific, A-21065, dilution 1:40,000) for 1 hour in 1% skim milk with TBS/T. Membranes were washed three times for five minutes in TBS/T to remove unbound antibody. To detect GFP, membranes were probed with anti-GFP (Living Colors A.v. monoclonal antibody (JL-8) against GFP) antibody (Clontech, 632381,

dilution 1:1000) for 1 hour in 1% skim milk with TBS/T. Membranes were washed, probed with secondary antibody, and washed according to the above steps. To detect the loading control protein Pgk1, membranes were probed with anti-Pgk1 (Phosphoglycerate Kinase Monoclonal antibody, mouse (clone 22C5D8)) antibody (Life Technologies, 459250, dilution 1:20,000) for 1 hour in 1% skim milk with TBS/T. Membranes were washed, probed with secondary antibody, and washed according to the steps above. Membranes were imaged with Li-Cor Odyssey imaging software.

Flow Cytometry

Flow cytometry was performed using cells expressing the indicated GFP-tagged protein. Cells were cultured until they reached mid-logarithmic growth and were then subjected to experimental treatments as indicated. Mean GFP fluorescence of 10,000 cells in synthetic-defined medium was measured using the MACSquant Analyzer X. A two-tailed unpaired *t*-test was used to determine significance.

RESULTS

UPR and ERSU are not required for ERAD-T or its impairment by ER stress

Cycloheximide chase analysis was performed to determine if the UPR or ERSU pathways are required for stabilization of *Deg1*^{*}-Sec62 during ER stress (**Figure 4**). Wild type, *ire1Δ* (i.e. UPR-defective), and *slt2Δ* (i.e. ERSU-defective) yeast expressing *Deg1*^{*}-Sec62 were cultured in the presence or absence of 6 mM DTT for one hour followed by cycloheximide chase and western blot analysis. The rate of degradation of *Deg1*^{*}-Sec62 in the *ire1Δ* and the *slt2Δ* strains was compared to the degradation rate in the wild type strain. *Deg1*^{*}-Sec62 was rapidly degraded in wild type cells. Degradation was strongly impaired by treatment with DTT. Deletion of *IRE1* and *SLT2* did not substantially stabilize *Deg1*^{*}-Sec62 during non-stress conditions or restore degradation under ER stress, indicating that neither pathway is required for ERAD-T or its impairment by ER stress.

SHRED is not required for ERAD-T or its impairment by ER stress

Cycloheximide chase analysis was performed to determine if the SHRED pathway is required for stabilization of *Deg1*^{*}-Sec62 during ER stress (**Figure 5**). Wild type and *ubr1Δ* (i.e. SHRED-defective) yeast expressing *Deg1*^{*}-Sec62 were cultured in the presence or absence of 6 mM DTT for one hour followed by cycloheximide chase and western blot analysis. The rate of degradation of *Deg1*^{*}-Sec62 in the *ubr1Δ* strain was compared to the degradation rate in the wild type strain. *Deg1*^{*}-Sec62 was rapidly degraded in wild type cells. Degradation was strongly impaired by treatment with DTT. Deletion of *UBR1* did not stabilize *Deg1*^{*}-Sec62 during non-stress conditions or restore

degradation under ER stress, indicating that the pathway is not required for ERAD-T or its impairment by ER stress.

RESET is not required for ERAD-T or its impairment by ER stress

Cycloheximide chase analysis was performed to determine if the RESET pathway is required for stabilization of *Deg1*^{*}-Sec62 during ER stress (**Figure 6**). Wild type and *emp24Δ* (i.e. predicted to be RESET-defective) yeast expressing *Deg1*^{*}-Sec62 were cultured in the presence or absence of 6mM DTT for one hour followed by cycloheximide chase and western blot analysis. The rate of degradation of *Deg1*^{*}-Sec62 in the *emp24Δ* strain was compared to the degradation rate in the wild type strain. *Deg1*^{*}-Sec62 was rapidly degraded in wild type cells. Degradation was strongly impaired by treatment with DTT. Deletion of *EMP24* did not stabilize *Deg1*^{*}-Sec62 or restore degradation under ER stress, indicating that the pathway is not required for ERAD-T or its impairment by ER stress.

*Deg1-Sec62 induces the UPR in *ubc7Δ* cells*

Flow cytometry was performed to determine if ER stress (as indicated by the UPR reporter GFP driven by the unfolded protein response element, or UPRE) is induced by expression of *Deg1*-Sec62. Wild type and *ubc7Δ* yeast expressing an empty vector or *Deg1*-Sec62 were incubated in the absence (vector and *Deg1*-Sec62) or presence (vector) of 6 mM DTT followed by flow cytometry analysis (**Figure 7**). DTT treatment caused a robust (5-7 fold) induction of the UPR in wild type and *ubc7Δ* cells. *Deg1*-Sec62 expression resulted in a modest ~2-fold induction of the UPR in *ubc7Δ* cells.

Inositol limitation does not impair ERAD-T

Cycloheximide chase analysis was performed to determine if inositol limitation (which induces the UPR) impairs degradation of *Deg1*^{*}-Sec62 similar to other forms of ER stress (**Figure 8**). Mid-logarithmic-phase wild type yeast expressing *Deg1*^{*}-Sec62 were cultured in standard growth media, media containing 6mM DTT for one hour, or media lacking inositol for five hours, followed by cycloheximide chase and western blot analysis. In the absence of stress, *Deg1*^{*}-Sec62 was rapidly degraded. In the presence of DTT, *Deg1*^{*}-Sec62 was strongly stabilized. Inositol limitation did not stabilize *Deg1*^{*}-Sec62, indicating that ER stress caused by membrane perturbation does not impair ERAD-T.

Cells were co-transformed with a plasmid encoding GFP under the control of the UPRE as a control for ER stress induction. GFP abundance increased in the DTT supplementation and inositol limitation treatment groups. This confirms that the UPR was induced and that ER stress occurred during these treatments.

Heat shock does not impair ERAD-T

Cycloheximide chase analysis was performed to determine if heat shock (which is predicted to globally disrupt protein folding) impairs degradation of *Deg1*-Sec62 (**Figure 9**). Mid-logarithmic-phase wild type and *hrd1Δ* yeast expressing *Deg1*-Sec62 were cultured at 30°C (preferred growth temperature) or 42°C (heat shock conditions) for one hour, followed by cycloheximide chase and western blot analysis. *Deg1*-Sec62 was rapidly degraded in cells cultured at 30°C. Heat shock did not stabilize *Deg1*-Sec62, indicating that ERAD-T is not sensitive to acute heat shock.

Oxidative stress does not impair ERAD-T

Cycloheximide chase analysis was performed to determine if oxidative stress (which is predicted to globally damage protein molecules) impairs degradation of *Deg1*^{*}-Sec62. Mid-logarithmic-phase wild type yeast expressing *Deg1*^{*}-Sec62 were cultured in the presence or absence of 0.4 mM hydrogen peroxide (H₂O₂) for one hour, followed by cycloheximide chase and western blot analysis (**Figure 10A**). *Deg1*^{*}-Sec62 was rapidly degraded in the absence of oxidative stress. Oxidative stress did not result in stabilization of *Deg1*^{*}-Sec62, further indicating that conditions associated with globally disrupted proteostasis do not impair ERAD-T. To confirm oxidative stress treatment was successful, parallel cultures of yeast expressing Rtc3-GFP (which is induced following oxidative stress [39]) was subjected to flow cytometry analysis. As expected, fluorescence increased significantly following H₂O₂ treatment, confirming H₂O₂ activity (**Figure 10B**).

Neither glucose limitation nor overload impairs ERAD-T

Cycloheximide chase analysis was performed to determine if glucose limitation or overload impairs degradation of *Deg1*^{*}-Sec62. Mid-logarithmic-phase wild type yeast expressing *Deg1*^{*}-Sec62 were cultured in 0.05%, 2%, or 8% glucose for two hours, followed by cycloheximide chase and western blot analysis (**Figure 11A**). *Deg1*^{*}-Sec62 was rapidly degraded in glucose limitation (0.05%) and glucose overload (8%). Neither glucose limitation or overload resulted in stabilization of *Deg1*^{*}-Sec62. To confirm glucose stress treatments were successful, parallel cultures of yeast expressing Adh2-GFP (the expression of which is inversely correlated to glucose abundance [44]) was subjected

to flow cytometry analysis. As expected, fluorescence intensity decreased with increasing glucose concentrations (**Figure 11B**).

DISCUSSION

ER stress is known to increase abundance of Hrd1 ERAD machinery and degradation of Hrd1 ERAD-M substrates proceeds unperturbed during ER stress [10, 45]. One might expect degradation of an ERAD-T substrate like *Deg1*-Sec62 to rapidly increase. The initial observation that ERAD-T is impaired during ER stress [45] was therefore surprising.

We tested whether known ER stress-sensing mechanisms (UPR, ERSU, SHRED, and RESET) are required for ERAD-T or its impairment during ER stress. The results show that none of the characterized ER stress-sensing pathways tested were required for ERAD-T or its impairment by ER stress. This suggests the existence of a novel ER stress-response mechanism that impairs ERAD-T. We also determined that elevated abundance of an ERAD-T substrate induces ER stress in yeast lacking the ubiquitin-conjugating enzyme Ubc7, suggesting translocon clogging may generally induce mild ER stress. Finally, we examined the impact of different forms of stress at the ER membrane. We compared the effects of ER stress caused by protein misfolding to ER stress caused by lipid perturbation on ERAD-T using the translocon-clogging substrate *Deg1*^{*}-Sec62. We observed that ER stress caused by protein misfolding impaired ERAD-T. Conversely, ERAD-T was unaffected by lipid perturbation. Next we compared the effects of different forms of stress (oxidative stress, heat shock, nutrient limitation, and glucose overload) on ERAD-T. We observed that these different forms of stress did not impair degradation of *Deg1*(^{*})-Sec62.

How does ER stress impair ERAD-T?

It remains unclear how ER stress impairs ERAD-T. We hypothesized that one of several characterized ER stress-sensing pathways is required for impairment of ERAD-T during ER stress. However, our results show that impairment of *Deg1*^{*}-Sec62 degradation during ER stress does not require the characterized ER stress-sensing pathways UPR, ERSU, RESET, and SHRED, unless they function redundantly. To test if these pathways function redundantly in impairing *Deg1*(^{*})-Sec62 degradation during stress would require production of a series of mutant yeast strains defective in two, three, or all four of these pathways followed by assessment of ERAD-T during stress and non-stress conditions.

Deg1-Sec62 becomes extensively modified following translocon engagement. Treatment with the glycosylation inhibitor Tm prevents all modifications detectable by SDS-PAGE [45], and DTT partially impairs glycosylation. Since impaired modification of *Deg1*-Sec62 correlates with impaired degradation during ER stress, one plausible explanation for impaired *Deg1*-Sec62 degradation is compromised translocon engagement of *Deg1*-Sec62 during stress. If ER stress prevents translocon-clogging, the protein would no longer be targeted for degradation by Hrd1. However, results show that *Deg1*-Sec62 is N-glycosylated in the presence of DTT (albeit more slowly than in the absence of DTT), which requires translocation of the clogging portion of *Deg1*-Sec62 into the ER lumen [45]. In addition, mutations that prevent aberrant translocon engagement have been shown to cause a reversion of *Deg1*-Sec62 degradation from Hrd1 to Doa10 dependency for degradation [12]. Therefore, if ER stress prevented translocon engagement by *Deg1*-Sec62, the protein would be expected to be targeted by Doa10 and degraded quickly, even during ER stress. This is not observed. This shows that lack of

aberrant translocon engagement does not explain impaired *Deg1*-Sec62 degradation during ER stress.

Another possibility is that ER stress prevents one or more post-translational modifications (PTMs) required for recognition by Hrd1. *Deg1*-Sec62 modification is strongly impaired by tunicamycin and DTT. Glycosylation is also required for degradation of a subset of Hrd1 substrates [10]. It has been shown however, that neither glycosylation nor acetylation are needed for *Deg1*-Sec62 degradation [10, 25]. N-glycosylation sites were mutated (N90D and N153D) in cells expressing *Deg1*-Sec62. These mutations did not affect Hrd1-mediated degradation of *Deg1*-Sec62 suggesting that glycosylation was not required for degradation [10]. Degradation of variants of *Deg1**-Sec62 with mutated acetylation sites showed that acetylation was also not required for Hrd1-dependent degradation of *Deg1**-Sec62 [25]. There may be uncharacterized PTMs that contribute to *Deg1*-Sec62 degradation, but there is no evidence to suggest so.

Direct inhibition of Hrd1 function during ER stress is unlikely since degradation of a subset of Hrd1 substrates remains unperturbed by ER stress. ERAD-M, which is one ERAD pathway targeted by the E3 ligase Hrd1, is unaffected by ER stress. On the contrary, degradation of ERAD-L substrates also targeted by Hrd1 is sensitive to ER stress. Similarly, degradation of the translocon-clogging protein Clogger, which is targeted by Ste24 and Hrd1, is also inhibited during ER stress. ERAD-C, which requires the E3 ligase Doa10, is not sensitive to ER stress. Overall this suggests that ERAD and ERAD-like pathways are differentially affected by ER stress. However, since ERAD-M (which requires Hrd1) is unaffected by ER stress, Hrd1 function is not directly impaired.

We speculate that a novel mechanism is involved in impairment of *Deg1*-Sec62

degradation during ER stress. We hypothesize that one or more proteins sense ER stress and specifically inhibit Hrd1-dependent degradation of translocon-associated proteins. Ongoing efforts in the Rubenstein lab are directed toward identifying proteins that may be involved in ERAD-T. (Christopher Indovina, Samantha Turk, Danielle Overton, Cade Orchard, Julia Niekamp, Kyle Richards, Courtney Broshar, and Eric Rubenstein, unpublished results). A genome-wide screen recently revealed potential roles in ERAD-T for proteins with a variety of functions. Biochemical function of these proteins in ERAD-T is currently being validated by cycloheximide chase and western blot experiments. Such proteins may exhibit stress-sensitive function in ERAD-T. Future experiments will determine if expression or function of genes found from the genome-wide screen are regulated by ER stress.

“Why” does ER stress impair ERAD-T?

During ER stress, proteostasis machinery is burdened. Translocon function is impaired during ER stress, as shown by slowed translocation of a subset of proteins [33]. This slowed translocation might be a protective mechanism to reduce the burden of this machinery. There are two characterized mechanisms by which protein import into the ER is slowed during stress. The first is reduced chaperone availability. The essential ER luminal molecular chaperone Kar2 performs multiple roles, including assisting protein folding, delivering terminally misfolded substrates to the ERAD machinery for degradation, and promoting translocation of proteins into the ER [7, 38, 45]. During stress, the Kar2 chaperone is saturated by misfolded proteins already in the lumen and is titrated away from the translocon. The second characterized mechanism is through ER

stress-induced pre-emptive quality control (ERpQC), which has been demonstrated in mammalian cells [45]. There is increased association of HRD1 with the translocon during ER stress. This is believed to preemptively promote degradation of secretory proteins before translocation. Our results suggest a third mechanism by which protein import may be slowed: undegraded channel-engaged proteins might hinder translocation of other proteins into an already stressed ER. We therefore speculate that specific impairment of translocon-clogging protein degradation is protective during ER stress.

Specificity of ER stress in ERAD-T impairment

ER stress caused by misfolded proteins impaired ERAD-T. Thus, we hypothesized that several different forms of stress associated with impaired general proteostasis or ER homeostasis would also impair ERAD-T, but the opposite was discovered. The fact that inositol limitation, glucose limitation, and glucose overload did not impair ERAD-T indicates that impaired degradation may be specifically stimulated by misfolded proteins in the ER. Failure of oxidative stress and heat shock to impair ERAD-T suggests that impaired degradation is not due to global protein misfolding or general disruption of ER homeostasis. Therefore, the signal regulating ERAD-T is likely a specific increase in the abundance of misfolded proteins in the ER.

Induction of UPR by translocon-clogging proteins

The engineered protein Clogger clogs the translocon in a manner that is distinct from the way in which *Deg1*-Sec62 clogs the translocon [10, 18]. Clogger is targeted for degradation primarily by the metalloprotease Ste24, but Hrd1 has also been shown to

contribute (Avery Kirschbaum and Eric Rubenstein, unpublished results). Interestingly, degradation of Clogger is also impaired during ER stress similarly to *Deg1*-Sec62 [45]. Overexpression of Clogger was shown to mildly induce the UPR ER stress-sensing pathway [18]. We wished to test if UPR induction was specific to Clogger or if clogging proteins generally had this effect. We found that both *Deg1*-Sec62 and Clogger overexpression modestly induce the UPR. Therefore, overexpression of two distinct translocon-clogging proteins induces the UPR pathway.

It should be noted that ER stress induction by *Deg1*-Sec62 only occurred in *ubc7Δ* cells. It is likely we saw no effect in wild type cells because the ERAD machinery was fully functional to target *Deg1*-Sec62 for degradation even during overexpression. By contrast, in *ubc7Δ* cells, the ERAD machinery is impaired. On its own, loss of *UBC7* mildly induces UPR, consistent with previous reports [18]. Overexpression of the translocon-clogging substrate *Deg1*-Sec62 in these cells more robustly induces ER stress and the UPR.

Induction of the UPR by translocon-clogging proteins appears to be contrary to the idea that persistent translocon engagement protects against ER stress by reducing the amount of normal proteins translocating inward. However, one model is that translocon clogging may increase stress during basal (non-stress) conditions by preventing the passage of proteostasis machinery through the translocon [45]. By contrast, during stress, the luminal burden of unfolded proteins may be reduced by non-specifically stemming ER import.

Artificial substrates and artificial stress?

It will be important to identify endogenous translocon-clogging substrates in yeast. Although *Deg1*-Sec62 and Clogger have been used as model translocon-clogging proteins, they are engineered and therefore not found naturally in yeast or other eukaryotes. The only characterized physiological translocon-clogging protein is mammalian apoB [19, 20, 21, 22], which is toxic to yeast (Eric Rubenstein, unpublished results). It could be argued the stress-regulated degradation of these artificial proteins may not accurately model a physiologically relevant process. However, degradation of these proteins following clogging is strong evidence for the existence of such quality control mechanisms operating under physiological conditions. Further, engineered proteins have long served as model substrates for a host of protein quality control mechanisms, leading to insights later found to be applicable to physiological substrates [14, 45]. Nonetheless, it will be important to identify naturally occurring translocon-clogging proteins in yeast to enhance and generalize our biological understanding of translocon quality control under physiological conditions. Further, identification of native translocon-clogging proteins will allow us to search for features that make proteins more likely to clog the translocon. Identifying these native proteins will require isolating the translocon from *hrd1Δ* cells where degradation is impaired. Proteins found engaged with the translocon (i.e. potential translocon-clogging proteins) of these cells can then be identified using mass spectrometry.

Some may question the applicability of using DTT and tunicamycin to study the effects of ER stress. The benefits of using these treatments are that they are convenient to administer and they have the ability to induce potent ER stress. Their mechanisms of action are also known and well characterized. However, DTT and tunicamycin do not

precisely mimic ER stress under physiological conditions. Because of this, accumulation of misfolded proteins caused by other means in eukaryotic cells may or may not have similar effects as DTT or tunicamycin. However, one study showed that expression of polyQ-expanded huntingtin protein in yeast both induced ER stress and increased *Deg1*-*Sec62* abundance, suggesting that accumulation of a misfolded protein in yeast has a similar effect as DTT and tunicamycin, supporting the use of these chemicals to model ER stress under physiological conditions [46].

Concluding thoughts

ER stress is present in certain cell types such as professional secretory cells [30]. ER stress is also characteristic of a number of conditions including heart disease, neurodegeneration, and cancer [21, 22, 23, 24]. It is not understood if stress is a contributing cause or consequence of these diseases. It will be important to learn if impaired translocon quality control is found in these cells, and if this is protective or detrimental to these cells. Pharmacological interventions that modulate translocation in cells under perpetual ER stress may be helpful in alleviating symptoms of diseases associated with ER stress. Understanding how the cell responds to ER stress may inform the treatment of such diseases associated with stress.

TABLES

Table 1: Plasmids used for these experiments.

Plasmid Number	Plasmid Name	Description	Yeast Selection Marker	Bacterial Selection Marker
pVJ26	pRS313	Empty vector	<i>HIS3</i>	ampR
pVJ27	pRS316	Empty vector	<i>URA3</i>	ampR
pVJ122	p415- <i>P_{MET25}</i>	Empty vector	<i>LEU2</i>	ampR
pVJ317	p416- <i>P_{MET25}</i> - <i>Deg1</i> *-Sec62-2XProtA	<i>Deg1</i> *-Sec62 driven by <i>MET25</i> promoter; <i>Deg1</i> * = F18S, I22T	<i>URA3</i>	ampR
pVJ411	p415- <i>P_{MET25}</i> - <i>Deg1</i> *-Sec62-2XProtA	<i>Deg1</i> *-Sec62 driven by <i>MET25</i> promoter; <i>Deg1</i> * = F18S, I22T	<i>LEU2</i>	ampR
pVJ463	p413- <i>P_{GDP}</i> - <i>Deg1</i> *-Sec62-2XProtA	<i>Deg1</i> -Sec62 driven by <i>TDH3</i> (<i>GPD</i>) promoter	<i>HIS3</i>	ampR
pVJ552	pRS314-UPRE-GFP	GFP driven by unfolded Protein Response Element (UPRE)	<i>TRP1</i>	ampR

Table 2: Yeast strains used for these experiments.

Yeast Strain	Genotype	Background
VJY6	<i>MATa his3-Δ200 leu2-3,112 ura3-52 lys2-801 trp1-1 gal2</i>	MHY500
VJY38	<i>MATa his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 slt2Δ::kanMX4</i>	BY4741
VJY50	<i>MATa his3-Δ200 leu2-3,112 ura3-52 lys2-801 trp1-1 ubc7::LEU2</i>	MHY550
VJY173	<i>MATa his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 ire1Δ::kanMX4</i>	BY4741
VJY380	<i>MATa leu2-3,112 his3-11, 15 trp1-1 ura3-1::URA3-UPRE-GFP* ade2-1 can1-100</i>	W303
VJY476	<i>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0</i>	BY4741
VJY469	<i>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 ubr1Δ::kanMX4</i>	BY4741
VJY511	<i>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 hrd1Δ::kanMX4</i>	BY4741
VJY536	<i>MATa ADE2 leu2-3,112 trp1-1 ura3-1 his3-11,15</i>	W303
VJY538	<i>MATa ADE2 leu2-3,112 trp1-1 ura3-1 his3-11,15 hrd1::HIS3 doa10::TRP1</i>	W303
VJY539	<i>MATa ADE2 leu2-3,112 trp1-1 ura3-1 his3-11,15 hrd1::HIS3 doa10::TRP1 ubr1::nat</i>	W303
VJY540	<i>MATa ADE2 leu2-3,112 trp1-1 ura3-1 his3-11,15 ubr1::HIS3</i>	W303
VJY616	<i>MATa his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 RTC3-GFP:his5Sp</i>	BY4741
VJY620	<i>MATa his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 emp24Δ::kanMX4</i>	BY4741
VJY731	<i>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 ADH2-GFP: HIS5sp</i>	BY4741

FIGURES

Figure 1: Overview of the UPS. An E1 transfers a ubiquitin molecule (purple circle) to an E2, which associates with the E3. The E3 then transfers the ubiquitin molecule from the E2 to the target protein. Once the polyubiquitin chain is formed, the proteasome will target the protein for degradation.

Figure 2: ER quality control pathways. Ste24-mediated degradation, ERAD-T, ERAD-M, ERAD-C, and ERAD-RA, are distinct branches of protein quality control at the ER membrane. Yellow star, degron. Purple circle, ubiquitin.

Figure 3: *Deg1*-Sec62 before and after translocon clogging. *Deg1*-Sec62 is an engineered transmembrane protein. The two transmembrane-segments of Sec62 are co-translationally inserted into the ER membrane. Subsequently, a segment post-translationally loops into the translocon causing it to become clogged. Two copies of the Protein A tag are present at the C-terminus of *Deg1*-Sec62 to facilitate detection (not pictured).

Figure 4: The UPR and ERSU ER stress-sensing pathways do not regulate ERAD-T in the presence or absence of ER stress. Cycloheximide chase analysis of yeast of the indicated genotypes expressing *Deg1^{*}*-Sec62 or harboring an empty vector (Vec) cultured in the presence or absence of 6 mM DTT. DTT was maintained at the same concentration during incubation with cycloheximide. Pgk1 served as a loading control. The percentage of *Deg1^{*}*-Sec62 remaining at each time point (normalized to Pgk1) is indicated below the image.

Figure 5: The SHRED ER stress-sensing pathway does not regulate ERAD-T in the presence or absence of ER stress. Cycloheximide chase analysis of yeast of the indicated genotypes expressing *Deg1*^{*}-Sec62 or harboring an empty vector (Vec) cultured in the presence or absence of 6 mM DTT. DTT was maintained at the same concentration during incubation with cycloheximide. Pgk1 served as a loading control. The percentage of *Deg1*^{*}-Sec62 remaining at each time point (normalized to Pgk1) is indicated below the image.

Figure 6: The RESET ER stress-sensing pathway does not regulate ERAD-T in the presence or absence of ER stress. Cycloheximide chase analysis of yeast of the indicated genotypes expressing *Deg1^{*}*-Sec62 or harboring an empty vector (Vec) cultured in the presence or absence of 6 mM DTT. DTT was maintained at the same concentration during incubation with cycloheximide. Pgk1 served as a loading control. The percentage of *Deg1^{*}*-Sec62 remaining at each time point (normalized to Pgk1) is indicated below the image.

Figure 7: *Deg1*-Sec62 induces the UPR. Abundance of the UPR reporter GFP (driven by the UPRE) was analyzed by flow cytometry in WT and *ubc7Δ* yeast. Mid-logarithmic-phase cells harboring an empty vector or a plasmid encoding *Deg1*-Sec62 under the control of the GPD promoter were incubated in the absence (vector and *Deg1*-Sec62) or presence (vector) of 6 mM DTT for 1 h. The mean fluorescence intensity of 10,000 cells from each of nine cultures for each condition was normalized to the average mean fluorescence intensity of nine cultures of untreated WT cells harboring a vector. Mean fluorescence intensity $\pm$ standard error of the mean is presented. A two-tailed unpaired *t*-test was performed to determine the significance of the difference between *ubc7Δ* cells harboring an empty vector and those expressing *Deg1*-Sec62.

Figure 8: ERAD-T is not sensitive to inositol limitation. Cycloheximide chase analysis of wild type yeast harboring an empty vector (Vec) or expressing *Deg1**-Sec62 cultured in inositol-rich medium in the presence or absence of 6 mM DTT for 1 h or shifted to inositol-free medium for 5 h. DTT concentration and inositol abundance were maintained during incubation with cycloheximide. Pgk1 served as a loading control. The percentage of *Deg1**-Sec62 remaining at each time point (normalized to Pgk1) is indicated below the image.

Figure 9: ERAD-T is not sensitive to heat shock. Cycloheximide chase analysis of wild type yeast expressing *Deg1-Sec62* cultured at 30°C or shifted to 42°C for 1 h. Temperatures were maintained during incubation with cycloheximide. *Pgk1* served as a loading control. This experiment was performed in collaboration with Brian Snow.

Figure 10: ERAD-T is not sensitive to oxidative stress. (A) Cycloheximide chase analysis of wild type yeast harboring an empty vector or expressing *Deg1*-Sec62* in the presence of 0.4 mM hydrogen peroxide (H₂O₂), or no treatment for 1 h. Pgk1 served as a loading control. H₂O₂ concentrations were maintained during incubation with cycloheximide. The percentage of *Deg1*-Sec62* remaining at each time point

(normalized to Pgk1) is indicated below the image. (B) In parallel to experiment depicted in (A), mid-logarithmic-phase yeast expressing oxidant-responsive Rtc3-GFP were analyzed by flow cytometry following incubation in the presence of 0.4 mM H₂O₂ for 1 h. The mean fluorescence intensity for each culture was normalized to the average mean fluorescence intensity of untreated cells. Mean fluorescence intensity $\pm$ standard error of the mean is presented for 3 repeats of 10,000 cells for each condition.

A

B

Figure 11: ERAD-T is not sensitive to glucose limitation or overload. (A)

Cycloheximide chase analysis of wild type yeast expressing *Deg1*-Sec62* cultured in 0.05%, 2%, or 8% glucose for 2 hours. Concentrations were maintained during incubation with cycloheximide. Pgk1 served as a loading control. (B) In parallel to

experiment depicted in (A), yeast cultures expressing Adh2-GFP (the expression of which is inversely proportional to glucose concentration) were analyzed by flow cytometry following incubation in the presence of 0.05%, 2%, or 8% glucose for 2 hours. The mean fluorescence intensity for each culture was normalized to the average mean fluorescence intensity of untreated cells. Mean fluorescence intensity $\pm$ standard error of the mean is presented for 3 repeats of 10,000 cells for each condition.

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