Video Article

Growth-based Determination and Biochemical Confirmation of Genetic Requirements for Protein Degradation in *Saccharomyces cerevisiae*

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Abstract

Regulated protein degradation is crucial for virtually every cellular function. Much of what is known about the molecular mechanisms and genetic requirements for eukaryotic protein degradation was initially established in *Saccharomyces cerevisiae*. Classical analyses of protein degradation have relied on biochemical pulse-chase and cycloheximide-chase methodologies. While these techniques provide sensitive means for observing protein degradation, they are laborious, time-consuming, and low-throughput. These approaches are not amenable to rapid or large-scale screening for mutations that prevent protein degradation. Here, a yeast growth-based assay for the facile identification of genetic requirements for protein degradation is described. In this assay, a reporter enzyme required for growth under specific selective conditions is fused to an unstable protein. Cells lacking the endogenous reporter enzyme but expressing the fusion protein can grow under selective conditions only when the fusion protein is stabilized (*i.e.* when protein degradation is compromised). In the growth assay described here, serial dilutions of wild-type and mutant yeast cells harboring a plasmid encoding a fusion protein are spotted onto selective and non-selective medium. Growth under selective conditions is consistent with degradation impairment by a given mutation. Increased protein abundance should be biochemically confirmed. A method for the rapid extraction of yeast proteins in a form suitable for electrophoresis and western blotting is also demonstrated. A growth-based readout for protein stability, combined with a simple protocol for protein extraction for biochemical analysis, facilitates rapid identification of genetic requirements for protein degradation. These techniques can be adapted to monitor degradation of a variety of short-lived proteins. In the example presented, the His3 enzyme, which is required for histidine biosynthesis, was fused to *Deg1*-Sec62. *Deg1*-Sec62 is targeted for degradation after it aberrantly engages the endoplasmic reticulum translocon. Cells harboring *Deg1*-Sec62-His3 were able to grow under selective conditions when the protein was stabilized.

Video Link

The video component of this article can be found at https://www.jove.com/video/52428/

Introduction

Selective protein degradation is essential for eukaryotic life, and altered protein degradation contributes to a number of medical conditions, including several types of cancer, neurodegenerative disease, cardiovascular disease, and cystic fibrosis[1-5]. The ubiquitin-proteasome system (UPS), which catalyzes selective protein degradation, is an emerging therapeutic target for these conditions[6-10]. Ubiquitin ligases covalently attach polymers of the 76-amino acid ubiquitin to proteins[11]. Proteins that have been marked with polyubiquitin chains are recognized and proteolyzed by the ~2.5 megadalton 26S proteasome[12]. Studies initiated in the model eukaryotic organism *Saccharomyces cerevisiae* (budding yeast) have been foundational in the elucidation of protein degradation mechanisms in eukaryotic cells. The first demonstrated physiological substrate of the UPS was the yeast transcriptional repressor MATα2[13,14], and many highly conserved components of the UPS were first identified or characterized in yeast (*e.g.* [15-26]). Discoveries made in this versatile and genetically tractable model organism are likely to continue to provide important insights into conserved mechanisms of ubiquitin-mediated degradation.

Recognition and degradation of most UPS substrates require the concerted action of multiple proteins. Therefore, an important goal in characterizing the regulated degradation of a given unstable protein is to determine the genetic requirements for proteolysis. Classical approaches (*e.g.* pulse-chase and cycloheximide-chase experiments[27]) for monitoring protein degradation in mammalian or yeast cells are laborious and time-consuming. While these types of methodology provide highly sensitive means for detecting protein degradation, they are not suitable for rapid analysis of protein degradation or large-scale screening for mutations that prevent protein degradation. Here, a yeast growth-based assay for the rapid identification of genetic requirements for the degradation of unstable proteins is presented.

In the yeast growth-based method for analyzing protein degradation, an unstable protein of interest (or degradation signal) is fused, in frame, to a protein that is required for yeast growth under specific circumstances. The result is an artificial substrate that may serve as a powerful tool

to determine the genetic requirements of protein degradation of the unstable protein of interest. Conveniently, most commonly used laboratory yeast strains harbor a panel of mutations in genes encoding metabolic enzymes involved in the biosynthesis of particular amino acids or nitrogenous bases (*e.g.* [20,28-30]). These enzymes are essential for cellular proliferation in the absence of exogenously provided metabolites in whose synthesis the enzymes participate. Such metabolic enzymes may thus function as growth-based reporters for the degradation of unstable proteins to which they are fused. The genetic requirements for protein degradation can be readily elucidated, since mutations that prevent proteolysis will allow cells harboring the degradation reporter to grow under selective conditions.

A growth advantage is an indirect indication that a particular mutation increases the abundance of the protein of interest. However, direct biochemical analysis is required to confirm that a mutation permits growth through increased protein levels rather than via indirect or artifactual causes. The effect of a mutation on protein abundance may be confirmed by western blot analysis of steady-state protein levels in cells that do and do not harbor the particular mutation. A method for the rapid and efficient extraction of yeast proteins (sequential incubation of yeast cells with sodium hydroxide and sample buffer) in a form suitable for analysis by western blotting is also presented[31]. Together, these experiments facilitate the rapid identification of candidate regulators of protein degradation.

Protocol

1. Yeast Growth Assay to Identify Candidate Mutants Defective in Protein Degradation

1. Transform wild-type and mutant yeast cells with a plasmid encoding an unstable protein fused, in frame, to a reporter metabolic enzyme.
2. Inoculate transformants in 5 ml of synthetic defined (SD) minimal medium that is selective for cells harboring plasmid molecules. Incubate overnight at 30 °C, rotating.
3. Measure the optical density at 600 nm (OD_{600}) of each overnight culture.
   NOTE: Following overnight incubation, cells in culture may be in either logarithmic or stationary growth phase but should have reached a minimal OD_{600} of 0.2. Very slow-growing yeast strains may require incubation times longer than one night, or inoculation of a greater number of cells, as determined empirically.
4. Prepare six-fold serial dilutions of transformed yeast cells in a sterile 96-well plate, beginning with cells diluted to an OD_{600} of 0.2. Place each yeast transformant to be assayed in a different row in the 96-well plate.
   1. For each transformant, calculate the volume of overnight culture required to dilute cells to an OD_{600} of 0.2 in a final volume of 200 µl. Add this volume of overnight culture to the corresponding well in Column 1. Add the appropriate amount of sterile water to bring the volume to 200 µl.
   2. For each row of yeast, add 125 µl of sterile water to the wells in Columns 2, 3, and 4.
      NOTE: Individually wrapped sterile 96-well plates may be packaged with sterile lids. The lids may be used as reservoirs for the sterile water that is distributed in this step. This allows simultaneous transfer of sterile water to all wells in a given column with a multichannel pipettor.
   3. Mix the contents of the first column (yeast diluted to an OD_{600} of 0.2) by pipetting up and down with a multichannel pipettor.
   4. Transfer 25 µl of yeast from Column 1 to Column 2, using a multichannel pipettor. Mix by pipetting up and down. Transfer 25 µl from Column 2 to Column 3, and 25 µl from Column 3 to Column 4 (mixing well at each step).
5. Mix each sample with a multichannel pipettor. Proceeding from most dilute to least dilute columns of yeast, pipette 4 µl of each sample onto two plates containing the appropriate selective medium. Use one plate with medium that maintains plasmid selection (this plate serves as a yeast spotting and growth control). Use a second plate with medium that selects for plasmid maintenance and expression of the unstable protein fused to the reporter enzyme. Because yeast settle rapidly, mix cells by pipetting up and down at regular intervals.
   NOTE: Drier plates will more readily absorb liquid than freshly prepared plates and are therefore recommended for these experiments. Damp plates may be dried by incubation at room temperature in low humidity for 1 – 2 days or shorter incubations in a laminar flow hood. Plates may dry unevenly if the laminar air flow is parallel to the bench. Use of a template makes it easier to spot yeast cells at regular distances. Two sample templates are provided in **Figure 1**. These may be printed, cut out, and affixed to the inside of a Petri dish lid.
6. Allow plates to dry on the bench top.
7. Incubate plates at 30 °C for 2 – 6 days.
8. Photograph each plate after incubation.

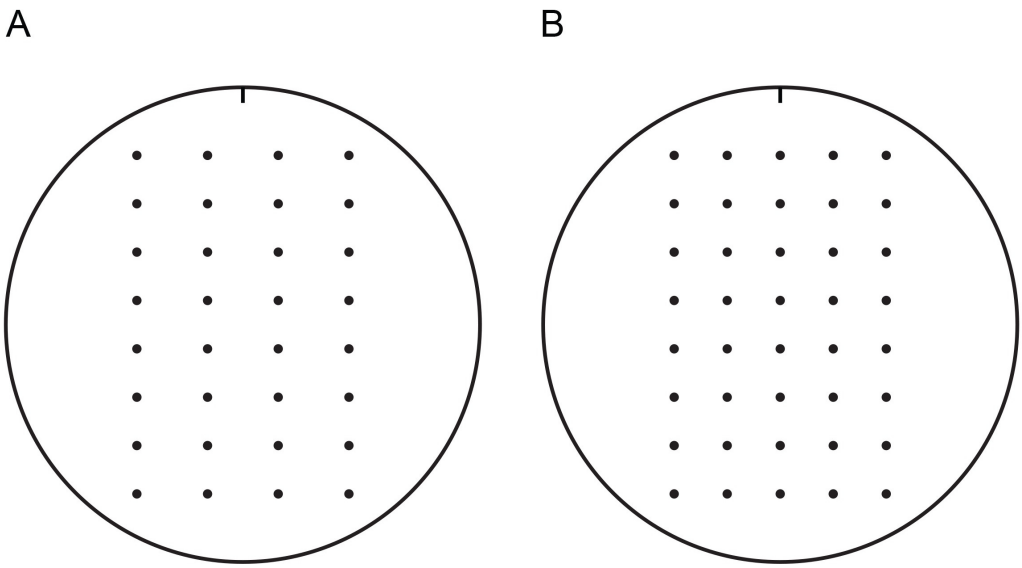

Figure 1. Templates for spotting yeast cells onto 100-mm agar plates. These templates may be used to facilitate spotting yeast at regular distances with a multichannel pipettor. Templates may be printed, cut out, and affixed to the inside of a Petri dish lid. Place Petri dish with growth medium inside lid with template affixed. Templates are marked with a notch to track orientation. It is recommended that plates used in growth assays be similarly marked with a notch to track orientation. Templates for spotting four **(A)** or five **(B)** serial dilutions of yeast cells are provided. Please click here to view a printable version of this figure with 100-mm templates.

2. Biochemical Confirmation of Yeast Growth Assay

1. Growth of Yeast Cells and Post-Alkaline Protein Extraction (modified from [31])
    1. Transform wild-type and mutant yeast cells with a plasmid encoding the unstable protein.
    2. Inoculate transformants in 5 ml of SD medium that is selective for cells harboring plasmid molecules. Incubate overnight at 30 °C, rotating.
    3. Measure the OD_{600} of each overnight culture.
    NOTE: Following overnight incubation, cells may be in either logarithmic or stationary growth phase but should have reached an OD_{600} that will permit dilution to an OD_{600} of 0.2 in 10 ml fresh selective medium (step 2.1.4). Very slow-growing yeast strains may require incubation times longer than one night, or inoculation of a greater number of cells, as determined empirically.
    4. Dilute yeast cells to an OD_{600} of 0.2 in 10 ml fresh selective medium.
    5. Continue to incubate cells at 30 °C, rotating or shaking, until cultures reach an OD_{600} between 0.8 and 1.2 (*i.e.* are in mid-logarithmic growth).
    NOTE: If the unstable protein of interest is under the control of a regulatable promoter, the optimal timing of induction of protein expression and cell harvest may vary according to previous studies or empirical observations.
    6. Collect 2.5 OD_{600} units of culture in a 15-ml conical tube by centrifugation at 5,000 x g for 5 min at room temperature. Remove supernatant by pipetting or aspiration.
    NOTE: One OD_{600} unit is defined as the amount of yeast present in 1 ml of culture at OD_{600} of 1.0. The volume of culture (in ml) required to harvest 2.5 OD_{600} units (V) can be determined using the following equation: V = 2.5 OD_{600} units / Measured OD_{600}
    7. Resuspend cells in 1 ml distilled water. Transfer suspended cells to a microcentrifuge tube.
    8. Pellet cells by centrifugation at 6,500 x g for 30 sec at room temperature. Remove supernatant by pipetting or aspiration.
    9. Resuspend cells in 100 µl distilled water by pipetting up and down or vortexing, and add 100 µl 0.2 M NaOH. Mix by pipetting up and down. Incubate samples for 5 min at room temperature.
    10. Pellet cells (most of which have not yet released proteins and are still viable) by centrifugation at 18,000 x g for 5 min. Remove supernatant by pipetting or aspiration.
    11. Resuspend pellet in 50 – 100 µl 1x Laemmli sample buffer, which will lyse cells, by pipetting up and down or vortexing.
    NOTE: Removal of the alkaline supernatant following centrifugation and subsequent resuspension of cells in Laemmli sample buffer extracts proteins at a pH compatible with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a Tris-glycine running buffer system and western blotting.
    12. To fully denature proteins, incubate lysates at 95 °C for 5 min.
    NOTE: Aggregation-prone proteins (*e.g.* proteins with several transmembrane segments) may become insoluble when incubated at 95 °C. Therefore, lysates should be incubated at lower temperatures (*e.g.* 37 °C – 70 °C) for 10 – 30 min, as empirically determined, for the analysis of such proteins.
    13. Cool lysates by placing on ice for 5 min.
    14. Centrifuge lysates at 18,000 x g for 1 min at room temperature to pellet insoluble material. Separate the supernatant (solubilized extracted protein) by SDS-PAGE prior to subsequent western blot analysis (section 2.2). Alternatively, store lysates at -20 °C.

2. Representative Western Blotting Protocol
    1. Load empirically determined volume of lysates in an SDS-PAGE gel.
    2. Run gel at 200 V until dye front has reached the bottom of the gel.
    3. Transfer proteins from gel to polyvinylidene fluoride (PVDF) membrane by wet transfer at 20 V for 60 – 90 min at 4 °C.

4. Block membrane by incubating in 5% skim milk in Tris-Buffered Saline (TBS), rocking, for 1 hr at room temperature or overnight at 4 °C.
5. Decant blocking solution.
6. Incubate membrane with primary antibody specific for protein of interest (or epitope tag thereof) in 1% skim milk in TBS with 0.1% Tween-20 (TBS/T) for 1 hr at room temperature, rocking.
7. Decant antibody solution, and wash membrane 3 x 5 min with TBS/T at room temperature, rocking.
8. Incubate membrane with appropriate fluorophore-conjugated secondary antibody in 1% skim milk in TBS/T for 1 hr at room temperature, rocking.
   NOTE: Because fluorophores are light-sensitive, dilutions of fluorophore-conjugated antibodies should be prepared in the dark. Additionally, incubation of membranes in the presence of fluorophore-conjugated antibodies should occur in lightproof containers. This can be accomplished by wrapping incubation trays in aluminum foil.
9. Decant antibody solution, and wash membrane 3 x 5 min with TBS/T at room temperature, rocking.
10. Acquire image of membrane using Li-Cor Odyssey CLx and Image Studio software (or comparable imaging equipment and software), according to manufacturer recommendations.
11. After imaging membrane, incubate the membrane with a primary antibody specific for a loading control protein in 1% skim milk in TBS/T for 1 hr at room temperature, rocking.
12. Decant antibody solution, and wash membrane 3 x 5 min with TBS/T at room temperature, rocking.
13. Incubate membrane with appropriate fluorophore-conjugated secondary antibody in 1% skim milk in TBS/T for 1 hr at room temperature, rocking.
14. Decant antibody solution, and wash membrane 3 x 5 min with TBS/T at room temperature, rocking.
15. Acquire image of membrane using Li-Cor Odyssey CLx and Image Studio software (or comparable imaging equipment and software), according to manufacturer recommendations.

Representative Results

To illustrate this methodology, the His3 enzyme has been fused to the carboxy-terminus of the model endoplasmic reticulum (ER)-associated degradation (ERAD) substrate, *Deg1*-Sec62 (**Figure 2A**) to create *Deg1*-Sec62-His3 (**Figure 3**). *Deg1*-Sec62 represents a founding member of a novel class of ERAD substrates that are targeted following persistent, aberrant association with the translocon, the channel primarily responsible for moving proteins across the ER membrane[32-34]. Such unstable proteins have provisionally been called ERAD-T (for translocon-associated) substrates. Previous studies indicate that, upon aberrant translocon engagement, *Deg1*-Sec62 is targeted for degradation by the Hrd1 ubiquitin ligase (**Figure 2B-D**)[32,34,35]. Factors required for the degradation of other Hrd1 substrates appear to be dispensable for *Deg1*-Sec62 degradation, suggesting a novel degradation mechanism[32]. Under conditions of impaired lipid binding and prolonged translocon association, apolipoprotein B, the protein component of mammalian low-density lipoproteins, appears to be degraded by a related mechanism[36-38]. Therefore, *Deg1*-Sec62 may provide a useful model for degradation of medically relevant translocon-associated proteins.

Wild-type and *hrd1Δ* yeast cells that lack the chromosomal *HIS3* gene were transformed with an empty vector[39] or a plasmid encoding *Deg1*-Sec62-His3 and spotted onto selective growth medium (**Figure 4**). To confirm that equal numbers of transformed yeast cells were transferred to plates, cells were spotted onto medium lacking tryptophan (which selects for cells harboring the plasmid) but containing histidine. Similar growth was observed for all transformed yeast cells. Cells that express *Deg1*-Sec62-His3 were expected to grow in the absence of histidine only when the fusion protein is stabilized (i.e. when ERAD-T is compromised). Indeed, *hrd1Δ* yeast expressing *Deg1*-Sec62-His3 exhibited a growth advantage relative to wild-type cells expressing *Deg1*-Sec62-His3 on medium lacking tryptophan and histidine. However, marked fusion-protein-dependent growth in the absence of histidine was observed even in the presence of Hrd1. In order to increase the stringency of the assay, medium lacking histidine was supplemented with 3-amino-1H-1,2,3-triazole (3-AT), a competitive inhibitor of the His3 enzyme[40]. Yeast expressing Hrd1 grew very poorly on medium lacking histidine supplemented with 1 – 2 mM 3-AT; when *HRD1* was deleted, cell growth was restored. Inclusion of 3-AT at a concentration of 3 mM dramatically inhibited growth of all cells, regardless of the presence or absence of Hrd1. These results are consistent with Hrd1-dependent substrate degradation.

Next, the steady-state abundance of the *Deg1*-Sec62-His3 protein in yeast expressing or lacking the Hrd1 enzyme was directly tested. Western blotting analysis indicated a comparable increase in *Deg1*-Sec62-His3 and *Deg1*-Sec62 protein in *hrd1Δ* yeast relative to wild-type cells (**Figure 5**). This confirms a role for Hrd1 in the regulation of levels of both proteins. Hrd1-dependent degradation of *Deg1*-Sec62 proceeds after the protein aberrantly engages the ER translocon[32]. Importantly, *Deg1*-Sec62-His3 aberrantly engages the translocon in a similar manner (unpublished data), further validating the use of *Deg1*-Sec62-His3 as a growth-based reporter for degradation of *Deg1*-Sec62 specifically and translocon-associated proteins generally.

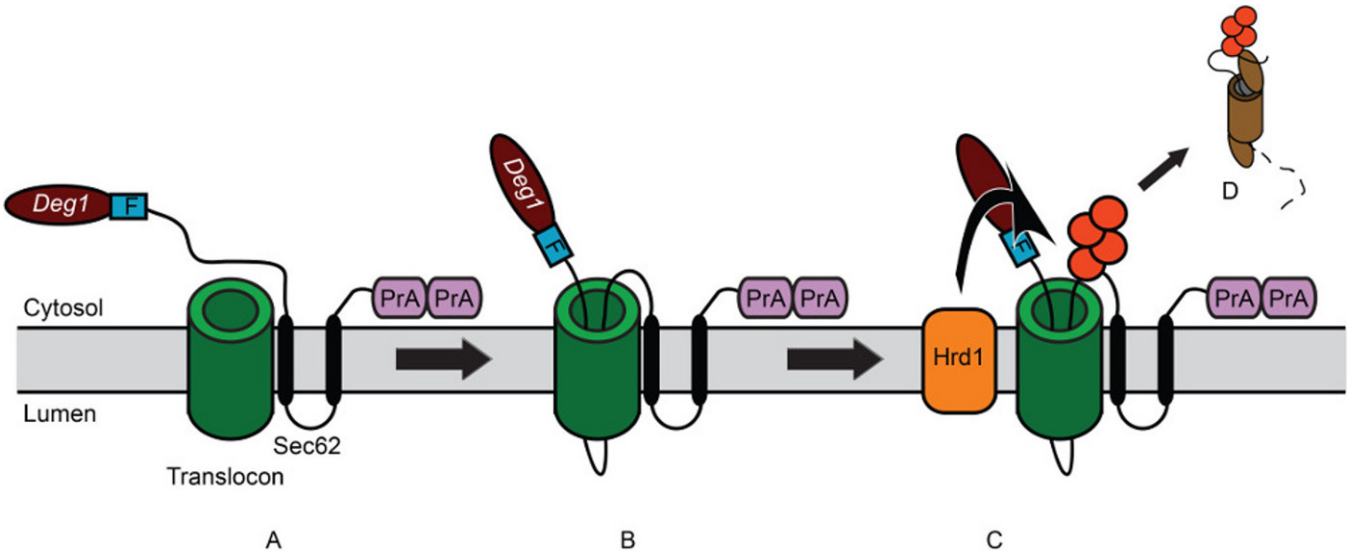

Figure 2: Model for degradation of *Deg1*-Sec62 following aberrant translocon engagement. (A) Schematic depiction of *Deg1*-Sec62. *Deg1* (the amino-terminal 67 amino acids from MATα2) is followed, in sequence, by the Flag (F) epitope, the 2-transmembrane endoplasmic reticulum (ER) protein Sec62, and two copies of the *S. aureus* Protein A (PrA). For clarity, the fusion protein is referred to as *Deg1*-Sec62. **(B)** Following normal insertion of its two transmembrane segments into the ER membrane, persistent interaction of *Deg1*-Sec62 with the translocon triggers abnormal, *Deg1*-dependent translocon engagement. A portion of the initially cytosolic amino-terminal tail aberrantly enters—and likely remains within—the translocon. **(C)** Following abnormal translocon engagement, Hrd1 recognizes and ubiquitylates *Deg1*-Sec62. Red circles indicate ubiquitin molecules. **(D)** *Deg1*-Sec62 is then extracted from the ER membrane and degraded by the proteasome, likely relieving translocon obstruction.

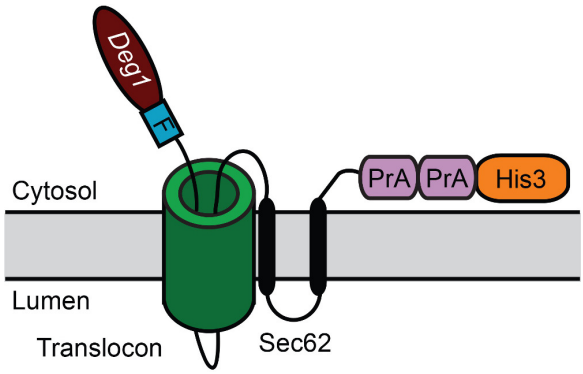

Figure 3: Schematic depiction of *Deg1*-Sec62-His3 following aberrant translocon engagement. *Deg1* is followed, in sequence, by the Flag (F) epitope, the 2-transmembrane ER protein Sec62, two copies of the *S. aureus* Protein A (PrA), and the yeast His3 enzyme. For clarity, the fusion protein is referred to as *Deg1*-Sec62-His3.

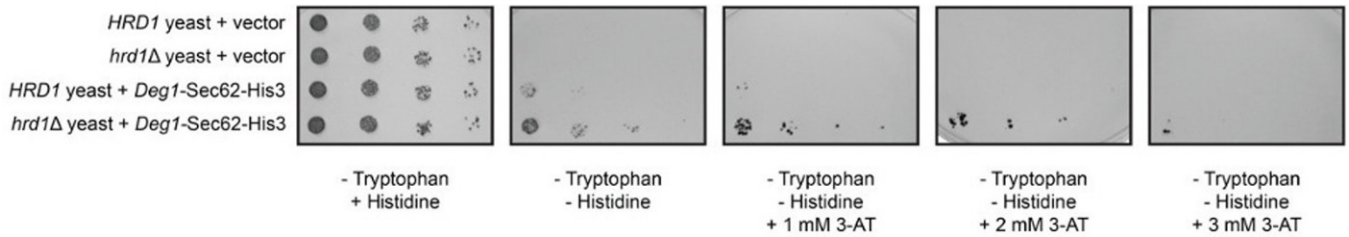

Figure 4: Fusing His3 to *Deg1*-Sec62 permits selection of degradation-defective mutants. Serial dilutions of wild-type (*HRD1*) and *hrd1Δ* yeast transformed with an empty vector or a plasmid encoding *Deg1*-Sec62-His3 were spotted onto medium lacking tryptophan, medium lacking tryptophan and histidine, and medium lacking tryptophan and histidine supplemented with 3-amino-1H-1,2,3-triazole (3-AT), a competitive inhibitor of His3, at the indicated concentrations. Please click here to view a larger version of this figure.

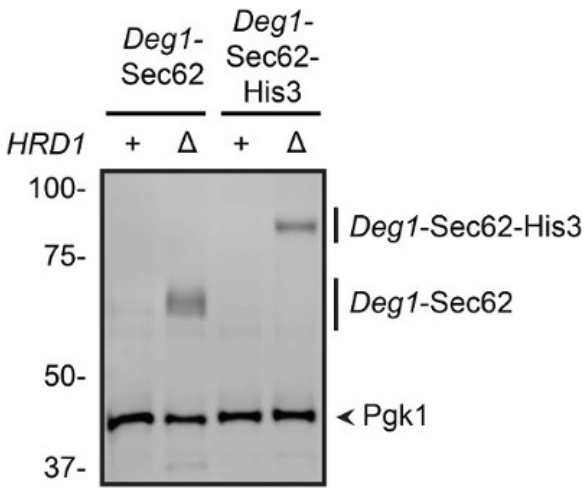

Figure 5: Increased abundance of *Deg1*-Sec62 and *Deg1*-Sec62-His3 in cells lacking Hrd1. Protein extracts were prepared from wild-type (+) and *hrd1Δ*(Δ) yeast expressing *Deg1*-Sec62 or *Deg1*-Sec62-His3. Proteins (equivalent to 0.125 OD_{600} units) were separated by SDS-PAGE, followed by western blotting with rabbit anti-mouse secondary antibodies, which directly bind the Protein A epitopes of the fusion proteins. Subsequent western blotting with antibodies specific to Pgk1 provides a loading control.

Solution	Components	Comments
Synthetic Defined (SD) Minimal Yeast Medium	2 % dextrose, 0.67 % yeast nitrogen base without amino acids, 0.002 % adenine, 0.004 % uracil, 0.002 % arginine, 0.001 % histidine, 0.006 % isoleucine, 0.006 % leucine, 0.004 % lysine, 0.001 % methionine, 0.006 % phenylalanine, 0.005 % threonine, 0.004 % tryptophan. For solid (plate) medium, include 2 % agar.	1. Selective medium is prepared by omitting appropriate amino acid(s) or nitrogenous bases.
		2. For convenience, these ingredients may be maintained as concentrated stock solutions as follows. Amino acids may be maintained as 100X stock solution containing all desired amino acids. Yeast nitrogen base may be maintained in a 20X stock solution (13.4 %). Dextrose may be maintained in a 40 % stock solution. Adenine and uracil may be maintained as 1 % stock solutions in 0.1 M NaOH.
		3. Sterilize medium by autoclaving.
1X Laemmli Sample Buffer	2 % SDS, 10 % glycerol, 5 % β-mercaptoethanol, 60 mM Tris HCl pH 6.8, 0.008 % bromophenol blue	1. 1X Sample buffer is often prepared by diluting a more concentrated (e.g. 5X) stock.
		2. The dye bromophenol blue may be added to desired intensity. A "pinch" (very small amount tapped from the edge of a spatula) is typically sufficient.
0.2 M Sodium Hydroxide		Prepare in water. Sodium hydroxide reacts with glass. Therefore, for long-term storage, 0.2 M sodium hydroxide should be maintained in plastic containers.
Laemmli Running Buffer (5X)	125 mM Tris, 960 mM glycine, 0.5 % SDS	To prepare 1 L of 1X Laemmli Running Buffer, dilute 1:5 in dH_2O
Tris Acetate-SDS Transfer Buffer (5X)	125 mM Tris acetate (pH 8.8), 960 mM glycine, 0.05 % SDS	To prepare 20 L of 1X Tris Acetate-SDS Transfer Buffer, combine 4 L of 5X stock, 4 L of methanol, and 12 L of dH_2O
10X Tris-Buffered Saline (TBS)	500 mM Tris, 1.5 M NaCl; pH adjusted to 7.5	To prepare 1 L of 1X TBS, dilute 1:10 in dH_2O. 1X TBS may be supplemented with the detergent Tween-20 and powdered skim milk, as appropriate.

Table 1: Solutions and buffers used in this study.

Strain Name	Alias	Relevant Genotype	Figures	Source
VJY6	MHY500	*MATa*	4 and 5	Chen et al., 1993
		his3-Δ200		
		leu2-3,112		
		ura3-52		
		lys2-801		
		trp1-1		
		gal2		
VJY10		*MATa*	4 and 5	This study
		his3-Δ200		
		leu2-3,112		
		ura3-52		
		lys2-801		
		trp1-1		
		gal2		
		hrd1::kanMX4		

Table 2: Yeast strains used in this study. Details of construction are available upon request.

Plasmid Number	Full Plasmid Name	Figure	Source
pVJ30	pRS414-P_{MET25}-*Deg1*-Flag-Sec62-2xProtA	5	Rubenstein et al., 2012
pVJ121	pRS414-P_{MET25} (empty vector with *MET25* promoter)	4	Mumberg et al., 1994
pVJ467	pRS414-P_{MET25}-*Deg1*-Flag-Sec62-2xProtA-His3	5	This study
pVJ477	pRS414-P_{GAL4}-*Deg1*-Flag-Sec62-2xProtA-His3	4	This study

Table 3: Plasmids used in this study. Note that all plasmids contain a yeast centromere to allow replication in yeast cells, the *TRP1* gene for selection in yeast cells, and the *AmpR* gene for maintenance in bacterial cells. Plasmid maps, sequences, and details of construction are available upon request.

Discussion

The methodology presented here allows for the rapid determination and biochemical confirmation of genetic requirements for protein degradation in yeast cells. These experiments highlight the utility and power of yeast as a model eukaryotic organism (several excellent reviews of yeast biology and compilations of protocols for handling, storing, and manipulating yeast cells (*e.g.* [41-44]) are available for investigators new to the organism). The techniques can readily be applied to investigate the degradation and abundance of a variety of classes of proteins. For example, others have employed this strategy to characterize the degradation mechanisms of unstable cytosolic, nuclear, and ER luminal and transmembrane proteins[45-49].

A few factors must be considered in the choice of metabolic enzyme to fuse to an unstable protein. First, it is essential that a functional version of the gene encoding the enzyme not be present in the host genome. To minimize false positive results (*i.e.* growth under selective conditions when the unstable protein is actually degraded), it is recommended to work with strains that harbor non-reverting mutant alleles of the reporter gene (preferably complete gene deletions)[50]. Another consideration for reporter enzyme selection is the availability of competitive inhibitors, which can be included in the selective growth medium to reduce background growth and enhance assay stringency. This may be useful in cases of proteins with relatively low turnover rates even in the presence of fully functional degradation mechanisms. In the representative experiments presented here, the inclusion of 3-AT, which competitively inhibits the His3 enzyme, reduces background growth[40]. Similarly, the compound 6-azauracil inhibits Ura3, an enzyme required for uracil biosynthesis[51]. The inhibitor concentration at which growth occurs in degradation-defective, but not wild-type, cells must be determined empirically. Some metabolic enzymes may also be counter-selected against. Under counter-selective conditions, cells may grow only when the unstable protein is degraded (and the fused metabolic enzyme is not present). For example, Ura3 converts the compound 5-fluoroorotic acid (5-FOA) to the toxic compound, 5-fluorouracil[52]. Cells expressing a Ura3-fusion protein will only grow in the presence of 5-FOA if the Ura3 fusion protein is degraded. Similarly, the compound 5-fluoroanthranilic acid (5-FAA) is toxic to cells with a functional tryptophan biosynthesis pathway. 5-FAA may thus be used to counter-select for cells expressing Trp1-fusion proteins[53]. Counter-selection strategies may be useful for the identification of suppressors of degradation-impairing mutations.

The promoter used to drive expression of a degradation reporter must also be carefully selected. As low levels of biosynthetic enzymes may be sufficient to support growth in the absence of exogenously supplied metabolite, a weak promoter is recommended[54]. In the representative

experiments presented here, the *GAL4* promoter, which is repressed in the presence of glucose[55], is used to promote the transcription of *Deg1*-Sec62-His3. Basal expression of this fusion protein under repressing conditions (*i.e.* 2% glucose) is sufficient to support growth under selective conditions (*i.e.* absence of histidine and presence of 1 – 2 mM 3-AT) when the degradation mechanism is disabled. However, protein levels sufficient to support growth under selective conditions are likely to be below the threshold of detection by western blotting. Therefore, it may be necessary to drive expression with a weak promoter for the growth assay and a stronger promoter for biochemical confirmation. In the case of *Deg1*-Sec62 (with or without the His3 fusion), a more robust promoter (here, the *MET25* promoter[39]) is required for protein visualization by western analysis.

As described here, the yeast-based growth assay may be performed on a small-scale, candidate-based approach. Serial dilutions of yeast cells are prepared in a 96-well plate and transferred by pipetting to solid growth medium; an alternative method for the efficient and reproducible transfer of diluted yeast cell suspensions onto solid medium is the use of a multi-pin replicator, commonly referred to as a "frogger"[56]. The ideal time to photograph plates will vary with yeast strains and conditions. It is recommended to photograph a given plate when colonies from the fastest growing culture first become visible at the most dilute spot. This is typically the point at which differences in growth rates among samples are most obvious. It may be advisable to take photographs on multiple days, particularly in the case of yeast exhibiting a wide range of growth rates.

The growth-based reporter assay may also be adapted for large-scale analyses. For example, a degradation reporter may be introduced into a commercially available library of ~5,000 viable haploid yeast gene deletion strains using Synthetic Genetic Array (SGA) technology[46,57]. In this technique, a haploid yeast strain with a chromosomally integrated metabolic reporter fusion protein is mated to each strain of the gene deletion library. The resultant diploid cells are induced to sporulate (undergo meiosis) and subjected to selection for haploid meiotic progeny harboring both the metabolic reporter and individual gene deletions. These strains are then transferred en masse to medium selective for cells in which the protein has been stabilized. As for small-scale analyses, if a gene with a role in protein degradation is deleted, the fusion protein will be stabilized, and cell growth will be enhanced. Comparable approaches have been devised that allow for simultaneous transformation of a large collection of strains with extra-chromosomally maintained plasmids; this strategy obviates chromosomal integration, mating, sporulation, and meiotic selection[54].

When a mutation is found to confer a growth advantage to cells harboring a metabolic reporter, it is necessary to biochemically confirm that the mutation increases the abundance of the protein of interest. A rapid and reliable yeast lysis procedure, closely adapted from the method of Kuhsnirov[31], is presented. This protocol allows for the extraction of proteins in a form directly suitable for analysis by western blotting. For the analysis of a given protein, the amount of lysate to be loaded per well, acrylamide gel properties, choice of membrane, antibodies used and dilutions thereof, and method of detection must be empirically determined. The representative western blotting protocol described utilizes secondary antibodies conjugated to fluorescent dyes; other commonly used protocols rely on chemiluminescence dependent on antibody-conjugated enzymes[58]. As described here, the membrane used to detect the protein of interest may be directly reprobed with an antibody for a loading control protein. If the primary antibodies used to detect the protein of interest and loading control protein have been raised from the same species, reprobing the membrane is possible as long as the bands arising from these proteins do not co-migrate. If, however, the primary antibodies used to detect the protein of interest and loading control protein have been raised from different species, the same membrane may be sequentially probed, even if bands co-migrate, if the secondary antibodies have been conjugated to fluorophores with different emission wavelengths. In the case that the protein of interest and loading control protein co-migrate and the respective primary antibodies have been raised from the same species, samples may be resolved on two SDS-PAGE gels, transferred to PVDF membrane, and probed separately with antibodies specific for the protein of interest and loading control protein. Alternatively, loading consistency may be judged by incubating membranes with non-specific protein stains (*e.g.* Coomassie or Ponceau S). Further, the representative western blotting protocol assumes a protein or epitope that is detected by sequential incubation of primary and secondary antibodies, as is typical. The fusion proteins analyzed in the representative results contain two epitopes derived from *Staphylococcus aureus* Protein A (PrA in **Figures 2** and **3**). Protein A binds directly to mammalian immunoglobulins and therefore can be detected using secondary antibody alone (*i.e.* no primary antibody incubation step is required)[59]. It is possible that fusion of a reporter enzyme may influence protein abundance or degradation. It is therefore advisable to biochemically confirm the results using a version of the substrate unencumbered by the reporter protein. Finally, both assays described here rely on differences in steady-state protein levels as a proxy for differences in protein stability. Because protein abundance reflects the integration of rates of protein synthesis and degradation, further biochemical analysis (*e.g.* cycloheximide chase or pulse chase experiments) must be employed to directly analyze a protein's dynamic degradation profile.

The representative results establish a novel application of this protocol for the determination of genetic requirements for degradation of a protein that aberrantly engages the ER translocon. *Deg1*-Sec62-His3 conferred a yeast growth advantage under selective conditions when the degradation pathway was inactivated (*i.e.* in the absence of the Hrd1 ubiquitin ligase). A fast and reliable protein extraction method followed by western blotting confirmed an increase in abundance of *Deg1*-Sec62 (with or without His3) in the absence of Hrd1. Previous studies indicate that the mechanism of Hrd1-dependent degradation of translocon-associated proteins differs from those of other Hrd1 ER luminal or transmembrane substrates[32]. Future work will employ the *Deg1*-Sec62-His3 fusion protein in large-scale genetic screens to identify novel genes required for this unique degradation mechanism.

Disclosures

The authors have nothing to disclose.

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References

1. Goldberg, A. L. Protein degradation and protection against misfolded or damaged proteins. *Nature.* **426**, 895-899 (2003).
2. Guerriero, C. J., Brodsky, J. L. The delicate balance between secreted protein folding and endoplasmic reticulum-associated degradation in human physiology. *Physiological Reviews.* **92**, 537-576 (2012).
3. Pagan, J., Seto, T., Pagano, M., Cittadini, A. Role of the ubiquitin proteasome system in the heart. *Circulation Research.* **112**, 1046-1058 (2013).
4. Rubinsztein, D. C. The roles of intracellular protein-degradation pathways in neurodegeneration. *Nature.* **443**, 780-786 (2006).
5. Turnbull, E. L., Rosser, M. F., Cyr, D. M. The role of the UPS in cystic fibrosis. *BMC Biochemistry.* **8**, Suppl 1. S11 (2007).
6. Bedford, L., Lowe, J., Dick, L. R., Mayer, R. J., Brownell, J. E. Ubiquitin-like protein conjugation and the ubiquitin-proteasome system as drug targets. *Nature Reviews Drug Discovery.* **10**, 29-46 (2011).
7. Kar, G., Keskin, O., Fraternali, F., Gursoy, A. Emerging role of the ubiquitin-proteasome system as drug targets. *Current Pharmaceutical Design.* **19**, 3175-3189 (2013).
8. Paul, S. Dysfunction of the ubiquitin-proteasome system in multiple disease conditions: therapeutic approaches. *BioEssays: News and Reviews in Molecular, Cellular and Developmental Biology.* **30**, 1172-1184 (2008).
9. Shen, M., Schmitt, S., Buac, D., Dou, Q. P. Targeting the ubiquitin-proteasome system for cancer therapy. *Expert Opinion on Therapeutic Targets.* **17**, (9), 1091-1108 (2013).
10. Finley, D., Ulrich, H. D., Sommer, T., Kaiser, P. The ubiquitin-proteasome system of Saccharomyces cerevisiae. *Genetics.* **192**, 319-360 (2012).
11. Scheffner, M., Nuber, U., Huibregtse, J. M. Protein ubiquitination involving an E1-E2-E3 enzyme ubiquitin thioester cascade. *Nature.* **373**, 81-83 (1995).
12. Ravid, T., Hochstrasser, M. Diversity of degradation signals in the ubiquitin-proteasome system. *Nature Reviews Molecular Cell Biology.* **9**, 679-690 (2008).
13. Hochstrasser, M., Ellison, M. J., Chau, V., Varshavsky, A. The short-lived MAT alpha 2 transcriptional regulator is ubiquitinated in vivo. *Proceedings of the National Academy of Sciences of the United States of America.* **88**, 4606-4610 (1991).
14. Hochstrasser, M., Varshavsky, A. In vivo degradation of a transcriptional regulator: the yeast alpha 2 repressor. *Cell.* **61**, 697-708 (1990).
15. Bays, N. W., Gardner, R. G., Seelig, L. P., Joazeiro, C. A., Hampton, R. Y. Hrd1p/Der3p is a membrane-anchored ubiquitin ligase required for ER-associated degradation. *Nature Cell Biology.* **3**, 24-29 (2001).
16. Hampton, R. Y., Gardner, R. G., Rine, J. Role of 26S proteasome and HRD genes in the degradation of 3-hydroxy-3-methylglutaryl-CoA reductase, an integral endoplasmic reticulum membrane protein. *Molecular Biology of the Cell.* **7**, 2029-2044 (1996).
17. Swanson, R., Locher, M., Hochstrasser, M. A conserved ubiquitin ligase of the nuclear envelope/endoplasmic reticulum that functions in both ER-associated and Matalpha2 repressor degradation. *Genes & Development.* **15**, 2660-2674 (2001).
18. Goebl, M. G., *et al.* The yeast cell cycle gene CDC34 encodes a ubiquitin-conjugating enzyme. *Science.* **241**, 1331-1335 (1988).
19. Bachmair, A., Finley, D., Varshavsky, A. In vivo half-life of a protein is a function of its amino-terminal residue. *Science.* **234**, 179-186 (1986).
20. Chen, P., Johnson, P., Sommer, T., Jentsch, S., Hochstrasser, M. Multiple ubiquitin-conjugating enzymes participate in the in vivo degradation of the yeast MAT alpha 2 repressor. *Cell.* **74**, 357-369 (1993).
21. Varshavsky, A. Discovery of the biology of the ubiquitin system. *JAMA: The Journal of the American Medical Association.* **311**, 1969-1970 (2014).
22. Heinemeyer, W., Kleinschmidt, J. A., Saidowsky, J., Escher, C., Wolf, D. H. Proteinase yscE, the yeast proteasome/multicatalytic-multifunctional proteinase: mutants unravel its function in stress induced proteolysis and uncover its necessity for cell survival. *The EMBO Journal.* **10**, 555-562 (1991).
23. Sommer, T., Jentsch, S. A protein translocation defect linked to ubiquitin conjugation at the endoplasmic reticulum. *Nature.* **365**, 176-179 (1993).
24. Hiller, M. M., Finger, A., Schweiger, M., Wolf, D. H. ER degradation of a misfolded luminal protein by the cytosolic ubiquitin-proteasome pathway. *Science.* **273**, 1725-1728 (1996).
25. Seufert, W., Jentsch, S. In vivo function of the proteasome in the ubiquitin pathway. *The EMBO Journal.* **11**, 3077-3080 (1992).
26. Knop, M., Finger, A., Braun, T., Hellmuth, K., Wolf, D. H. Der1, a novel protein specifically required for endoplasmic reticulum degradation in yeast. *The EMBO Journal.* **15**, 753-763 (1996).
27. Zattas, D., Adle, D. J., Rubenstein, E. M., Hochstrasser, M. N-terminal acetylation of the yeast Derlin Der1 is essential for Hrd1 ubiquitin-ligase activity toward luminal ER substrates. *Molecular Biology of the Cell.* **24**, 890-900 (2013).
28. Brachmann, C. B., *et al.* Designer deletion strains derived from Saccharomyces cerevisiae S288C: a useful set of strains and plasmids for PCR-mediated gene disruption and other applications. *Yeast.* **14**, 115-132 (1998).
29. Sikorski, R. S., Hieter, P. A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in Saccharomyces cerevisiae. *Genetics.* **122**, 19-27 (1989).
30. Ralser, M., *et al.* The Saccharomyces cerevisiae W303-K6001 cross-platform genome sequence: insights into ancestry and physiology of a laboratory mutt. *Open Biology.* **2**, 120093 (2012).
31. Kushnirov, V. V. Rapid and reliable protein extraction from yeast. *Yeast.* **16**, 857-860 (2000).
32. Rubenstein, E. M., Kreft, S. G., Greenblatt, W., Swanson, R., Hochstrasser, M. Aberrant substrate engagement of the ER translocon triggers degradation by the Hrd1 ubiquitin ligase. *The Journal of Cell Biology.* **197**, 761-773 (2012).
33. Mayer, T. U., Braun, T., Jentsch, S. Role of the proteasome in membrane extraction of a short-lived ER-transmembrane protein. *The EMBO Journal.* **17**, 3251-3257 (1998).
34. Scott, D. C., Schekman, R. Role of Sec61p in the ER-associated degradation of short-lived transmembrane proteins. *The Journal of Cell Biology.* **181**, 1095-1105 (2008).
35. Kim, I., *et al.* The Png1-Rad23 complex regulates glycoprotein turnover. *The Journal of Cell Biology.* **172**, 211-219 (2006).

36. Fisher, E. A., *et al.* The degradation of apolipoprotein B100 is mediated by the ubiquitin-proteasome pathway and involves heat shock protein 70. *The Journal of Biological Chemistry.* **272**, 20427-20434 (1997).
37. Pariyarath, R., *et al.* Co-translational interactions of apoprotein B with the ribosome and translocon during lipoprotein assembly or targeting to the proteasome. *The Journal of Biological Chemistry.* **276**, 541-550 (2001).
38. Yeung, S. J., Chen, S. H., Chan, L. Ubiquitin-proteasome pathway mediates intracellular degradation of apolipoprotein. *B. Biochemistry.* **35**, 13843-13848 (1996).
39. Mumberg, D., Muller, R., Funk, M. Regulatable promoters of Saccharomyces cerevisiae: comparison of transcriptional activity and their use for heterologous expression. *Nucleic Acids Research.* **22**, 5767-5768 (1994).
40. Bruckner, A., Polge, C., Lentze, N., Auerbach, D., Schlattner, U. Yeast two-hybrid, a powerful tool for systems biology. *International Journal of Molecular Sciences.* **10**, 2763-2788 (2009).
41. Amberg, D. C., Burke, D., Strathern, J. N., Burke, D. *Methods in Yeast Genetics: A Cold Spring Harbor Laboratory Course Manual.* Cold Spring Harbor Laboratory Press Cold Spring Harbor, NY (2005).
42. Duina, A. A., Miller, M. E., Keeney, J. B. Budding yeast for budding geneticists: a primer on the Saccharomyces cerevisiae model system. *Genetics.* **197**, 33-48 (2014).
43. Guthrie, C., Fink, G. R. *Guide to Yeast Genetics and Molecular and Cell Biology.* Elsevier San Diego (2004).
44. Sherman, F. Getting started with yeast. *Methods in Enzymology.* **350**, 3-41 (2002).
45. Xie, Y., Rubenstein, E. M., Matt, T., Hochstrasser, M. SUMO-independent in vivo activity of a SUMO-targeted ubiquitin ligase toward a short-lived transcription factor. *Genes & Development.* **24**, 893-903 (2010).
46. Ravid, T., Kreft, S. G., Hochstrasser, M. Membrane and soluble substrates of the Doa10 ubiquitin ligase are degraded by distinct pathways. *The EMBO Journal.* **25**, 533-543 (2006).
47. Metzger, M. B., Maurer, M. J., Dancy, B. M., Michaelis, S. Degradation of a cytosolic protein requires endoplasmic reticulum-associated degradation machinery. *The Journal of Biological Chemistry.* **283**, 32302-32316 (2008).
48. Medicherla, B., Kostova, Z., Schaefer, A., Wolf, D. H. A genomic screen identifies Dsk2p and Rad23p as essential components of ER-associated degradation. *EMBO Reports.* **5**, 692-697 (2004).
49. Kohlmann, S., Schafer, A., Wolf, D. H. Ubiquitin ligase Hul5 is required for fragment-specific substrate degradation in endoplasmic reticulum-associated degradation. *The Journal of Biological Chemistry.* **283**, 16374-16383 (2008).
50. Crouse, G. F. Mutagenesis assays in yeast. *Methods.* **22**, 116-119 (2000).
51. Le Douarin, B., Pierrat, B., vom Baur, E., Chambon, F., Losson, R. A new version of the two-hybrid assay for detection of protein-protein interactions. *Nucleic Acids Research.* **23**, 876-878 (1995).
52. Boeke, J. D., Trueheart, J., Natsoulis, G., Fink, G. R. 5-Fluoroorotic acid as a selective agent in yeast molecular genetics. *Methods in Enzymology.* **154**, 164-175 (1987).
53. Toyn, J. H., Gunyuzlu, P. L., White, W. H., Thompson, L. A., Hollis, G. F. A counterselection for the tryptophan pathway in yeast: 5-fluoroanthranilic acid resistance. *Yeast.* **16**, 553-560 (2000).
54. Schafer, A., Wolf, D. H. Endoplasmic reticulum-associated protein quality control and degradation: genome-wide screen for ERAD components. *Methods in Molecular Biology.* **301**, 289-292 (2005).
55. Griggs, D. W., Johnston, M. Regulated expression of the GAL4 activator gene in yeast provides a sensitive genetic switch for glucose repression. *Proceedings of the National Academy of Sciences of the United States of America.* **88**, 8597-8601 (1991).
56. Duennwald, M. L. Growth assays to assess polyglutamine toxicity in yeast. *The Journal of Visualized Experiments.* (61), e3791 (2012).
57. Baryshnikova, A., *et al.* Synthetic genetic array (SGA) analysis in Saccharomyces cerevisiae and Schizosaccharomyces pombe. *Methods in Enzymology.* **470**, 145-179 (2010).
58. Szymanski, E. P., Kerscher, O. Budding yeast protein extraction and purification for the study of function, interactions, and post-translational modifications. *The Journal of Visualized Experiments.* (80), e50921 (2013).
59. Hjelm, H., Sjodahl, J., Sjoquist, J. Immunologically active and structurally similar fragments of protein A from Staphylococcus aureus. *European Journal of Biochemistry.* **57**, 395-403 (1975).

