Video Article
Reporter-based Growth Assay for Systematic Analysis of Protein Degradation

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Abstract

Protein degradation by the ubiquitin-proteasome system (UPS) is a major regulatory mechanism for protein homeostasis in all eukaryotes. The standard approach to determining intracellular protein degradation relies on biochemical assays for following the kinetics of protein decline. Such methods are often laborious and time consuming and therefore not amenable to experiments aimed at assessing multiple substrates and degradation conditions. As an alternative, cell growth-based assays have been developed, that are, in their conventional format, end-point assays that cannot quantitatively determine relative changes in protein levels.

Here we describe a method that faithfully determines changes in protein degradation rates by coupling them to yeast cell-growth kinetics. The method is based on an established selection system where uracil auxotrophy of *URA3*-deleted yeast cells is rescued by an exogenously expressed reporter protein, comprised of a fusion between the essential *URA3* gene and a degradation determinant (degron). The reporter protein is designed so that its synthesis rate is constant whilst its degradation rate is determined by the degron. As cell growth in uracil-deficient medium is proportional to the relative levels of Ura3, growth kinetics are entirely dependent on the reporter protein degradation.

This method accurately measures changes in intracellular protein degradation kinetics. It was applied to: (a) Assessing the relative contribution of known ubiquitin-conjugating factors to proteolysis (b) E2 conjugating enzyme structure-function analyses (c) Identification and characterization of novel degrons. Application of the degron-*URA3*-based system transcends the protein degradation field, as it can also be adapted to monitoring changes of protein levels associated with functions of other cellular pathways.

Video Link

The video component of this article can be found at http://www.jove.com/video/52021/

Introduction

The ubiquitin-proteasome degradation system is a major regulatory machine, which has been implicated in the maintenance of protein homeostasis in all eukaryotes. The UPS initially conjugates multiple ubiquitin molecules to a target protein after which the poly-ubiquitin-tagged protein is degraded by the 26S proteasome. In most cases, the rate limiting step for ubiquitin mediated degradation is substrate ubiquitylation, mediated by E2 conjugating enzymes and E3 ligating enzymes (E3 Ligases)[1]. Consequently, intracellular stability of specific proteins reflects their susceptibility to ubiquitin-conjugation and the activity of their cognate ubiquitylation enzymes.

E3 ligases are the principal substrate recognition components of the UPS. As such, these enzymes recognize degrons within their substrates that are either absent or not exposed in their stable counterparts [2]. For example, many regulators of the cell cycle must be synthesized and degraded in a temporally specific manner in order to keep cell cycle progression in order. The degradation of these proteins is often controlled by phosphorylation, mediated by cell-signaling regulated kinases [3,4]. On the other hand, aberrantly-folded proteins are recognized through cryptic degrons. These are regions that are normally hidden in the native structure and are exposed upon structure perturbation. Such degrons include hydrophobic domains [5-7] and intrinsically disordered segments[8].

Since the seminal discovery of the ubiquitin-system for protein degradation and characterization of its fundamentals in reticulocyte lysates[9], yeast genetics was instrumental in discovering many of the components of the ubiquitin system[10]. The success of yeast as a model organism for systematic analysis of protein degradation by the UPS is mainly due to the fact that the UPS is highly conserved in all eukaryotes[4], coupled with their amenability as an experimental system. Indeed, yeast-based systems are commonly employed to decipher the mechanisms of action of the ubiquitylation machinery.

Studying protein degradation by biochemical means usually requires preparation of cell extracts. While animal cell proteins can be extracted under relatively mild conditions that preserve protein interactions and function, the presence of a robust cell wall in yeast[11] requires considerably harsher disruption conditions which may affect protein recovery. Indeed, different procedures for yeast cell disruption vary considerably in their capacity to recover intact proteins in amounts that correctly represent their relative cellular abundance. Further inaccuracy is inherent in the different methods employed for determining degradation rates of specific proteins: Metabolic labeling-based 'pulse-chase' experiments followed by immunoprecipitation, to isolate specific proteins[12], is often not strictly quantitative. Thus, when protein degradation is compared by this

method, extra caution should be exercised in interpreting the results. To circumvent this drawback, an alternative cycloheximide (CHX) chase assay can be employed[12]. In this assay, the translation inhibitor is added to cell cultures and temporal changes in protein steady-state levels are subsequently monitored. Nevertheless, the usage of CHX is limited to proteins with relatively short half-lives (< 90 min), as long-term inhibition of protein synthesis is cytotoxic. Notably, both of the above-mentioned assays require the use of protein-specific antibodies, which are not always available.

To overcome these technical limitations, researchers have developed several approaches that do not require cell extraction and direct protein handling. One approach is based on the establishment of auxotrophic yeast strains, obtained by the deletion of genes encoding essential metabolic enzymes. Such genes include *HIS3, LEU2, LYS2* and *TRP1*, encoding for enzymes required for amino acid biosynthesis, as well as *URA3* that encodes OMP decarboxylase (Ura3), an essential enzyme of pyrimidine ribonucleotide biosynthesis. Ura3 has been widely used in protein degradation studies. In these assays, constitutive expression of Ura3 rescues growth of *ura3* cells in uracil-deficient medium[13]. Consequently, destabilizing Ura3 through the fusion of a degron can diminish cell growth on minimal medium lacking uracil. This method has been used in various protein degradation studies, including the identification of degradation determinants[5], E3 ligases[14] and auxiliary ubiquitylation factors[15] and the discovery of novel UPS degrons[16]. All of these methods employed cell growth on agar plates as assay readout. However, the growth criterion (positive/negative growth), while robust and efficient, is mostly qualitative and does not provide quantitative information that is important for evaluating a degron's potency or the relative contribution of various auxiliary degradation factors.

We have therefore developed and utilized yeast vectors and screening methods enabling systematic and quantitative analysis of protein degradation by the *URA3*-degron fusion system. The protocol is based on an easy-to-handle assay that measures Growth kinetics in Liquid culture under Selective conditions (GiLS) and on the generation of standard growth curves. Yeast growth kinetics are characterized by three main phases — the lag, the exponential (log) and the stationary phase. Calculation of yeast replication kinetics during the log phase under selective conditions, which is determined by the levels of expression of the Ura3-degron, provides an unbiased quantitative measurement of protein degradation. This method can be applied to measuring and comparing degradation rates of multiple UPS substrates simultaneously in multiple strains and under various conditions.

Protocol

1. Cell Culture

1. Transform the appropriate auxotrophic yeast cells, such as Try467 (**Table 2**), with a plasmid containing (a) a *URA3*-degron fusion and (b) an additional metabolic marker for plasmid selection and maintenance.
   NOTE: An example of a suitable plasmid is *YDpK-MET25p-Deg1-FLAG-Vma12-Ura3 (LYS2)* (*Deg1*-UV). This is a yeast integrative plasmid containing a fusion protein comprising of *Deg1* — a degron derived from the yeast transcription factor Mat2[17], FLAG epitope — for detection by immunoblotting, Vma12 — a stable ER protein, and Ura3[15,18] (**Figure 3A**). (See **Table 3** for plasmids used in this study and for additional examples of suitable plasmids.)
2. For plasmid selection and maintenance, keep cells in agar plates under a suitable Synthetic Defined (SD) medium lacking amino acid selection markers[19] (For example, *Deg1*-UV is selected and maintained under SD-LYS selective media).
3. Grow cells O/N at 30 °C in a suitable selective SD medium for plasmid maintenance until a stationary phase is reached. Grow the cells in test tubes or in a 96-well plate, depending on the number of samples to be examined (section 2).

2. Cell Preparation for Analysis

1. When a small number of samples (N≤15) are to be assayed, dilute the cells and replace the medium as follows:
   1. Dilute 50 µl of cells from an O/N culture with 150 µl of SD medium per well in a 96-well plate (clear bottom). Note, the first sample is designated as blank contains medium only.
   2. Determine the OD_{600} values of the samples in the 96-well plate using a microplate reader.
   3. Calculate the actual OD_{600} in each well by multiplying the OD_{600} reading by the dilution factor (x4), from which the value of the blank reading is subtracted. Normalize the resulting values by dividing by 0.55 to obtain a calculated path-length of 1 cm according to the Beer-Lambert law. Note, this value is constant when measuring the OD_{600} of 200 µl of cells in a standard 96-well plate.
   4. Calculate the exact volume needed for obtaining yeast cells at amount equivalent to OD_{600} of 0.25 and transfer it to an Eppendorf tube.
   5. Spin down the cells at high speed (12,000 x g) for 1 min.
   6. Remove the supernatant and resuspend the cells in 1 ml of the appropriate selective medium (see section 3.1) to obtain OD_{600} of 0.25.
   7. Transfer 200 µl of the diluted strains to a new well in a 96-well plate.

2. When a large number of samples (N>15) are to be tested, dilute the cells and replace the medium without prior OD_{600} measurement, as follows:
   1. Transfer 10 µl of stationary cells grown O/N in a 96-well plate into a new 96-well plate containing 190 µl (dilution 1:20) of the appropriate selective media (see section 3.1).

3. Growth Kinetics in Liquid Culture Under Selective Conditions (GiLS)

1. Use the following media for growth measurements using the Ura3-degron reporter:
   1. For positive controls of yeast growth under non-restrictive conditions, use plasmid selective SD media (see section 1.2). If no plasmid selection is required, for example, when using an integrative plasmid such as *Deg1*-UV, SD medium containing all necessary amino acids (SD complete) can be used.
   2. For experimental samples measuring growth under restrictive conditions, use SD-URA medium.

2. Set a multimode microplate reader to: an incubation temperature of 30 °C, OD_{600} measurement intervals of 15 min, and an orbital and linear shaking cycles of 1 min every seven min.
   NOTE: This dual-mode shaking was optimized to obtain homogenous distribution of cells; however, other shaking modes or even no shaking may work as well.
3. Incubate the cells at 30 °C in a microplate reader O/N or until cells have reached the stationary phase (12-24 hr).
4. Export the raw data to a spreadsheet file.
5. Determine yeast growth kinetics using MDTcalc, a customized software program that calculates the minimal time (in hr) for cells to double their population size (Minimal Doubling Time (MDT); for details see section 4).

4. Calculation of Minimal Doubling Time (MDT): Principles of the MDTcalc Algorithm

NOTE: Principles of the MDTcalc algorithm, using a representative sample of Try467 yeast cells (**Table 2**) grown in SD complete media, are illustrated in **Table 1** and **Figure 1**, showing a data spreadsheet and growth plots, respectively.

1. Subtract OD_{600} blank values, obtained in the respective well, from each of the readings obtained in the sample well. Divide the resulting differences by 0.55 (for 96-well plates) to correct for the light path length. Plot the final values (**Table 1**, Trans.) against time to produce the actual yeast growth curve (OD_{600}/hr) (**Figure 1A**).
2. Calculate $\log_2 (OD_{600})$ for each of the transformed values to convert the logarithmic growth phase into a linear curve (**Figure 1B**).
3. Calculate the slope of a 10 time-points interval (N=10) starting at time 0 and moving one time-point at a time until N<10 (**Table 1**, Slope; **Figure 1C**).
4. Calculate the inverse value of each slope to obtain the time required for the cell population to double (**Table 1**, 1/Slope; **Figure 1C**). Calculate the minimal 1/Slope value to determine the MDT (**Table 1**, black rectangle; **Figure 1C**). Extract the calculated MDT for cells in the sample (**Table 1**, 1/Slope, black rectangle) from the selected time interval (**Table 1**, outlined by red rectangle, **Figures 1B, 1D**).
   NOTE: The rate of cell growth on SD-URA medium is inversely proportional to the MDT.

5. Using MDTcalc

1. Copy the supplemented program files into a designated folder in the computer.
2. Make sure the spreadsheet file containing the growth data is saved and closed. Open the MDTcalc application program to see the start screen appears.
   1. Insert the required information, as demonstrated in **Figure 2**:
      1. Under 'File path', click on the rectangle on the right to locate the spreadsheet file.
      2. Under 'Sheet name', write the spreadsheet name where the raw data is located.
      3. Under 'Starting position', insert the spreadsheet coordinate of time 0 of the first sample.
      4. Under 'Time column', insert the letter defining the location of the time point column (time should be provided in seconds).
      5. Under 'Blank column', insert the letter defining the location of the blank column (usually but not necessarily in well A1 of the 96-well plate).
      6. Under 'OD value', choose the minimal transformed OD_{600} value required for the initiation of MDT calculation (usually 0.15-0.25). Setting this value prevents calculations of MDT for cultures in lag phase due to excessive dilution so that MDT is calculated only for samples that reach the defined OD_{600} value or higher.
   2. Once the last value has been entered, press the 'calculate' button at the bottom of the screen. Ensure that the progress bar on the right gradually changes to green. Do not open the spreadsheet file during the calculation process.
   3. Export the two sets of data that appear automatically on the screen at the completion of the MDT calculation into a spreadsheet. Ensure that the data includes: (a) the MDT value for each well that passes the minimal OD value test (as set in section 5.2.1.6) and (b) the initial time point of the interval from which the MDT was calculated (Start time).

Representative Results

Investigating the role of enzymes of the Doa10 pathway in the degradation of a reporter substrate

To test the validity of the GiLS method it was compared to a traditional degradation assay. This experiment assesses the relative contribution of components of the ER-membrane localized Doa10 E3-ligase complex[20,21] to the degradation of the protein quality control reporter substrate *Deg1*-VU (**Figure 3A**). The *Deg1*-VU plasmid was integrated into *wild type* cells or into cells lacking the gene for the E2 conjugating enzyme, *UBC7* (*ubc7*) and protein stability was subsequently determined by a CHX chase assay[6]. Anti-FLAG immunoblotting revealed a stable *Deg1*-VU protein band in *ubc7* cells that was absent in *wild type* cells (**Figure 3B**). The absence of *Deg1*-VU in *wild type* cells is attributed to its continuous degradation that was abolished in *ubc7* cells, indicating that *Deg1*-VU stability is strongly dependent on Ubc7.

Obviously, the degradation of *Deg1*-VU is rapid. However, the relative rate of degradation cannot be determined by a CHX assay since the protein is undetectable in *wild type* cells from the outset. Consequently, the newly-developed GiLS assay method was employed, in order to compare the relative differences in the kinetics of protein degradation between wild-type and various mutant strains (**Figure 3C**). Initially, *wild type, ubc6, ubc7* or *doa10* cells expressing *Deg1*-VU were grown on SD complete medium. To measure GiLS, cells were washed and incubated in SD-URA. MDT calculation was executed as exemplified in the protocol. **Figures 3C, 3D** show similar growth curves and calculated MDT (~2.5 hr) for all strains grown in SD complete, excluding the possibility that the deletion of any of the examined genes affects cell growth. In contrast,

incubation in SD-URA resulted in poor growth of *wild-type* cells (MDT=6.34), whereas only a minor growth defect was observed in the mutant strains (MDT ~3 hr).

Examining the effect of different mutants of the E2 conjugating enzyme Ubc7 on the degradation of *Deg1*-VU

The fact that Ubc7 is absolutely required for *Deg1*-VU degradation enables accurate assessment of even partial effects of various E2 mutants, since the experimental range of the system is very wide. Accordingly, plasmids containing *wild type* or mutant *UBC7* were integrated into *Ubc7* cells expressing *Deg1*-VU and their contribution to degradation was determined by GiLS. As expected, fast growth kinetics were observed in cells expressing Ubc7 with the active-site mutants C89S and N81A[22] (**Figure 4**). In addition, two mutants predicted to indirectly hinder *Deg1*-VU degradation (V25G and H94K) were tested. These mutations also enhanced cell growth, compared to *wild-type* Ubc7, albeit to a lesser degree than the active-site mutants (average MDTs of 3.4 hr for V25G and H94K compared to average MDTs of 2.7 hr for C89S and N81K) (**Figure 4**). Thus, the GiLS method can be used for accurate measurement of the relative contribution of various degradation factors to the stability of a reporter substrate.

Isolation and evaluation of novel degrons

The GiLS system was also employed in a high throughput screen format to identify novel degrons and quantify their relative potency, *i.e.*, the degree by which they induce degradation (**Figure 5**). To optimize the identification of bona fide degrons, an additional selection step, which is growth in the presence of Fluoroorotic Acid (5-FOA), was added. Ura3 efficiently converts 5-FOA into a toxic compound (5-fluorouracil) that can serve as a positive selection for the isolation of yeast strains where Ura3 is destabilized[16,23]. To identify novel degrons, a library of reporter plasmids was generated by fusing random fragments derived from a yeast cDNA library to Ura3-GFP plasmid[24] (**Figure 5A**). The GFP moiety was added in order to enable a secondary screen and provide information about the localization of the fusion degron (see discussion). The library was transformed into yeast, then selected in the presence of 5-FOA (**Figure 5B**) and positive clones were isolated and re-seeded on agar plates in a 96-well plate format. Each colony was transferred into 96-well plate, incubated O/N, and diluted in a new 96-well plate as described in section 2.2. It is expected that growth of cells in SD-URA medium will correlate with the degree of Ura3 expression, which is the outcome of the degron's potency to induce degradation. This was subsequently confirmed by applying GiLS (**Figure 5C**). We confirmed that all randomly-picked colonies that were pre-selected with 5-FOA showed similar MDT values, on SD-LEU medium (**Figure 5C**, empty bars). In contrast, all clones showed variable growth rates on SD-URA, which were significantly slower than cells expressing control Ura3-GFP (Data not shown). As indicated, there is an inverse relationship between growth rate on SD-URA and MDT. Thus, the higher the MDT, the more potent is the degron. Indeed, all MDTs derived from positively selected clones were higher than that of the control (above the red line) (**Figure 5C**, filled bars).

Time (hr)	Blank	Sample	Trans.	Log$_2$	Slope	1/Slope
0.00	0.16	0.28	0.22	-2.19	0.463	2.16
0.27	0.16	0.28	0.21	-2.23	0.524	1.91
0.54	0.16	0.28	0.22	-2.18	0.552	1.81
0.81	0.15	0.29	0.24	-2.05	0.560	1.79
1.08	0.15	0.30	0.26	-1.93	0.564	1.77
1.35	0.15	0.31	0.29	-1.79	0.560	1.79
1.62	0.15	0.33	0.32	-1.63	0.551	1.82
1.89	0.15	0.35	0.36	-1.46	0.538	1.86
2.17	0.15	0.37	0.40	-1.31	0.525	1.90
2.44	0.15	0.40	0.45	-1.17	0.510	1.96
2.71	0.15	0.42	0.50	-1.01	0.490	2.04
2.98	0.15	0.45	0.55	-0.86	0.470	2.13
3.25	0.15	0.49	0.61	-0.71	0.451	2.22
3.52	0.15	0.52	0.68	-0.56	0.429	2.33
3.79	0.15	0.56	0.74	-0.42	0.409	2.44
4.06	0.15	0.60	0.82	-0.29	0.390	2.56
4.33	0.15	0.64	0.89	-0.17	0.373	2.68
4.60	0.15	0.68	0.97	-0.05	0.356	2.81
4.87	0.15	0.73	1.05	0.07	0.339	2.95
5.14	0.15	0.77	1.13	0.18	0.320	3.13
5.41	0.15	0.82	1.22	0.29	0.295	3.38
5.68	0.15	0.87	1.31	0.39	0.267	3.75
5.95	0.15	0.92	1.40	0.48		
6.23	0.15	0.97	1.49	0.58		
6.50	0.15	1.02	1.58	0.66		
6.77	0.15	1.07	1.68	0.75		
7.04	0.15	1.12	1.77	0.82		
7.31	0.15	1.17	1.86	0.90		
7.58	0.15	1.21	1.94	0.95		
7.85	0.15	1.24	1.99	0.99		
8.12	0.15	1.26	2.03	1.02		

Table 1. Illustration of the calculation of yeast MDT. *Time* units are hours (hr). *Blank* and *sample* are from OD$_{600}$ reads. *Transformation* (Trans.) is the OD$_{600}$ value after blank subtraction and path-length correction. *Log$_2$* is calculated from the transformed data. *Slope* is the Log$_2$ (OD$_{600}$) values within consecutive of intervals of 10 time points divided by time. *1/Slope* is the time required for the cell population to duplicate itself. *Black rectangle* marks the MDT value. *Red rectangle* marks the time interval from which the MDT was calculated.

Yeast	Genotype	Source
TRy467	α, his3Δ1, lys2Δ0, ura3Δ0, leu2Δ0	Euroscarf
TRy508	α, his3-Δ200::pRS303/Ubc7prom-Ubc7-3HA::HIS, leu2-3,112, ura3-5, lys2-801::Met25-Deg1-Flag-Vma12-Ura3::LYS, trp1-1, ubc7Δ::LEU2	This study
TRy528	α, his3-Δ200::pRS303/Ubc7prom-Ubc7[N81A]-3HA::HIS, leu2-3,112, ura3-5, lys2-801::Met25-Deg1-Flag-Vma12-Ura3::LYS, trp1-1, ubc7Δ::LEU2	This study
TRy530	α, his3-Δ200::pRS303/Ubc7prom-Ubc7[C89S]-3HA::HIS, leu2-3,112, ura3-5, lys2-801::Met25-Deg1-Flag-Vma12-Ura3::LYS, trp1-1, ubc7Δ::LEU2	This study
TRy532	α, his3-Δ200::pRS303/Ubc7prom-Ubc7[H94K]-3HA::HIS, leu2-3,112, ura3-5,	This study

	lys2-801::Met25-Deg1-Flag-Vma12-Ura3::LYS, trp1-1, ubc7Δ::LEU2	
TRy556	*α, his3-Δ200::pRS303::HIS, leu2-3,112, ura3-5, lys2-801::Met25-Deg1-Flag-Vma12-Ura3::LYS, trp1-1, ubc7Δ::LEU2*	This study
TRy563	*α, his3-Δ200::pRS303/Ubc7prom-Ubc7-3HA::HIS, leu2-3,112, ura3-5, lys2-801::Met25-Deg1-Flag-Vma12-Ura3::LYS, trp1-1, ubc7Δ::LEU2, ubc6Δ::KanMX*	This study
TRy633	*α, his3-Δ200::pRS303/Ubc7prom-Ubc7[V25A]-3HA::HIS, leu2-3,112, ura3-5, lys2-801::Met25-Deg1-Flag-Vma12-Ura3::LYS, trp1-1, ubc7Δ::LEU2*	This study
TRy786	*α, his3-Δ200::pRS303/Ubc7prom-Ubc7-3HA::HIS, leu2-3,112, ura3-5, lys2-801::Met25-Deg1-Flag-Vma12-Ura3::LYS, trp1-1, ubc7::LEU, doa10Δ::HIS3*	This study

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

Plasmids used in this study		
Plasmid	**Relevant markers**	**Source**
pTR717	*YDpK-MET25p-Deg1-Flag-Vma12-Ura3 (integrative plasmid containing LYS1)*	This study
pTR1412	*pRS315-CUP1p-URA3-HA-GFP (LEU)*	This study
Additional suitable reporter plasmids		
Plasmid	**Relevant markers**	**Source**
pOC9-CL1	*pOC9-CUP1p-HA-URA3 (TRP1)*	(17)
URA3-2(GFP)	*pPS414-TRP1p-MYC-ura3-2-GFP*	Lewis and Pelham

Table 3. List of plasmids used in this study. Reference: Lewis, M. J. & Pelham, H. R. Inefficient quality control of thermosensitive proteins on the plasma membrane. *PLoS One* **4**, e5038, doi:10.1371/journal.pone.0005038 (2009).
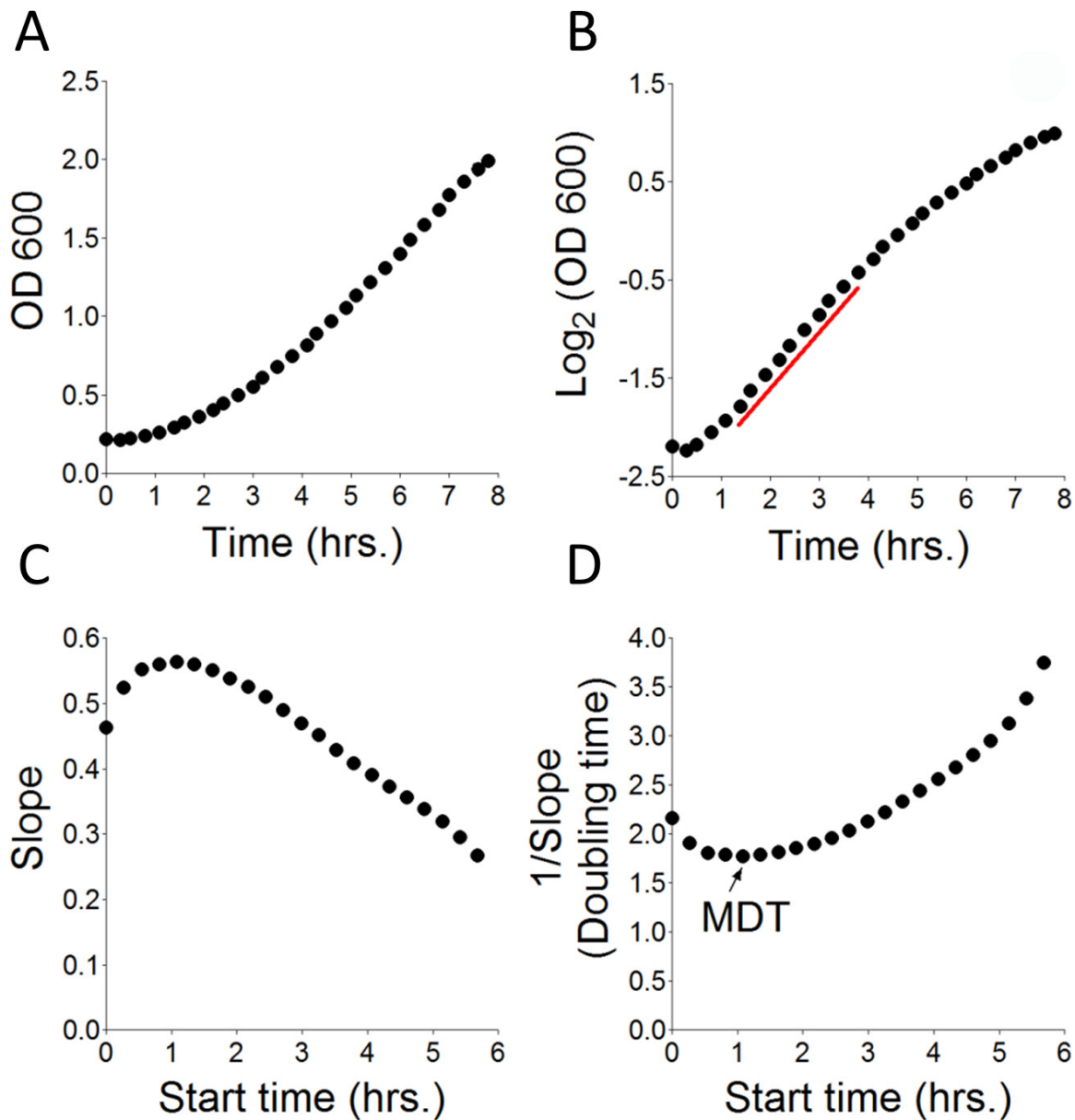
Figure 1. Principles of calculation of MDT. Try467 yeast cells at OD_{600} 0.22, were incubated in SD complete media for the indicated time period. OD_{600} measurements were taken every ~15 min and the data was collected in a spreadsheet. (**A**) Transformed OD_{600} values from **Table 1** plotted against time. (**B**) Log_2 of OD_{600} plotted against time. *Red line*: Marks the time interval from which the MDT was calculated (**D**). (**C**) Yeast growth rate (slope) was calculated by measuring changes in OD_{600} of consecutive 10 time points (assuming linearity) over time. *Start time*: The time from which calculation was initiated. (**D**) The time required for yeast to double is the inverse of the slope from (**C**) (1/Slope). *MDT*: The calculated Minimal Doubling time (in hr).
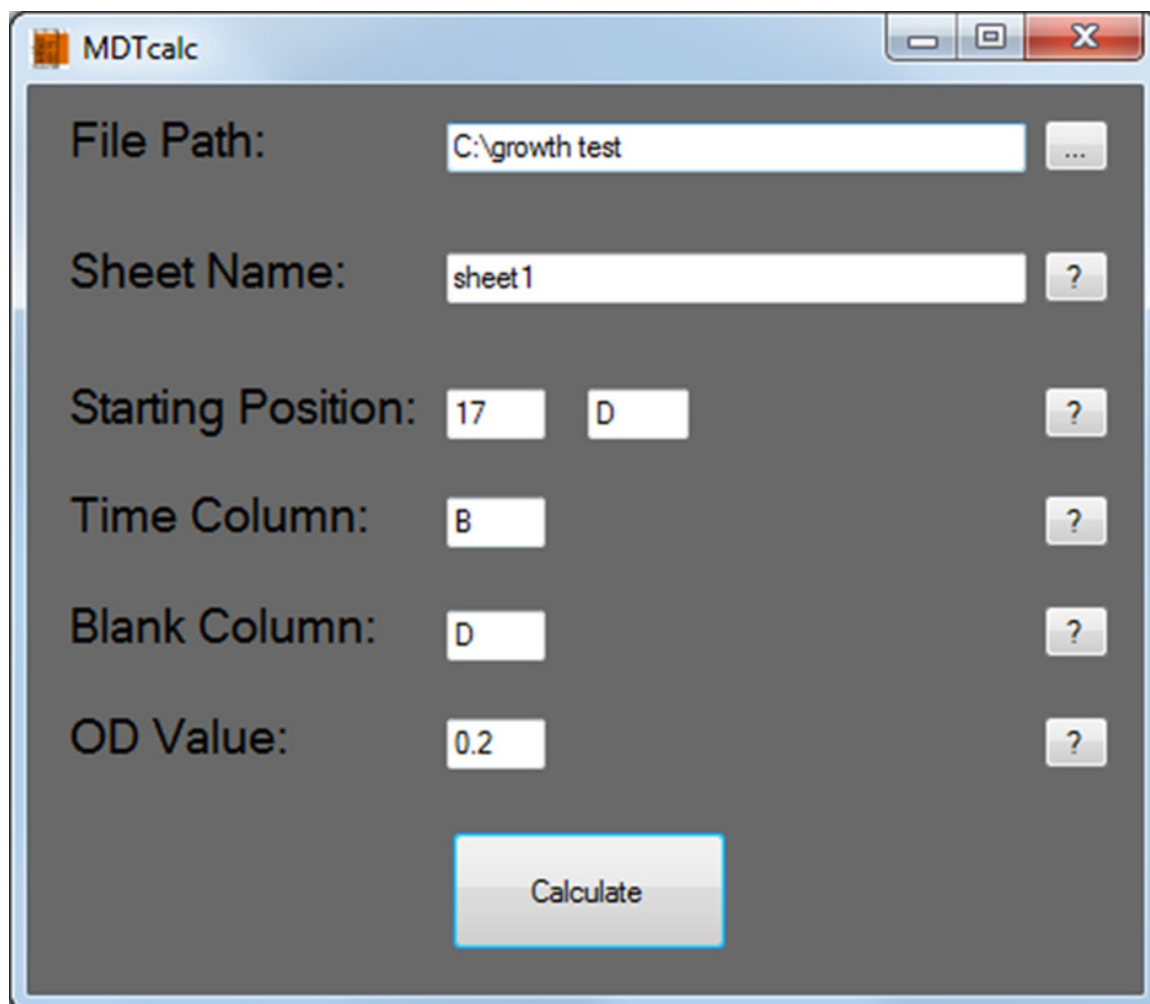
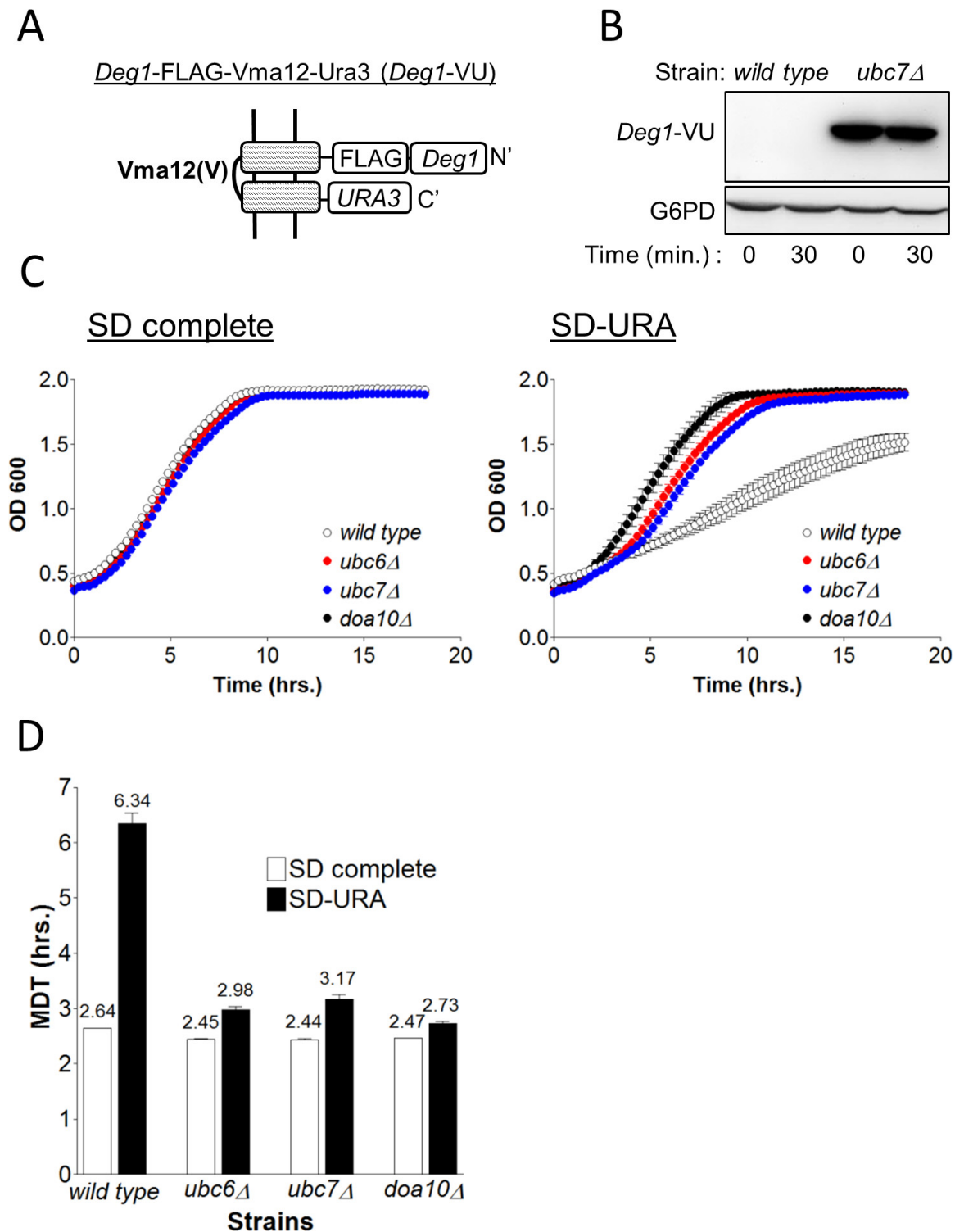**Figure 2. MDTcalc start screen:** Protocol section 5.1. Typical input values to be entered are shown.

Figure 3. Measuring the degradation of *Deg1*-VU. (**A**) Schematic presentation of the different protein elements of *Deg1*-VU and its organization at the ER membrane.(**B**) Determination of the degradation of *Deg1*-VU by the CHX assay in *wild type* and *ubc7* cells. Cells collected at time zero or after 30 min with CHX were lysed and proteins were separated by 5-15% SDS PAGE. *Deg1*-VU was visualized by immunoblotting using anti FLAG antibodies. G6PD staining served as a loading control. (**C**) The indicated strains, expressing *Deg1*-VU were incubated in SD-complete or SD-URA media for 20 hr and OD_{600} was measured every 15 min. OD_{600} are the transformed values. (**D**) The MDT for each strain was calculated using MDTcalc.
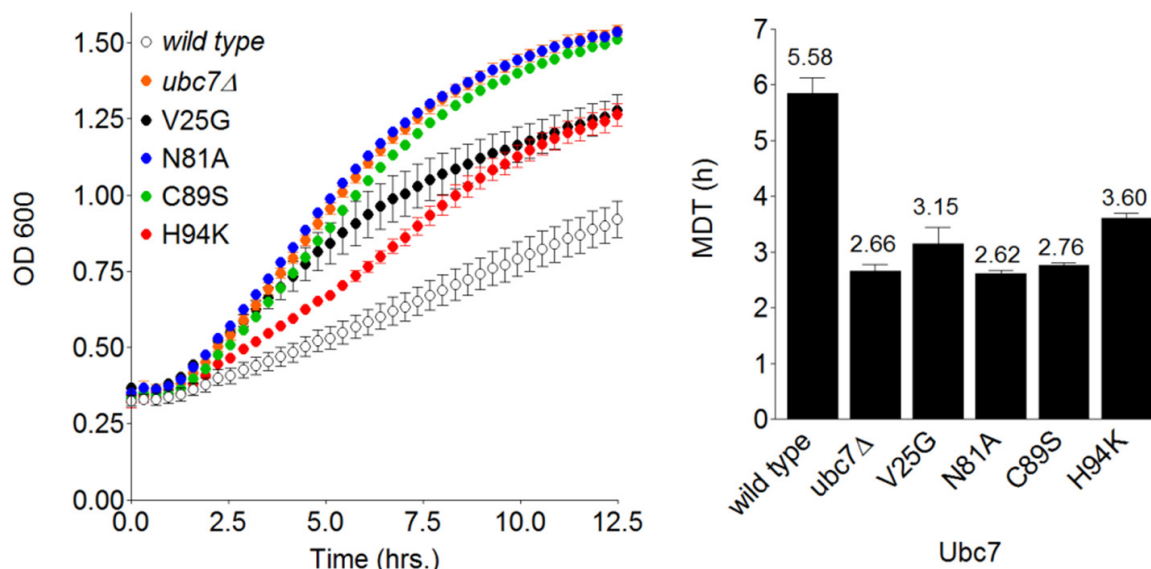
Figure 4. Measuring relative degradation rates of *Deg1*-VU in Ubc7 mutant strains. Left: Plot of the transformed OD$_{600}$ values, measured during the replication of w*ild-type, ubc7Δ* and the indicated Ubc7 mutants, versus time. Cells expressing *Deg1*-VU were grown on SD-URA medium and OD$_{600}$ was measured every ~15 min. Right: The MDT for each strain was calculated using MDTcalc.
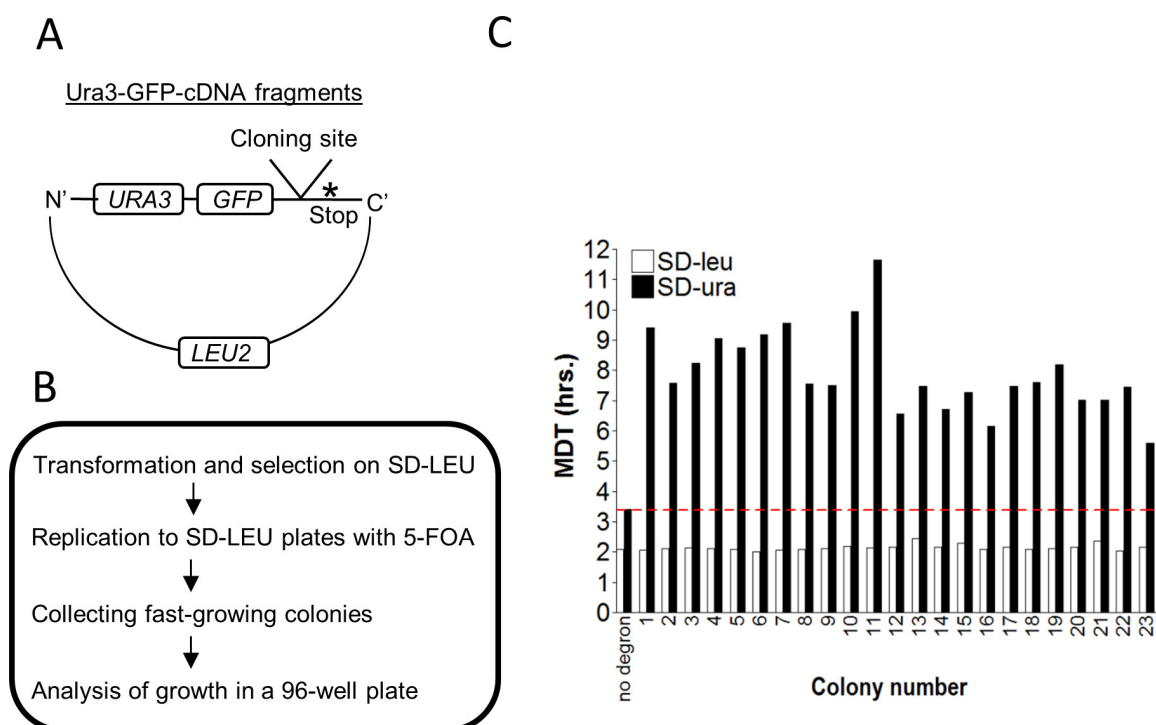

Figure 5. A high throughput assay for identification and isolation of novel degrons. (**A**) Schematic presentation of the features of the Ura3-GFP-cDNA reporter. (**B**) A flow chart describing the experimental steps for the isolation of yeast strains carrying a degron. The plasmid library described in A was transformed into Try467 cells, followed by selection on SD-LEU plates. Colonies were replicated to SD-LEU plates containing 5-FOA and fast-growing colonies were collected and organized on plates in an ordered array. (**C**) Colonies expressing Ura3-GFP-cDNA that grew on plates containing 5-FOA were incubated in either SD-LEU or SD-URA media for 20 hr. OD$_{600}$ was measured every ~15 min and the minimal yeast doubling time (MDT) was calculated using the MDTcalc software. *No degron*: a plasmid expressing Ura3-GFP without a degron, serves as a positive control for growth. *Red line*: threshold determined by the MDT value of the control.

Discussion

Here we describe an assay based on cell growth for determining relative protein degradation rates, termed 'Growth kinetics in Liquid culture under Selective conditions' (GiLS). The GiLS assay has several advantages: It is simple to set up, data acquisition and analysis is straightforward and it is extremely modular. Consequently, GiLS can be applied simultaneously to multiple samples in a user friendly multi-well plate format that can be adapted to automation for high throughput applications. Most importantly, GiLS provides highly reproducible,

quantitative and robust data and is more flexible than agar-plate based-growth assays that select for positive/negative growth. In addition, GiLS is highly sensitive in that it can detect small variations in protein degradation rates or steady-state levels, reflecting the activity of relevant UPS components or degrons. Finally, GiLS assays require significantly shorter growth periods (<12 hr) compared to growth assays on agar plates (typically 2-4 days).

While the GiLS assay is very useful for studying protein degradation, several important considerations should be taken into account. For example, it is essential to use an isogenic yeast strain background due to strain-specific growth characteristics. Furthermore, to ascertain assay validity, each assay should be repeated at least three times under identical conditions, most importantly, the initial cell density (OD_{600}), growth temperature, and cell culture shaking intensity should not be altered. It is therefore recommended that the specific assay protocol is stored in the microplate reader's software for repeated uses. An additional variable that should be considered is where the degron is positioned within Ura3. As the degron position may determine its function, positioning at either the C' or N' regions must be empirically tested.

Calibration of reporter protein expression level is also critical for successful application of GiLS. An optimal reporter is one that is efficiently degraded by the system under investigation and thus confers uracil auxotrophy. However, variations in auxotrophy should also faithfully mirror the reporter stability. This can be determined empirically by prior testing of the correlation between reporter protein degradation rates, determined by additional biochemical methods, and MDT values, determined by GiLS (**Figure 3B**). One common problem arises when the basal steady-state expression of a reporter does not reach a threshold level and is thus too low to enable growth on SD-URA even when it is completely stabilized. Such a problem can be overcome by placing the reporter expression under the control of an inducible promoter that increases basal protein expression without affecting degradation. Promoter activity in such systems should be finely calibrated to avoid permanent growth on SD-URA due to high protein levels and reporter enzyme activity even when it is partially degraded. The promoters and expression conditions described in this study were chosen because they allow sensitive regulation of protein expression: *MET25p* and *CUP1*p are metabolite-induced promoters the activity of which can be finely tuned through control of methionine and copper, respectively (**Table 3**).

Quality control reporter substrates (as those described in this study) can form intracellular aggregates that might sequester Ura3 and prevent its function. In such a case uracil auxotrophy can be mistakenly interpreted as the result of protein degradation. To test the tendency of different degrons to aggregate, an additional reporter should be employed. For example, the Ura3-GFP reporter designed for the degron screen (**Figure 5A**), enables visualization of intracellular aggregates by fluorescent microscopy of cells growing in the presence of 5-FOA. These aggregates appear as dense GFP foci that are clearly distinguishable from the diffuse appearance of the soluble protein (data not shown). The Ura3-GFP-degron reporter can be similarly used to determine the effect of the degron on protein co-localization and subcellular distribution. It can also be used for following the degradation of selected substrates by flow cytometry, as a secondary screen[25].

A most significant advantage of the GiLS method is that it is sufficiently flexible and thus can easily be adapted to a variety of applications in addition to those described in herein. For example, the method can also be used for examining the fitness of the proteasome under various conditions. In this case, a non-ubiquitylated proteasome substrate such as ornithine decarboxylase (ODC) is fused to Ura3. The method can also test protein degradation in distinct intracellular compartments when a specific localization signal is employed. In this study Vma12 anchored the reporter to the ER-membrane (**Figures 3, 4**). Similarly, a nuclear localization signal (NLS), ER retention signal (KDEL) or cytosol (absence of a specific signal) can be employed. A system enabling simultaneous analysis of protein degradation in various intracellular compartments could have profound impact on our understanding of how protein degradation networks operate. Finally, the principles of the selection method can be applied to research of protein degradation in other model cell-based systems, from bacteria to human cells, all of which require the *URA3* gene product for survival.

Disclosures

The authors have nothing to disclose.

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References

1. Hershko, A., & Ciechanover, A. The ubiquitin system. *Annual review of biochemistry.* **67**, 425-479 (1998).
2. Ravid, T., & Hochstrasser, M. Diversity of degradation signals in the ubiquitin-proteasome system. *Nature reviews. Molecular cell biology.* **9**, 679-689 (2008).
3. Willems, A. R. *et al.* SCF ubiquitin protein ligases and phosphorylation-dependent proteolysis. *Philos Trans R Soc Lond B Biol Sci.* **354**, 1533-1550, doi:10.1098/rstb.1999.0497 (1999).
4. Tyers, M., & Jorgensen, P. Proteolysis and the cell cycle: with this RING I do thee destroy. *Curr Opin Genet Dev.* **10**, 54-64, doi:S0959-437X(99)00049-0 [pii] (2000).
5. Johnson, P. R., Swanson, R., Rakhilina, L., & Hochstrasser, M. Degradation signal masking by heterodimerization of MATα2 and MAT **a**1 blocks their mutual destruction by the ubiquitin-proteasome pathway. *Cell.* **94**, 217-227 (1998).
6. Furth, N. *et al.* Exposure of bipartite hydrophobic signal triggers nuclear quality control of Ndc10 at the endoplasmic reticulum/nuclear envelope. *Molecular Biology of the Cell.* **22**, 4726-4739, doi:10.1091/mbc.E11-05-0463 (2011).
7. Fredrickson, E. K., Gallagher, P. S., Clowes Candadai, S. V., & Gardner, R. G. Substrate recognition in nuclear protein quality control degradation is governed by exposed hydrophobicity that correlates with aggregation and insolubility. *J Biol Chem.* doi:10.1074/jbc.M112.406710 (2013).

8. Rosenbaum, J. C. *et al.* Disorder Targets Misorder in Nuclear Quality Control Degradation: A Disordered Ubiquitin Ligase Directly Recognizes Its Misfolded Substrates. *Molecular cell.* **41**, 93-106 (2011).
9. Ciechanover, A., Hod, Y., & Hershko, A. A heat-stable polypeptide component of an ATP-dependent proteolytic system from reticulocytes. *Biochemica., & Biophysical Research Communications*. **81**, 1100-1105 (1978).
10. Finley, D., Ulrich, H. D., Sommer, T., & Kaiser, P. The ubiquitin-proteasome system of Saccharomyces cerevisiae. *Genetics.* **192**, 319-360, doi:10.1534/genetics.112.140467 (2012).
11. Klis, F. M., Boorsma, A., & De Groot, P. W. Cell wall construction in Saccharomyces cerevisiae. *Yeast.* **23**, 185-202, doi:10.1002/yea.1349 (2006).
12. Zhou, W., Ryan, J. J., & Zhou, H. Global analyses of sumoylated proteins in Saccharomyces cerevisiae. Induction of protein sumoylation by cellular stresses. *J Biol Chem.* **279**, 32262-32268 (2004).
13. Alani, E., & Kleckner, N. A new type of fusion analysis applicable to many organisms: protein fusions to the URA3 gene of yeast. *Genetics.* **117**, 5-12 (1987).
14. 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 Dev.* **15**, 2660-2674. (2001).
15. Ravid, T., Kreft, S. G., & Hochstrasser, M. Membrane and soluble substrates of the Doa10 ubiquitin ligase are degraded by distinct pathways. *EMBO J.* **25**, 533-543, doi:7600946 [pii] 10.1038/sj.emboj.7600946 (2006).
16. Gilon, T., Chomsky, O., & Kulka, R. G. Degradation signals for ubiquitin system proteolysis in Saccharomyces cerevisiae. *Embo J.* **17**, 2759-2766 (1998).
17. Hochstrasser, M., & Varshavsky, A. *In vivo* degradation of a transcriptional regulator: the yeast #2 repressor. *Cell.* **61**, 697-708 (1990).
18. Metzger, M. B., Maurer, M. J., Dancy, B. M., & Michaelis, S. Degradation of a cytosolic protein requires endoplasmic reticulum-associated degradation machinery. *J Biol Chem.* **283**, 32302-32316, doi:M806424200 [pii] 10.1074/jbc.M806424200 (2008).
19. Guthrie, C., & Fink, G. R. *Guide to Yeast Genetics and Molecular Biology.* **194** Academic Press (1991).
20. Carvalho, P., Goder, V., & Rapoport, T. A. Distinct ubiquitin-ligase complexes define convergent pathways for the degradation of ER proteins. *Cell.* **126**, 361-373 (2006).
21. Denic, V., Quan, E. M., & Weissman, J. S. A luminal surveillance complex that selects misfolded glycoproteins for ER-associated degradation. *Cell.* **126**, 349-359 (2006).
22. Wu, P. Y. *et al.* A conserved catalytic residue in the ubiquitin-conjugating enzyme family. *Embo J* **22**, 5241-5250 (2003).
23. Boeke, J. D., Trueheart, J., Natsoulis, G., & Fink, G. R. 5-Fluoroorotic acid as a selective agent in yeast molecular genetics. *Methods Enzymol.* **154**, 164-175 (1987).
24. Alfassy, O. S., Cohen, I., Reiss, Y., Tirosh, B., & Ravid, T. Placing a disrupted degradation motif at the C terminus of proteasome substrates attenuates degradation without impairing ubiquitylation. *J Biol Chem.* **288**, 12645-12653, doi:10.1074/jbc.M113.453027 (2013).
25. Cronin, S. R., & Hampton, R. Y. Measuring protein degradation with green fluorescent protein. *Methods in Enzymology.* **302**, 58-73 (1999).