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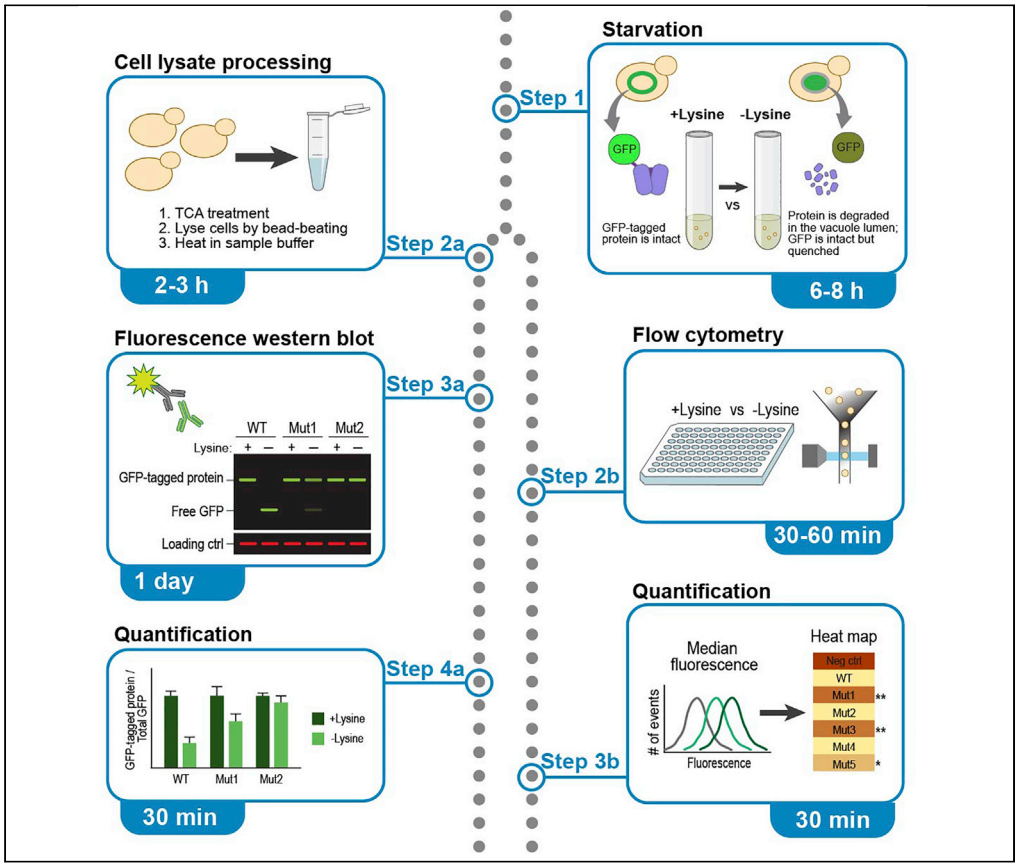
An optimized protocol to analyze membrane protein degradation in yeast using quantitative western blot and flow cytometry



Membrane proteins (MPs) are essential in many cellular functions. To maintain proteostasis, MPs are downregulated via ubiquitination and degradation. Here, we describe an optimized protocol to analyze MP degradation using quantitative western blot and flow cytometry-based approaches. We use the degradation of Ypq1, a vacuole membrane lysine transporter, to demonstrate the protocol, which can be adapted for other organelle MPs and thus provide useful tools to study MP regulation in yeast and other model organisms.

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Highlights

Two complementary protocols for quantifying membrane protein degradation in yeast

Measures the decrease of full-length protein by quantitative western blot

Quantifies the quenching of GFP fluorescence by flow cytometry

Adaptable to other fluorescent protein tags and mammalian cells

Arines & Li, STAR Protocols *3*, 101274
June 17, 2022
https://doi.org/10.1016/j.xpro.2022.101274

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An optimized protocol to analyze membrane protein degradation in yeast using quantitative western blot and flow cytometry

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https://doi.org/10.1016/j.xpro.2022.101274

SUMMARY

Membrane proteins (MPs) are essential in many cellular functions. To maintain proteostasis, MPs are downregulated via ubiquitination and degradation. Here, we describe an optimized protocol to analyze MP degradation using quantitative western blot and flow cytometry-based approaches. We use the degradation of Ypq1, a vacuole membrane lysine transporter, to demonstrate the protocol, which can be adapted for other organelle MPs and thus provide useful tools to study MP regulation in yeast and other model organisms.
For complete details on the use and execution of this protocol, please refer to Arines et al. (2021) and Yang et al. (2020).

BEFORE YOU BEGIN

To maintain proteostasis, cells undergo protein quality control, a process wherein misfolded, mislocalized, or orphan proteins are identified and degraded (Sun and Brodsky, 2019; Sardana and Emr, 2021; Natarajan et al., 2020). Furthermore, cells need to regulate the quantity of MPs in response to changing environmental cues. Many MPs are degraded in response to starvation or excess of their substrates (Arines et al., 2021; Gournas et al., 2010, 2017; Guiney et al., 2016; Keener and Babst, 2013; Li et al., 2015a, 2015b; Lin et al., 2008). For example, plasma membrane methionine transporter Mup1 and uracil transporter Fur4 are degraded when methionine and uracil, respectively, are in excess (Henne et al., 2012; Keener and Babst, 2013). Meanwhile, the lysine transporter Ypq1 and zinc transporter Cot1 at the vacuole membrane are degraded upon lysine or zinc starvation (Li et al., 2015a, 2015b). Furthermore, many membrane proteins were shown to be simultaneously degraded following nutrient starvation and autophagy (Muller et al., 2015; Yang et al., 2020). Ubiquitin ligases identify these proteins and tag them with ubiquitin to initiate the degradation through either the proteasome or by trafficking them into the vacuole (yeast lysosome) lumen.

In Arines et al. (2021), we studied how Ypq1 is selectively recognized by the vacuolar E3 ubiquitin ligase complex (Ssh4-Rsp5) only after lysine withdrawal (Figures 1A–1C). We generated a library of over 300 point mutants and used western blot and flow cytometry to quantify their degradation defects. The end goal was to identify what residues and regions on Ypq1 could be involved in its regulation. Here, we describe the details of these methods to aid researchers in studying membrane protein regulation.

Other applications of this method include:

1. Identifying genes that are involved in the degradation of the protein of interest (Li et al., 2015a, 2015b).

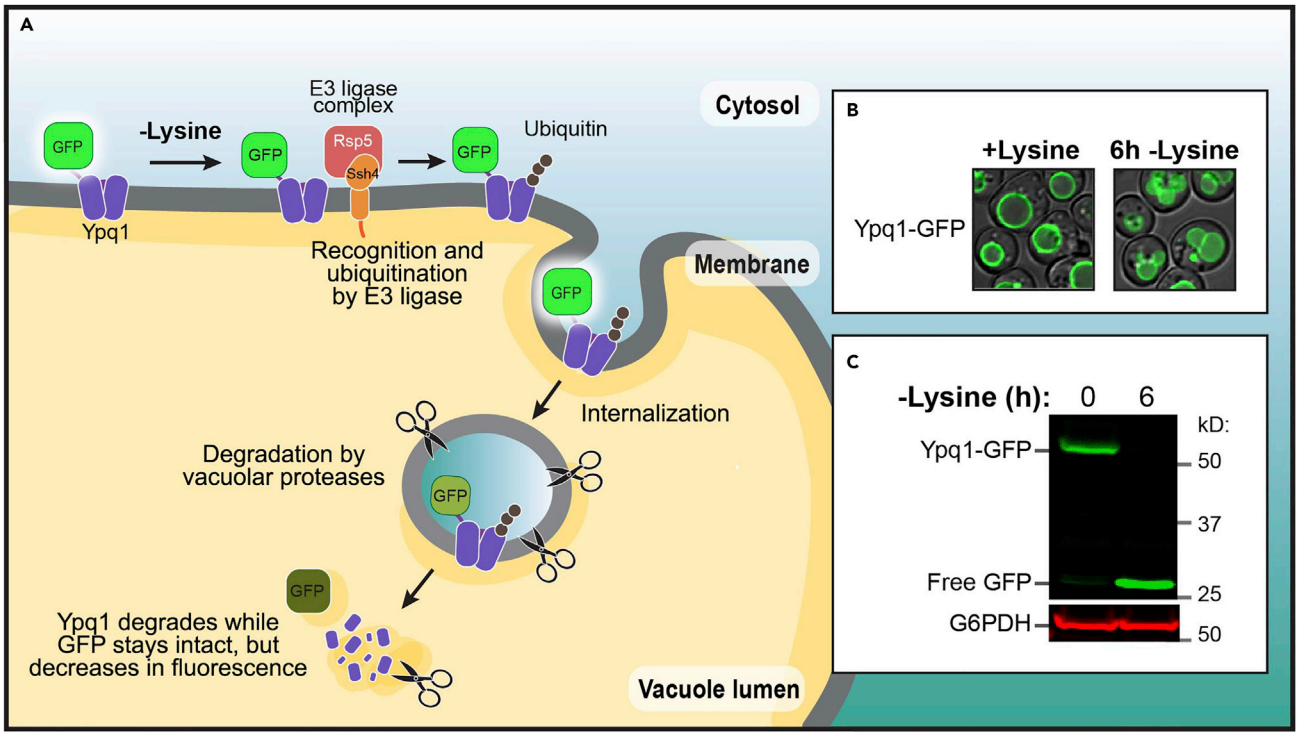

Figure 1. Ypq1-GFP degradation can be quantified using GFP fluorescence

(A) Ypq1 is a lysine transporter on the vacuole membrane. When cells are starved of lysine, Ypq1-GFP is ubiquitinated by an E3 ligase complex composed of Ssh4 and Rsp5. Ubiquitinated Ypq1-GFP is internalized into the vacuole lumen, where it is degraded by vacuolar proteases. Ypq1 is broken down, whereas GFP stays intact but decreases slightly in fluorescence.

(B) Before starvation, Ypq1-GFP localizes on the vacuole membrane. Upon lysine starvation, Ypq1-GFP is internalized and degraded.

(C) On a western blot, full-length Ypq1-GFP is visible as a ~64 kDa band. Upon lysine starvation, the full-length band disappears, whereas a smaller band accumulates, corresponding to free GFP. Glucose-6-phosphate dehydrogenase (G6PDH) is used as a loading control.

2. Testing for metabolic conditions that lead to the degradation of the protein of interest (Yang et al., 2021).

3. Studying the response of other MPs such as on the vacuole membrane (Yang et al., 2020), plasma membrane (Arines et al., 2021), and other membrane compartments.

Note: Other considerations for this method are as follows:

a. This protocol works well for MPs degraded by the vacuole. However, it is not suited for MPs that are partially or completely degraded through the proteasome because the GFP moiety will be destroyed. The following experiments can help determine if the GFP-tagged protein undergoes vacuolar or proteasomal degradation:
   i. Check the protein localization using fluorescence microscopy.
   ii. Delete PEP4, which encodes a major vacuolar protease. If the protein is degraded in wild-type but is intact in *PEP4Δ*, then the protein is degraded by the vacuole (Oku et al., 2017; Yang et al., 2020).
   iii. Treat cells with the proteasome inhibitor MG-132 (Lee and Goldberg, 1996).
b. When tagging a new protein with GFP, the researchers must test whether the degradation is due to the treatment and not simply due to a defective GFP fusion protein. Using microscopy, the non-treated cell should show no signs of delivery to the vacuole lumen.
c. Other GFP variants (e.g., pHluorin) and other fluorophores (e.g., mCherry, mKeima) may also work for western blot analysis, as long as a specific antibody is available. The protein of interest

can be tagged N-terminally or C-terminally, as long as the tag is facing the cytosol. Thus, the researcher should have some knowledge about the topology of the protein.

d. This protocol relies on a functional lytic vacuole and so background yeast strains should have the vacuolar proteases (e.g., Pep4, Prb1, and Prc1) intact. Standard background strains such as SEY6210 or BY4741 can be used. The vacuole should also be acidic and so modifications on genes that could affect vacuole acidification (e.g., v-ATPase genes) should be avoided.

e. This protocol has the potential to be adapted for degradation studies of MPs in mammalian cells.

⚠ CRITICAL: This protocol assumes the user has basic knowledge of yeast culture, western blot, and flow cytometry.

Prepare yeast strains

Timing: Operation (1–3 h), Incubation (1–2 days)

4. Tag your protein of interest with GFP using the following published methods:
   a. At the genomic locus, using the standard Longtine PCR and transformation procedures (Longtine et al., 1998).
   b. On a plasmid, using standard cloning and transformation methods (Gietz and Schiestl, 2007).
   c. To generate point mutants using plasmid site-directed mutagenesis, we recommend using KOD Hot Start DNA Polymerase (Millipore-Sigma, Cat# 71086) and follow the manufacturer's instructions.
5. Using a sterile (autoclaved) toothpick, pick and streak a total of three colonies from each transformation to represent three biological replicates.
6. Grow the plates in a 30°C incubator for 1–2 days.

Prepare media and reagents

Timing: 1–2 days (as needed)

7. Prepare the required buffers (see materials and equipment below).

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit polyclonal anti-GFP	Torrey Pines Biolabs	Cat#TP-401; RRID: AB_10013661
Mouse monoclonal anti-Pgk1 (22C5D8)	Thermo Fisher Scientific	Cat#459250; RRID: AB_2532235
IRDye® 800CW goat anti-rabbit IgG secondary antibody	LI-COR	Cat#926-32211; RRID: N/A
IRDye® 680LT goat anti-mouse IgG secondary antibody	LI-COR	Cat#926-68020; RRID: N/A
Chemicals, peptides, and recombinant proteins		
Trichloroacetic acid	Fisher Scientific	Cat#SA433-500; RRID: N/A
Biological samples		
SEY6210 (*MATα leu2-3,112 ura3-52 his3-Δ200 trp1-Δ901 suc2-Δ9 lys2-801; GAL*)	Laboratory stock	RRID: N/A; https://www.atcc.org/products/201392
Software and algorithms		
Image Studio Software	LI-COR	RRID: SCR_015795; https://www.licor.com/bio/empiria-studio/
iQue Forecyt® Software	Sartorius	RRID: N/A; https://www.sartorius.com/en/products/flow-cytometry/flow-cytometry-software

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Other		
Odyssey® CLx Infrared Imaging System	LI-COR	RRID: SCR_014579; https://www.licor.com/bio/odyssey-clx/index
IntelliCyt® iQue Screener PLUS flow cytometer	Sartorius	RRID: N/A; https://intellicyt.com/resources/ique-screener-plus-manual/
KOD Hot Start DNA Polymerase	MilliporeSigma	Cat#71086
Vortex for bead beating	Fisher Scientific	Cat#02-215-365
6-inch platform head and microtube foam insert for bead beating	Scientific Industries	Cat#146-6005-00 (head) Cat#504-0233-00 (foam insert)
2-Gel Tetra and Blotting Module	Bio-Rad	Cat# 1660827EDU
Glass beads, acid-washed (425–600 μm (30–40 US sieve))	Sigma-Aldrich	Cat#G8772
Precision Plus Protein™ All Blue Prestained Protein Standards	Bio-Rad	Cat#1610373
Amersham Protran Supported 0.45 Nitrocellulose (300 mm × 4 m)	Cytiva	Cat#10600016
96-well plate	Thermo Scientific	Cat#167008

MATERIALS AND EQUIPMENT

Stock solutions

+Lys amino acids mix (20×)

Reagent	Amount	Final concentration
L-Adenine	200 mg	0.4 mg/mL
L-Arginine	200 mg	0.4 mg/mL
L-Lysine (or L-Lysine-HCl)	2,300 mg (3,450 mg)	4.6 mg/mL (6.9 mg/mL)
L-Threonine	3,000 mg	6 mg/mL
L-Tyrosine	300 mg	0.6 mg/mL
ddH_2O	to 500 mL	n/a
Total	500 mL	**n/a**

Solution can be heated in a microwave to help dissolve hydrophobic amino acids, but should not be allowed to boil. Filter-sterilize and store at 20°C–25°C.

-Lys amino acids mix (20×)

Reagent	Amount	Final concentration
L-Adenine	200 mg	0.4 mg/mL
L-Arginine	200 mg	0.4 mg/mL
L-Threonine	3,000 mg	6 mg/mL
L-Tyrosine	300 mg	0.6 mg/mL
ddH_2O	to 500 mL	n/a
Total	500 mL	**n/a**

Solution can be warmed in a microwave to help dissolve hydrophobic amino acids, but should not be allowed to boil. Filter-sterilize and store at 20°C–25°C.

Histidine (100×)

Reagent	Amount	Final concentration
L-Histidine	500 mg	2 mg/mL
ddH_2O	to 250 mL	n/a
Total	250 mL	**n/a**

Filter-sterilize and store at 20°C–25°C. Wrap the bottle in foil and keep away from light.

Leucine (100×)		
Reagent	Amount	Final concentration
L-Leucine	1,500 mg	6 mg/mL
ddH_2O	to 250 mL	n/a
Total	250 mL	**n/a**
Filter-sterilize and store at 20°C–25°C.		

Tryptophan (100×)		
Reagent	Amount	Final concentration
L-Tryptophan	500 mg	2 mg/mL
ddH_2O	to 250 mL	n/a
Total	250 mL	**n/a**
Filter-sterilize and store at 20°C–25°C. Wrap the bottle in foil and keep away from light.		

Uracil (100×)		
Reagent	Amount	Final concentration
Uracil	500 mg	2 mg/mL
ddH_2O	to 250 mL	n/a
Total	250 mL	**n/a**
Filter-sterilize and store at 20°C–25°C.		

Methionine (1000×)		
Reagent	Amount	Final concentration
L-Methionine	1,000 mg	20 mg/mL
ddH_2O	to 50 mL	n/a
Total	50 mL	**n/a**
Filter-sterilize and store at 20°C–25°C.		

YNB media		
Reagent	Amount	Final concentration
Yeast Nitrogen Base (YNB) without Amino Acids	6.7 g	1×
Glucose	20 g	2% w/v
ddH_2O	to 1 L	n/a
Total	1 L	**n/a**
Aliquot into 400 mL in reagent bottles. Autoclave to sterilize.		

Yeast culture and lysine starvation media

+Lys YNB uracil dropout media			
Reagent	Stock concentration	Amount	Final concentration
YNB media	n/a	400 mL	n/a
+Lys amino acids mix	20×	22 mL	1×
Histidine	100×	4.4 mL	1×
Leucine	100×	4.4 mL	1×
Tryptophan	100×	4.4 mL	1×
Methionine	1000×	0.44 mL	1×
Total	**n/a**	**435.64 mL (~440 mL)**	**n/a**

⚠ CRITICAL: Different yeast strains have different selection markers. For example, SEY6210 (*MATα leu2-3,112 ura3-52 his3-Δ200 trp1-Δ901 suc2-Δ9 lys2-801; GAL*) is defective for synthesizing Leucine, Uracil, Histidine, Tryptophan, and Lysine. Adjust the amino acid dropout according to the selection markers of your plasmids and yeast strain. Here, we use a uracil dropout because Ypq1 is expressed from a pRS416 (uracil selection) vector in the SEY6210 background.

Alternatives: Pre-mixed amino acid dropout media are also available and can be prepared following the manufacturer's recommendations.

-Lys YNB uracil dropout media (starvation media)

Reagent	Stock concentration	Amount	Final concentration
Yeast nitrogen base (YNB) without amino acids	n/a	400 mL	n/a
-Lys amino acids mix	20×	22 mL	1×
Histidine	100×	4.4 mL	1×
Leucine	100×	4.4 mL	1×
Tryptophan	100×	4.4 mL	1×
Methionine	1000×	0.44 mL	1×
Total	**n/a**	**435.64 mL (~440 mL)**	**n/a**

Reagents for sample processing for western blot

Boiling Buffer (2×)

Reagent	Stock concentration	Amount	Final concentration
Tris, pH 7.5	1 M	2.50 mL	50 mM
EDTA, pH 8.0	0.5 M	1 mL	10 mM
Dithiothreitol (DTT)	1 M	10 mL	200 mM
SDS	n/a	2 g	4% w/v
Urea	n/a	18 g	6 M
ddH_2O	n/a	to 50 mL	n/a
Total	**n/a**	**50 mL**	**n/a**

Store at 4°C.

Urea Sample Buffer (2×)

Reagent	Stock concentration	Amount	Final concentration
Tris, pH 6.8	0.5 M	15 mL	150 mM
Urea	n/a	18 g	6 M
SDS	n/a	2 g	4% w/v
Bromophenol blue	1% w/v	0.5 mL	0.01% w/v
Glycerol	50% v/v	10 mL	10% v/v
ddH_2O	n/a	to 50 mL	n/a
Total	**n/a**	**50 mL**	**n/a**

Store at 4°C.

Homemade SDS-PAGE gel

Resolving gel, 15 mL (variable %)

Reagent	Stock concentration	Amount
ddH_2O	n/a	Refer to the table below
Tris, pH 8.8	1.5 M	3.75 mL

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Reagent	Stock concentration	Amount
SDS	10%	0.15 mL
Ammonium persulfate	10%	50 μL
Acrylamide-Bis (29:1) (Bio-Rad, Cat# 1610156)	30%	Refer to the table below
TEMED	n/a	14 μL
Total	n/a	**15 mL**

Amount of ddH2O and Acrylamide-Bis for Resolving gel

Reagent	Stock concentration	Amount			
		10%	11%	12%	15%
ddH_2O	n/a	6 mL	5.5 mL	5 mL	3.5 mL
Acrylamide-Bis (29:1) (Bio-Rad, Cat# 1610156)	30%	5 mL	5.5 mL	6 mL	7.5 mL

Stacking gel, 5 mL (5%)

Reagent	Stock concentration	Amount
ddH_2O	n/a	2.85 mL
Tris, pH 6.8	0.5 M	1.25 mL
SDS	10%	50 μL
Ammonium persulfate	10%	30 μL
Acrylamide-Bis (29:1) (Bio-Rad, Cat# 1610156)	30%	0.825 mL
TEMED	n/a	8 μL
Total	n/a	**5 mL**

Alternatives: Pre-cast gels are also available, such as Bio-Rad Cat#4561036 (10%) and Cat#4561046 (12%).

STEP-BY-STEP METHOD DETAILS

Quantification of protein degradation by western blot

Here, we describe a method that takes advantage of the stability of GFP, which resists proteolytic degradation inside the vacuole lumen. When Ypq1-GFP is sent to the vacuole lumen, Ypq1 is rapidly degraded, whereas GFP stays intact. This is visible as a band shift from a ~64 kDa full-length Ypq1-GFP band in +Lysine conditions to a 27-kDa free GFP band in –Lysine conditions (Figure 1C).

This method can be used to quantify the degradation efficiency of mutant strains compared to the wild-type. As an example, here we test point mutants of Ypq1-GFP, which have previously been shown to have a reduced degradation compared to the wild-type (Arines et al., 2021). The corresponding mutated regions were found to be part of putative regulatory regions that govern Ypq1 degradation.

Note: Prior to starting the experiment, Ypq1-GFP in mutant strains can be checked for proper localization and stability using standard microscopy and western blot methods.

Starter culture

Timing: Operation (30 min), Incubation (overnight)

1. In the morning, inoculate a fresh yeast colony in 5 mL +Lys Uracil dropout Yeast Nitrogen Base (YNB) media (refer to materials and equipment for recipe) and grow with shaking (250 rpm) at 26°C for 8–10 h.
2. In the late afternoon or evening, measure the cell density (optical density at 600 nm wavelength, OD_{600}) using a spectrometer.

3. Inoculate the appropriate volume of starter culture to a large culture (typically 30 mL) so that the cells will be at mid-log (exponential growing phase) the next morning. Use the following formula to calculate:

$$\text{Starter culture } OD_{600} \times \text{Volume to inoculate} = \frac{\text{Target } OD_{600}}{2^{\text{growth time/doubling time}}} \times \text{Large culture volume}$$

where

   a. *Starter culture* OD_{600} is the concentration from step 2.
   b. *Target* OD_{600} is mid-log (i.e., 0.5–0.8), we typically aim for an OD_{600} of 0.7.

   Note: In our hands, cells are not very adherent if OD_{600} is lower than 0.5, and so are difficult to pellet. A higher OD_{600} (i.e., OD_{600} = 0.5–0.8) is preferred so that the majority of the cells can be spun down and collected.

   c. *Large culture volume* is 15 mL × number of time points. For a standard starvation experiment, we collect samples at 0 h and 6 h. So, we need a 30 mL culture. Adjust this volume if you need to collect more time points.
   d. *Growth time* (in hours). For example, if you inoculate at 5 pm and start the next step at 9 am the following day, the total growth time is 16 h.
   e. *The doubling time* of cells in Yeast Nitrogen Base media is typically 2 h at 26°C. In Yeast Extract Peptone Dextrose (YEPD or YPD), the typical doubling time is 1.5 h. Some strains or mutants may have growth defects, so adjust this value accordingly.

4. Grow yeast culture with shaking (250 rpm) at 26°C for 16–20 h.

Starvation and sample collection

Timing: 8 h

Here, we subject cells to lysine starvation to trigger the degradation of Ypq1. From the previous section, cells are grown overnight in media containing lysine. In this section, mid-log cells are collected, washed with sterilized Milli-Q (MQ) water to remove any residual lysine, and incubated in YNB media without lysine for 6 h. An equivalent number of cells (7 OD_{600}) will be collected at 0 h and after 6 h.

Note: Different MPs degrade at different kinetics in response to their degradation triggers. A pilot experiment is necessary to empirically determine the appropriate incubation time for your protein of interest. From here, select the time point that shows complete protein degradation, where the full-length band disappears accompanied by the appearance of a free GFP band (refer to Figure 1C).

5. Label two sets of 15-mL Falcon tubes:
   a. One set will be for the collection of 0 h samples (details start at step 8).
   b. The second set will be for setting up the starvation (“6 h –Lys”) (details start at step 9).
6. Take out the overnight culture and measure OD_{600} using a spectrometer.
7. From the overnight culture, 7 OD_{600} of cells will be collected for western blot, while another 7 OD_{600} (or 14 OD_{600} if two time points are needed) of cells will be washed and resuspended in starvation media.

8. For 0 h sample:
   a. Calculate the volume of cells needed to get 7 OD_{600}.

$$\text{Volume of cells to be collected} = 7\ OD_{600} \div OD_{600} \text{ of overnight culture}$$

   As an example,

$$7\ OD_{600} \div \frac{0.5\ OD}{mL} = 14\ mL$$

   b. Pipette the appropriate volume into a labeled 15-mL Falcon tube. Centrifuge the tubes at 2,250 × *g* for 5 min.
   c. Carefully aspirate the supernatant to the 1-mL mark using a vacuum or pipette, making sure not to touch the pellet.
   d. Resuspend the pellet by pipetting and transfer it to a 1.5-mL tube. Centrifuge in a tabletop centrifuge at 13,500 × *g* for 2 min.
   e. Aspirate supernatant to the 100-μL mark using a vacuum. Make sure not to touch the pellet.
   f. Add 1 mL ice-cold MQ H_2O and add 100% trichloroacetic acid (TCA) to a final concentration of 10% (i.e., 120 μL 100% TCA). Resuspend by vortexing.

⚠ CRITICAL: TCA is categorized as an acid and/or corrosive. Wear gloves and goggles. Dispose according to your institute's regulations.

   g. Keep cells on ice for at least 30 min. These will be your 0 h sample.

Pause point: Once cells are in TCA, they can be kept at 4°C for several weeks.

9. For setting up the starvation:
   a. If only one starvation time point will be collected, use the same volume as in step 8a. If more time points are needed, multiply the volume accordingly.
   b. Pipette the calculated volume into the labeled 15-mL Falcon tubes. Centrifuge at 2,250 × *g* for 5 min.
   c. Carefully aspirate the supernatant to the 1-mL mark using a vacuum or pipette, making sure not to touch the pellet.
   d. Resuspend the pellet by pipetting and transfer it to a 1.5-mL Eppendorf tube. Centrifuge in a tabletop centrifuge at 13,500 × *g* for 2 min.
   e. Wash the pellet twice with 1 mL MQ H_2O, centrifuging at 13,500 × *g*, 1 min each time.
   f. Resuspend the washed pellet in 1 mL YNB-Ura-Lysine by vortexing.
   g. Inoculate the washed cells into 14 mL YNB-Ura-Lysine media in a culture tube or flask.
   h. Incubate the culture tube with shaking for 6 h at 26°C in a shaking incubator (250 rpm).
10. At 6 h, take out the culture tubes, measure the OD_{600} using a spectrometer, and collect 7 OD_{600} starved samples as in step 8.

Processing of samples for western blot

Timing: 2–3 h (as needed)

Here, TCA-precipitated samples are further processed for SDS-PAGE and western blot. Be careful with pipetting and supernatant removal.

11. Pellet the samples from steps 8 and 10.
   a. Centrifuge samples at 13,500 × *g*, 5 min, at 20°C–25°C.
   b. Aspirate supernatant to the 100-μL mark.

12. Wash the pellet:
    a. Add 500 μL of 0.1% TCA to the sample. Vortex to mix. Centrifuge at 13,500 × *g*, 2 min, at 20°C–25°C.
    b. Remove the supernatant using vacuum up to the 100-μl mark. Centrifuge at 13,500 × *g*, 1 min, at 20°C–25°C.
    c. Use a pipette (P200) to manually remove the remaining supernatant.
13. Lyse the cells in sample buffer:
    a. Add 70 μL 2× urea Boiling buffer and about ~50 μL of acid-washed glass beads.
    b. Using a multi-tube vortex or bead-beater (please refer to key resources table), vortex samples at maximum speed for 5 min to break the yeast cells.

    Note: Some samples might be stuck at the top of the tube after vortexing; centrifuge the tubes briefly.

    c. Incubate samples at 42°C, 5 min to aid in denaturing the proteins.

    Note: Using a low temperature such as 42°C minimizes membrane protein aggregation. Never boil membrane protein samples.

    d. While waiting, prepare 2× Urea sample buffer supplemented with DTT: In a 1.5-mL tube, combine 900 μL of 2× Urea sample buffer and 100 μL 1 M DTT. Vortex to mix.
    e. Add 70 μL of this solution to the sample.

    Note: If TCA was not completely removed in step 12, your sample could turn yellow. But this will not affect the SDS-PAGE.

    f. Using a multi-tube vortex or bead-beater, vortex samples at maximum speed for 5 min.
    g. Incubate tubes at 42°C, 5 min.

14. Centrifuge samples at 13,500 × *g*, 5 min. While spinning, prepare a new set of 1.5-mL tubes and label them accordingly.
15. Transfer as much supernatant (~100 μL) as possible to the new tubes. Samples can be used right away for SDS-PAGE.

Pause point: Samples can also be stored overnight at −20°C. But for longer storage, we recommend −80°C to minimize any residual protease cleavage.

Note: Frozen samples will need to be reheated at 42°C for 5 mins before gel loading.

SDS-PAGE and fluorescence western blot

Timing: 1 day

Here, samples are resolved by SDS-PAGE, transferred to a nitrocellulose (or PVDF) membrane, and probed using primary and secondary antibodies using standard procedures. For first-time users, we have detailed our western blot protocol below. However, any other western blot method and materials that have been optimized in your lab would also be sufficient.

For probing, we recommend using LI-COR fluorescent secondary antibodies, which allow us to simultaneously detect two different proteins (Ypq1-GFP and Pgk1). Using these secondary antibodies will also aid in quantification, as discussed in the next section. For more information, please refer to technical notes from LI-COR.

16. Load 20 μL (equivalent to 1 OD_{600}) of the processed samples in an 11% SDS-PAGE gel.

⚠ CRITICAL: A polyacrylamide concentration of 10% or higher is recommended to ensure that the free GFP band (27 kDa) is separated from the dye front since you need to quantify the free GFP signal.

17. Prepare the molecular weight marker in a 1.5-mL tube by mixing 5 μL Precision Plus Protein™ All Blue Prestained Protein Standards (Bio-Rad, Cat#1610373), 5 μL 2× Boiling Buffer, and 10 μL 2× Urea sample buffer supplemented with DTT (from step 13d). Load this mixture into the gel.

Note: We recommend using pre-stained molecular weight markers to allow monitoring of the separation of protein samples during electrophoresis.

18. Run the gel until the dye front is at the end of the gel. For a standard Ypq1-GFP assay, we run the gel in 1× Tris-Glycine-SDS buffer at 160 V for 1 h.
19. Using a standard wet transfer system (e.g., Bio-Rad 2-Gel Tetra and Blotting Module, Cat# 1660827EDU), blot the protein samples onto a nitrocellulose membrane (Cytiva, Cat# 10600016) at 120 V for 2–3 h on ice.

Note: The transfer time can be optimized based on the transfer efficiency of the pre-stained molecular weight marker.

Alternatives: The transfer can also be performed overnight at 75 V, 4°C.

Alternatives: Semi-dry transfer systems (e.g., Trans-Blot® Turbo™ Transfer System (Bio-Rad, Cat# 1704150)) can also be used and are usually quicker. Optimize the transfer time to ensure that the samples are completely transferred.

20. Perform western blot:
    a. Using forceps, transfer the membrane into a new container and block the membrane in 10 mL Blocking Solution (5% dry milk in 1× Tris-buffered Saline with 0.1% Tween (TBST)) with gentle shaking for 30 min at 20°C–25°C.
    b. Primary Antibody:
    Prepare 10 mL of Primary Antibody in Blocking Solution.

Reagent	Dilution	Add to 10 mL blocking solution
Rabbit Anti-GFP (Torrey Pines Biolabs, Cat# TP-401)	1:2500	4 μL
Mouse Anti-Pgk1 (Invitrogen, Cat# 459250)	1:5000	2 μL

    c. Replace the spent blocking solution with freshly prepared Primary Antibody solution. Incubate with shaking for 2 h at 20°C–25°C.

Alternatives: Membranes can also be incubated with shaking overnight at 4°C.

    d. Wash the membrane with 10 mL 1× TBST 5 times for 5 min with shaking.
    e. Secondary Antibody:
    Prepare 10 mL of Secondary Antibody in Blocking Solution.

Reagent	Dilution	Add to 10 mL blocking solution
IRDye 800CW (Green) Goat Anti-Rabbit (LI-COR, Cat.# 926-32211)	1:10000	1 μL
IRDye 680 LT (Red) Goat Anti-Mouse (LI-COR, Cat.# 926-68020)	1:10000	1 μL

f. Incubate the membrane in 10 mL Secondary Antibody with shaking for 1 h at 20°C–25°C.

⚠ CRITICAL: Because LI-COR secondary antibodies are coupled to light-sensitive fluorophores, membranes should be protected from light upon secondary antibody incubation.

g. Wash the membrane with 1× TBST at least 4 times for 5 min. Then, wash the membrane once in ddH_2O for 5 min to remove the residual salt.

⚠ CRITICAL: Keep the membrane wet by immediately pouring buffer onto the membrane at every wash or incubation step. Drying out the membrane may result in a background signal that may affect the band quantification.

Visualizing the membrane

Timing: 10–30 min

Here, the membrane is visualized using a LI-COR Odyssey® CLx scanner and Image Studio software. This system detects and quantifies the fluorescence signals from infrared dye-coupled secondary antibodies. Through this system, any image brightness and contrast adjustments will not affect the raw fluorescence data, making it suitable for quantitative western blot analysis.

Alternatives: Other fluorescent/chemiluminescent western blot detection systems such as Azure Biosystems and Bio-Rad ChemiDoc MP imaging systems can also be used.

21. Using forceps, place the membrane (protein side facing down) on the scanning plate.

22. Scan the membrane:
    a. Click the Acquire tab. Use the following parameters: μ = 169 μm, Q = Medium, f = 0.0 mm.
    b. Choose the region of interest that covers the membrane. Click Preview, which will do a quick and low-resolution scan.
    c. Adjust the region of interest, so it occupies all samples on the membrane. Click Start. A typical Ypq1-GFP degradation blot is shown in Figure 2.
    d. Image display settings can be adjusted to optimize sample and background signals.

Note: If adjusted through the LI-COR Image Studio software, brightness and contrast modifications affect visualization only and not the quantification.

    e. Export the image as a .tiff file. Save the following images: Membrane in both Green and Red channels, Membrane in Green channel only, Membrane in Red channel only.

23. After imaging:
    a. Strip the membrane in 2% SDS for at least 60 min with shaking at 20°C–25°C.
    b. Wash the membrane twice with 1× TBST shaking for 30 s each time.
    c. Transfer the wet membrane into a Ziploc bag. Label properly and store the bag in −20°C. The membrane can be re-blocked and probed with different antibodies in the future.

Densitometry using LI-COR Image Studio and quantification

Timing: 30 min

Here, we quantify the fluorescence signal coming from full-length Ypq1-GFP, free GFP, and Pgk1 bands, and normalize them to calculate the degradation efficiency of Ypq1-GFP after lysine withdrawal.

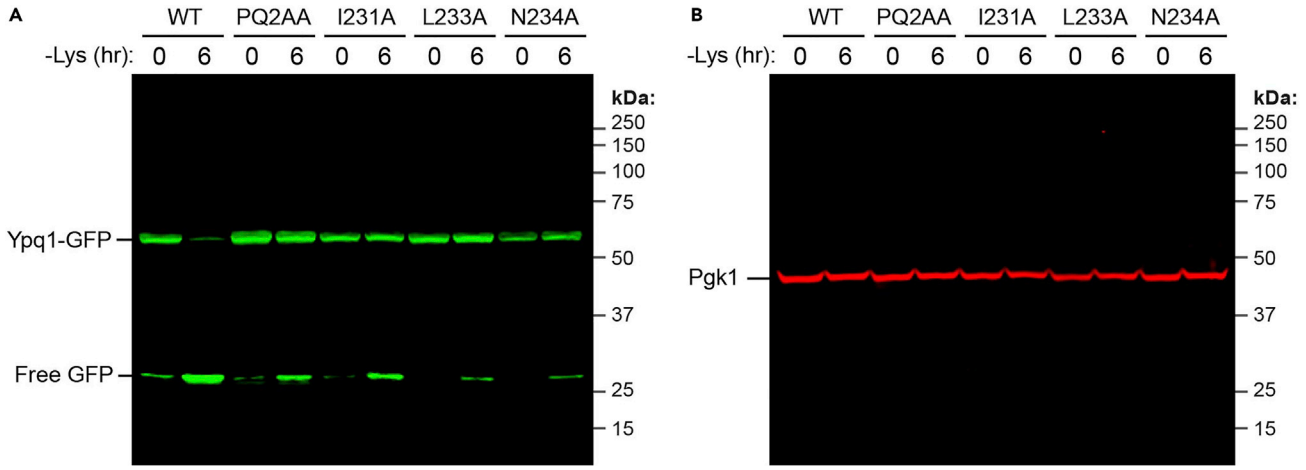

Figure 2. A typical Ypq1-GFP degradation blot

(A and B) GFP bands were viewed under the 800 nm (Green) channel (A), and Pgk1 bands were viewed under the 680 nm (Red) channel (B).

⚠ CRITICAL: We recommend using Image Studio instead of ImageJ. Adjusting the image brightness or contrast using Image Studio will not change the signal intensity of raw data, unlike ImageJ.

Alternatives: Other fluorescent/chemiluminescent western blot detection systems also have their analysis softwares such as Azurespot Pro (Azure Biosystems) and Image Lab (Bio-Rad) that can be used for the quantification.

24. To measure the band intensity,
    a. Click on the Image tab > Shapes > Add Rectangle. Click on a band corresponding to Full-length Ypq1-GFP on your image. Adjust the size of the rectangle so that it covers the whole band.
    b. Press Ctrl + C and Ctrl + V to duplicate the rectangle. Make rectangles for all full-length bands (Figure 3A).

Note: Copying and pasting ensure that the selected size will be equivalent among samples, providing a stringent quantification.

    c. Adjust the image brightness and contrast as necessary to be able to see and add boxes onto faint bands.

25. Repeat step 24 for bands corresponding to Free GFP (27 kDa), and for Pgk1 bands under the Red channel.
26. Export quantification values as an Excel file.
27. To quantify degradation,
    a. Calculate the degradation efficiency in terms of percentage of full-length Ypq1-GFP relative to total GFP (full-length Ypq1-GFP + free GFP):

$$\text{Full-length Ypq1-GFP}\ (\%) = \frac{\text{Full-length band}}{\text{Full-length band} + \text{Free GFP band}} \times 100\%$$

Note: The benefit of this quantification method is that it is internally controlled. We are comparing full-length vs. total GFP signal within the same sample, and so Pgk1 values will be canceled out. But probing for Pgk1 ensures that the loading is equivalent.

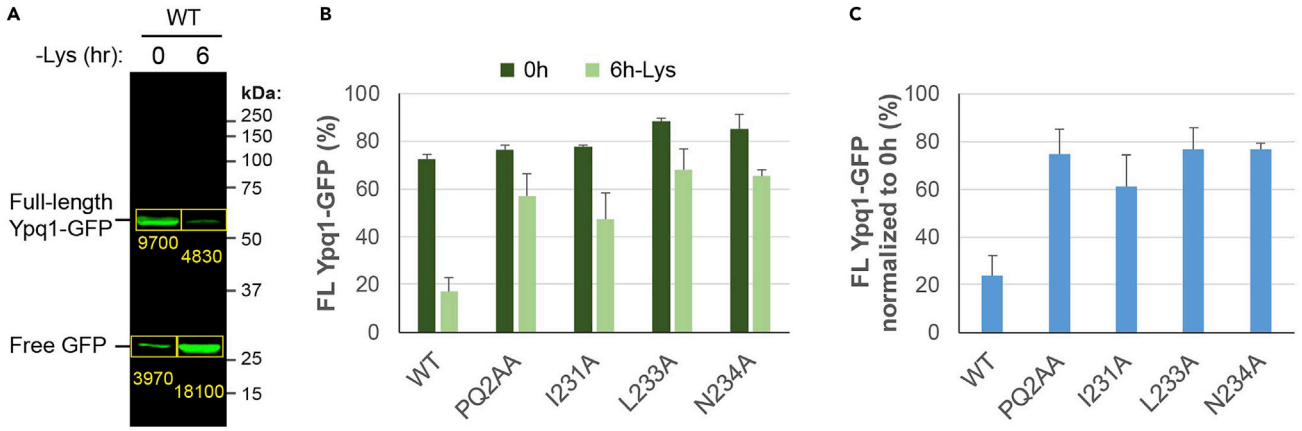

Figure 3. Quantification of Ypq1-GFP bands after lysine starvation

(A) During densitometry, full-length (FL) Ypq1-GFP and Free GFP bands are quantified with equally sized rectangles. Shown also are the raw fluorescence values obtained for each band using the Image Studio software.

(B) Final graph quantifying the degradation of Ypq1-GFP mutants vs. wild-type after 6 h lysine starvation. FL Ypq1-GFP was measured and normalized to total GFP (FL GFP + free GFP). A higher FL-GFP signal at 6 h-Lys corresponds to a lower degradation efficiency. Error bars denote standard deviation (n=3).

(C) An alternative way of quantification is to normalize the 6 h FL Ypq1-GFP levels to the values at 0 h.

b. Plot a bar graph comparing the protein levels at 0 h and 6 h –Lys (Figure 3B).

28. Repeat the process to obtain a total of 3 biological replicates for statistical analysis.

⚠ CRITICAL: A successful quantification relies on clean western blots that are free from stains, bubbles, or background signals. Assess the quality of your western blots and repeat the process as necessary before quantification.

Quantification of membrane protein degradation by flow cytometry

Upon lysine starvation, Ypq1-GFP is recognized by a ubiquitin ligase complex consisting of two proteins Ssh4 and Rsp5. Ubiquitinated Ypq1-GFP is then internalized into the vacuole lumen. Although GFP is proteolytically stable inside the vacuole lumen, the low luminal pH causes a decrease in GFP fluorescence (Arines et al., 2021; Patterson et al., 1997) (Figure 4). This decrease in fluorescence is detectable by flow cytometry and can be used as a quantitative readout for protein degradation.

This method can be used as an alternative to or corroborate data from the western blot protocol, and offers speed and higher throughput with a 96-well plate format. For this protocol, we used the IntelliCyt® iQue Screener PLUS flow cytometer (Sartorius) and the iQue Forecyt® Software (Sartorius).

Note: Other than GFP, the pH-sensitive pHluorin may also work, but for relatively abundant proteins. For red fluorophores, the pH-sensitive pHuji (Shen et al., 2014) may be suitable.

Alternatives: The details that we outline here can be easily adapted to other flow cytometry systems.

Starter culture

Timing: Operation (8–10 h), Incubation (overnight)

As in the western blot protocol, this pre-culture step ensures that all strains start at mid-log before starvation.

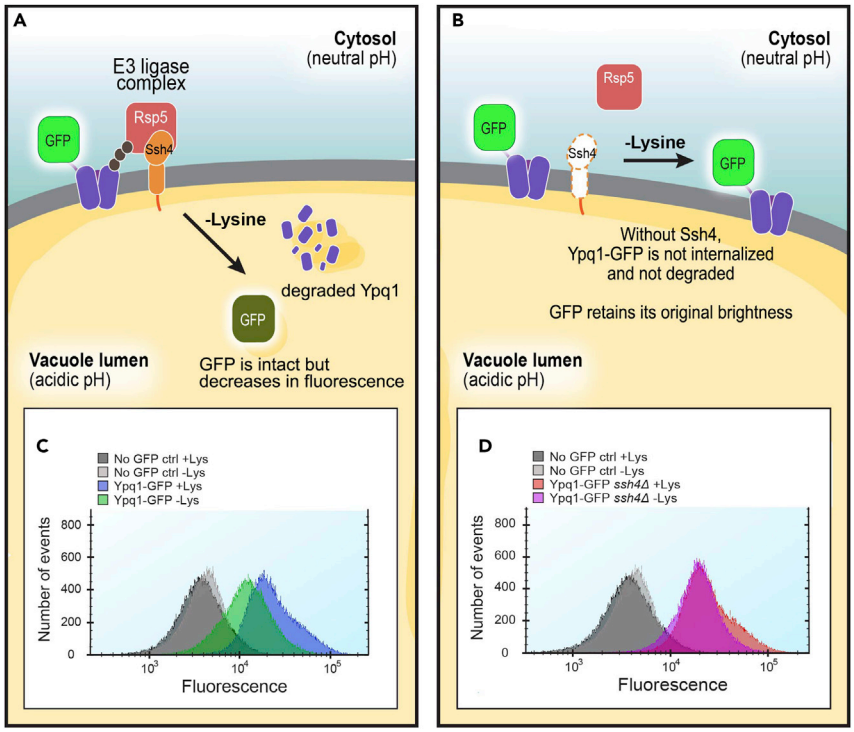

Figure 4. The rationale for flow-cytometry-based quantification of Ypq1-GFP degradation

(A) Upon lysine starvation, the E3 ligase complex (Rsp5-Ssh4) ubiquitinates Ypq1-GFP, leading to its internalization into the vacuole lumen. Ypq1 is degraded, while GFP stays intact. However, due to the low pH in the vacuole lumen, the fluorescence of GFP decreases.

(B) In the absence of Ssh4, the cytosolic E3 ligase Rsp5 cannot be recruited onto the vacuole membrane. In effect, Ypq1-GFP is retained on the vacuole membrane.

(C) The quenching of GFP in –Lys conditions is detectable as a shift in median fluorescence.

(D) When Ssh4 is deleted, there is no shift in median fluorescence.

29. In the morning before the day of the experiment, inoculate yeast in 5 mL YNB+Lys and grow with shaking at 26°C for 8–10 h.
    a. Include the following set of controls:
        i. No GFP control (e.g., SEY6210 + Empty vector).
        ii. Positive degradation control (e.g., SEY6210 + Ypq1-GFP).
        iii. Negative degradation control (e.g., SEY6210 *ssh4Δ* + Ypq1-GFP).

    Note: Here, our negative degradation control is a strain where Ssh4 (a component of the ubiquitin ligase complex that recognizes Ypq1) is deleted, so Ypq1-GFP does not degrade. For other membrane proteins, the following strains can be used as negative controls:

    b. A strain where the corresponding ubiquitin ligase is deleted or non-functional.
    c. If the protein is on the plasma membrane, an endocytosis-deficient strain (e.g., *end3Δ*) can be used (Munn, 2001).
    d. A strain where the vacuole sorting machinery is defective, for example, an ESCRT (endosomal sorting complexes required for transport) mutant (e.g., *vps4Δ*, *vps27Δ*) (Katzmann et al., 2001).
30. In the late afternoon or evening, measure the cell concentration (OD_{600}) using a spectrometer.
31. Inoculate the appropriate volume of starter culture to a 10-mL culture so that the cells will be at mid-log the next day. Use the following formula to calculate:

$$\text{Starter culture } OD_{600} \times \text{Volume to inoculate} = \frac{\text{Target } OD_{600}}{2^{\text{growth time/doubling time}}} \times 10 \text{ mL}$$

Note: For more details, refer to the western blot protocol (Starter Culture).

32. Culture yeast with shaking (250 rpm) at 26°C overnight.

Lysine starvation

Timing: Operation (30 mins to 1 h), Incubation (6 h)

Here, we separate the overnight culture into two halves: one half will be diluted and grown in the presence of lysine, while the second half will be diluted and grown in media without lysine. Depending on your protein, you may need to modify the starvation media and the duration of starvation.

33. Measure the cell concentration (OD_{600}) of the overnight cultures using a spectrometer. When the cells are at mid-log, proceed with lysine starvation.
34. Prepare two sets of culture tubes:
    a. Pipette 4 mL YNB-Ura into the set labeled as **"+Lys"**.
    b. Pipette 4 mL YNB-Ura-Lys into another set labeled as **"–Lys"**.

35. Prepare the cells for starvation:
    a. For each sample, measure out 0.94 OD and pipette into a tube labeled **+Lys.** Similarly, measure out 3.75 OD and label it as **-Lys**.

    Note: This calculation is based on the goal of reaching the final cell concentration of ~1.5 OD/mL after 6 h. In YNB+Lys, yeast cells have a doubling time of 2 h. Therefore, they will double three times after 6 h. However, in the absence of lysine, yeast cells double only once, and so you will need to inoculate more cells in **–Lys**.

    Note: Here, we determined 1.5 OD/mL as the optimal cell concentration for flow cytometry. To determine the optimal cell concentration for your flow cytometer, you need to dilute the yeast culture and check which concentration minimizes sample clogging and uneven cell flow. Consult with your flow cytometry core for further guidance.

    Note: Some strains may have a growth defect and so will have longer doubling times. Adjust your calculation accordingly.

    b. Centrifuge the cells at 2,250 × *g* for 5 min in a benchtop centrifuge.
    c. Aspirate the supernatant and wash the cells twice with 1 mL MQ H_2O.
    d. Finally, resuspend the cells in either 1 mL YNB-Ura or 1 mL YNB-Ura-Lys, and pipette them into the corresponding culture tubes. The final volume will be 5 mL.
36. Incubate both sets of tubes at 26°C with shaking for 6 h.

    Note: After 6 h of growth, the OD_{600} of samples can be measured by a spectrometer, but is optional. Any small differences in OD_{600} will not affect the quantification.

Plate setup for flow cytometry

Timing: 30 min to 1 h

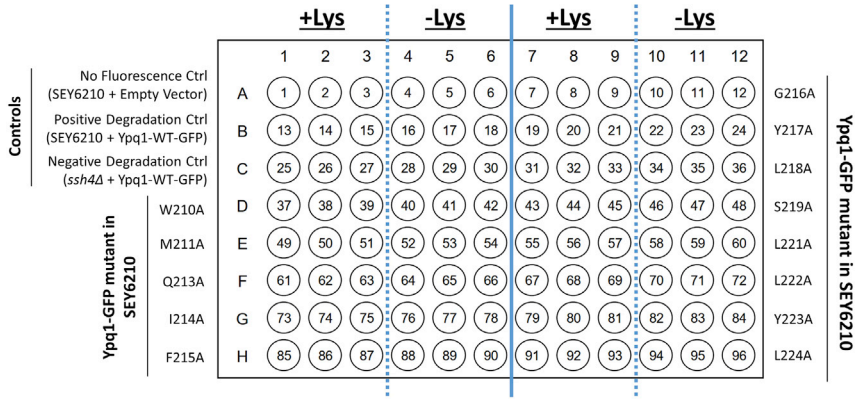

Figure 5. Sample loading arrangement in a 96-well plate

A typical setup for analyzing Ypq1-GFP mutants. All Ypq1-GFP mutants were expressed from a plasmid and transformed into the SEY6210 strain.

37. For each sample, pipette a triplicate of 200 μL cells into a 96-well plate, using Figure 5 as a guide.

⚠ CRITICAL: Each plate should contain all three controls (refer to step 29).

⚠ CRITICAL: All samples, including controls, should be treated with +Lys and –Lys conditions.

38. Proceed to flow cytometry.

⚠ CRITICAL: Proceed quickly to avoid further protein degradation. Also, yeast cells may settle to the bottom of the plate after waiting. This can lead to inaccurate readings due to non-uniform laminar flow (e.g., clogging and sample surging).

Flow cytometry

Timing: 30 min to 1 h

Here, we measure the GFP fluorescence using an IntelliCyt iQue Screener PLUS flow cytometer and the iQue Forecyt® software. The researchers may need to adjust steps according to their flow cytometer and software.

39. Load the plate into the flow cytometer equipped with all the necessary buffers. For the iQue Screener PLUS, we used the following buffers:
    a. Running buffer: Colorless media such as YNB or PBS should be suitable. In our experiments, we use YNB without nutrients.
    b. Decontamination solution: 1 vial of IntelliCyt Decontamination Concentrate (Sartorius, Cat#90077) in 180 mL of water.
    c. Cleaning solution: 3 mL of IntelliCyt Cleaning Concentrate (Sartorius, Cat#90079) in 197 mL of water.
    d. ddH_2O.
    e. Sheath fluid: 1 bottle of IntelliCyt Bacteriostatic Concentrate (Sartorius, Cat#90078) in 1 liter of water.
40. Open the Forecyt Standard Edition software.
41. Prime the system for 5 min using YNB as the running buffer.

42. While priming, set up the flow cytometry protocol on the "Protocol" tab as follows:

Prepare	Sample	Shake	Flush & Cleaning	Detector
Automatic Prime: 60 s (Cycle) Pre-plate Shake: 15 s at 1,000 rpm	Sample Order: E Mode (Row by Row) Sip Time: 4 s Additional Up Time: 1 s Sample Height: 1.50 Pump: 29 rpm	Shake 4 s at 1,000 rpm after every 3 wells (Rinse in Buffer while shaking) (*See Note below*)	Flush Duration: 30 s Post-plate Clean: Decon for 30 s Clean for 30 s Water for 60 s	Threshold: FSC-H < 50000 (*See Note below*)

Note: By setting the **SHAKE** step this way, we ensure that after every three samples, the flow cytometer will clean the probe, as well as shake the plate to prevent cells from settling to the bottom.

Note: The FSC-H threshold establishes which signals are most likely coming from samples and which are artifacts or debris. Here, any debris with FSC-H less than 50,000 will be omitted. For every set of experiments, we recommend doing a pilot run on cells expressing your GFP-tagged protein. Iteratively increase or decrease the FSC-H cut-off to test the effect on fluorescence. A reasonable FSC-H threshold minimizes artifacts without affecting fluorescence readings. Consult with your flow cytometry core for further advice.

Gating and analysis of flow cytometry data using Forecyt

43. When the run is finished, set the Noise ID filter by drawing a polygon around the cell population to gate out any debris (Figure 6A).
44. Match the signals to the samples:
    a. On the Well Identification toolbar, set the Algorithm to **Adaptive** and click on **Calculate Wells.** This will automatically draw boxes over cell populations coming from each well (Figure 6B).
    b. Zoom in to check if each selection box corresponds to events from each well (Figure 6C). Because we loaded triplicates for our samples, it will be easy to distinguish real cells from artifacts. Triplicates of the same sample should look similar in height and have a small gap in between. Note that there should be a gap between triplicates of different samples corresponding to the shake and probe wash step in step 42.
    c. If a selection box is misplaced, click on **Move Well** (hand icon), then click on the misplaced selection box to the proper column of events.
45. Plot FSC-H vs. SSC-H. Draw a polygon as shown to gate for **Yeast Cells** (Figure 7A).
46. Plot FSC-H vs. FSC-A. Draw a polygon as shown to gate for **Singlets** (Figure 7B).

Note: Clumps of two cells (doublets) will give high fluorescence readings. Similarly, budding cells will have a higher total fluorescence because they are essentially two cells. Since buds differ in size depending on the stage of cell division, this can lead to a wide range of fluorescence values. Therefore, we recommend gating for singlets to measure the fluorescence from single, non-budding cells. Singlets have proportionate FSC-A and FSC-H, while doublets and budding cells are slightly larger and will have a higher FSC-A value. Thus, singlets lie along the diagonal in FSC-H vs. FSC-A plot. Meanwhile, doublets and budding cells will have higher FSC-A values (Cattoglio et al., 2020; Wersto et al., 2001).

47. Plot BL1-H (i.e., Blue laser for GFP fluorescence) vs. SSC-H.

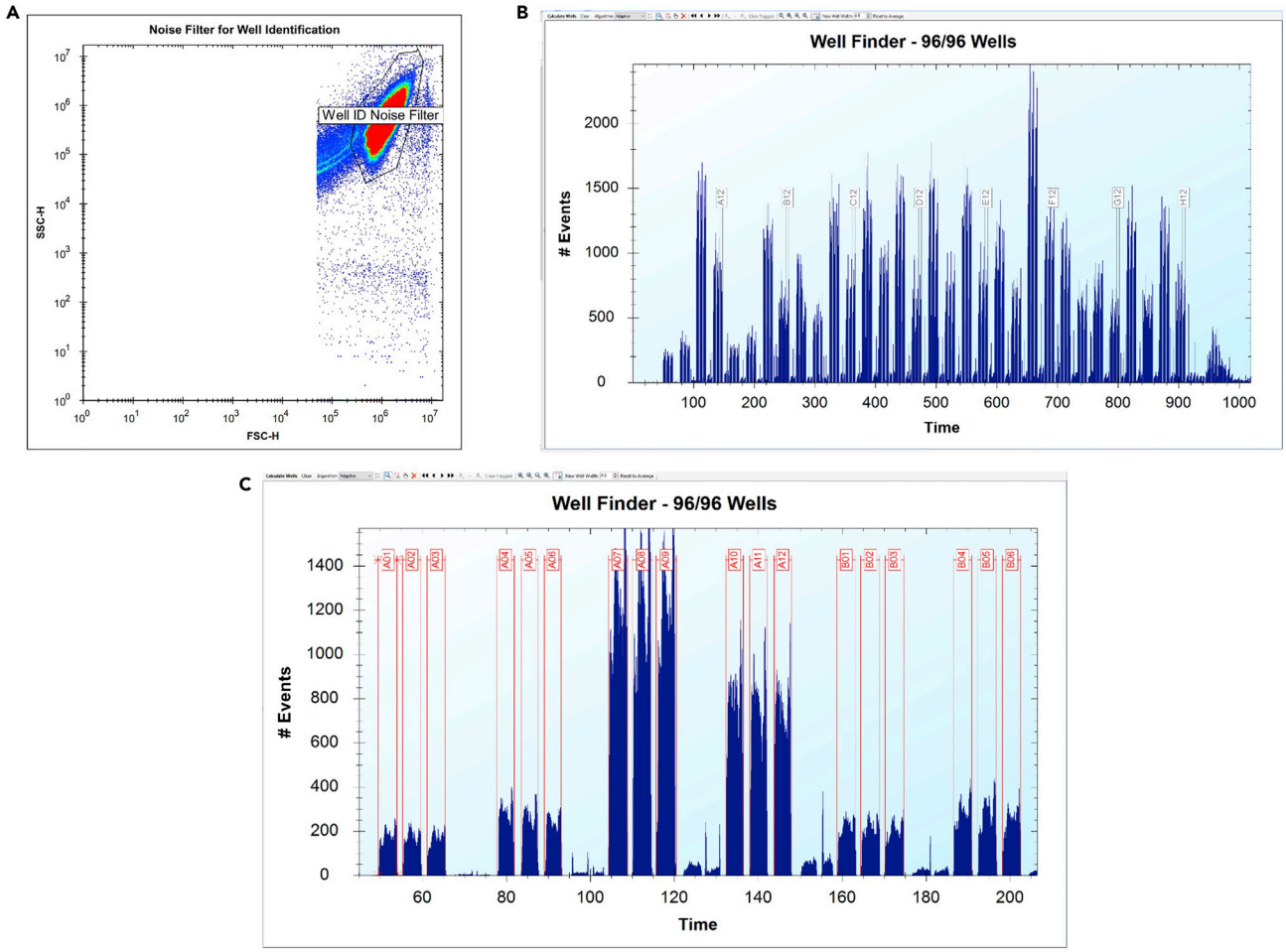

Figure 6. Setting up Well ID

(A) Set the Well ID Noise Filter to the select cells and gate out any debris.

(B) Wells, shown here as event columns, are automatically selected by Forecyt.

(C) Zoom in to confirm if the selections correspond to technical triplicates and manually adjust the boxes if necessary.

Quantification of GFP signals using Forecyt

48. Click on the **Analysis** tab.
49. On the Populations tab, click **Heat Map.** On the pop-up window, click **Metric**, then choose **Median BL1-H of Singlets.**
50. Right-click the resulting heat map, then click **Show** > **Actual Values.** The resulting heat map can be saved as an image and can be further processed for publication (Figure 8).
51. We can further process this data to quantify the degree of degradation of each sample. To do so, export the median fluorescence values as .csv or .xlsx files. Go to the **Metrics** tab, then click **Export Current Plate.**

Alternatives: Generating this heat map is optional but provides a quick glance to distinguish which samples could be having a somewhat normal or disrupted degradation. For flow cytometers and software that do not have this feature, export the median fluorescence values as .csv or .xlsx. Then, generate a heat map using Excel following the instructions in step 53. Set the Minimum value Type as "Lowest Value", and the Maximum value Type as "Highest Value".

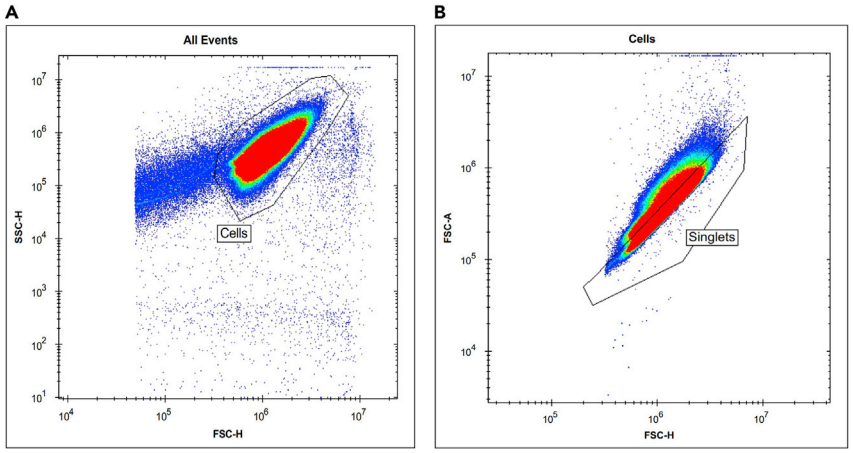

Figure 7. Gating for singlets in preparation for fluorescence measurement

(A) In a FSC-H vs SSC-H plot, draw a polygon around the cell population to gate for Yeast Cells.

(B) From this population, generate a FSC-H vs FSC-A plot and draw a polygon around the bottom half of the diagonal to gate for Singlets.

Fluorescence retention heat map generation

52. Calculate degradation efficiency in terms of fluorescence retention:
    a. Using Microsoft Excel, calculate the Average Fluorescence (AF) of the median fluorescence values from three technical replicates.
    b. To correct for any auto-fluorescence inherent in your cells, compute the Corrected Average Fluorescence (CAF) by subtracting the AF value of the No Fluorescence control (e.g., SEY6210 + Empty vector). A sample data set calculation is shown in Figure 9.
    c. Calculate the fluorescence change (FC) by dividing the CAF of cells from –Lys samples with the CAF of cells from +Lys samples.
    d. Finally, calculate the fluorescence retention (FR) score by normalizing the FC value of the mutant with the FC value of the Negative Degradation Control (e.g., SEY6210 *ssh4Δ* + Ypq1-GFP). This method sets the final FR score of the negative control to 100%; all other samples would have a lower score. Essentially, a higher FR score indicates a higher

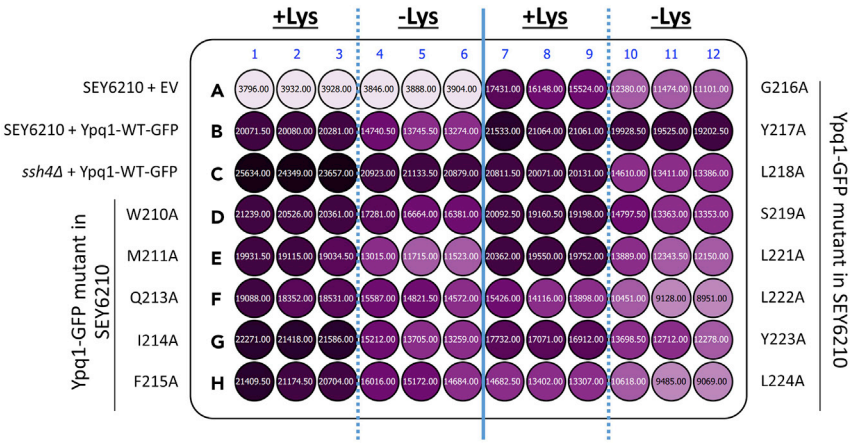

Figure 8. Median fluorescence values and heat map of Ypq1-GFP mutants after growing in +Lys and –Lys media

This heat map can be automatically generated from the Forecyt software. Here, the lightest wells correspond to cells with no fluorescence (SEY6210 + EV), while the darkest wells correspond to the highest levels of GFP fluorescence. Notice the difference in levels of GFP fluorescence between cells grown in +Lys vs. –Lys conditions.

	Average fluorescence (AF)		Corrected average fluorescence (CAF)		Fluorescence change (-Lys / +Lys)	Fluorescence retention (FR) score (%)
	+Lys	-Lys	+Lys	-Lys		
No Fluorescence ctrl	3885.3	3879.3	N/A	N/A	N/A	N/A
SEY6210 *ssh4Δ* + Ypq1-GFP *Negative ctrl*	24546.7	20978.5	20661.3	17099.2	0.83	100.0 ± 4.8
SEY6210 WT + Ypq1-GFP *Positive ctrl*	20144.2	13920.0	16258.8	10040.7	0.62	74.5 ± 6.0

Figure 9. Calculating the Fluorescence Retention score as a measure of degradation efficiency

Shown here are sample calculations for the controls. Generally, the median GFP fluorescence values from technical replicates are averaged. Next, the average fluorescence of the No Fluorescence control is subtracted to obtain the corrected fluorescence value. Then, the Fluorescence Change is calculated by dividing the values from –Lys with the values from +Lys. Lastly, the Fluorescence Change values are normalized to that of the Negative Degradation control to obtain the FR score.

fluorescence after lysine starvation, hence, a stronger block of degradation. Scores that approach 100% correspond to a complete block.

53. To further transform the FR scores into a heat map,
    a. Highlight the FR scores and click on Conditional Formatting > Color Scales > More Rules… (Figure 10A).
    b. Set the Format Style as "2-Color Scale" (Figure 10B).
    c. Set the Minimum value Type as "Lowest Value" and the Maximum value Type as 100.
    d. Set your preferred colors and click OK. The FR score heat map should appear as in Figure 11.

EXPECTED OUTCOMES

Western blot

After 6 h of lysine starvation, we expect to see a decrease in full-length Ypq1-GFP accompanied by an increase in free GFP. In wild-type samples, there should be a substantial difference between the values before and after starvation. In Figure 3B, the FL Ypq1-GFP signal was around 72% at 0 h, and 17% at 6 h. However, mutants that disrupt the degradation should have more full-length Ypq1-GFP signal remaining at 6 h. For example, L233A had 88% signal at 0 h and 68% at 6 h. This suggests that L233 could be important in the degradation of Ypq1-GFP, and so mutating it disrupts degradation. Microscope imaging shows that Ypq1(L233A)-GFP and other mutants failed to internalize into the vacuole lumen (Arines et al., 2021).

Alternatively, degradation can be shown as normalized full-length Ypq1-GFP signals (Figure 3C). For each sample, the FL Ypq1-GFP (%) value at 6 h is divided by the 0 h value.

Flow cytometry

When cells are grown in media without lysine, Ypq1-GFP is internalized and degraded in the vacuole lumen. GFP will be intact within the lumen but will have a decrease in fluorescence. If there is a mutation that impairs the degradation of Ypq1-GFP, the protein stays on the vacuole membrane, and so there will be very little to no decrease in fluorescence.

In Figure 11 we show a sample heat map generated from testing one transmembrane helix in Ypq1. We performed scanning mutagenesis, wherein we mutated each residue in the transmembrane helix to alanine. The first two boxes correspond to the negative degradation control (SEY6210 *ssh4Δ* + Ypq1-GFP) and positive degradation control (SEY6210 + Ypq1-GFP), while the rest correspond to the point mutants. Darker colors correspond to higher fluorescence retained, and so correspond to mutants that experienced a stronger disruption of degradation. Mutants that approach the value of the negative degradation control are considered to be "degradation-blocking mutants." In this

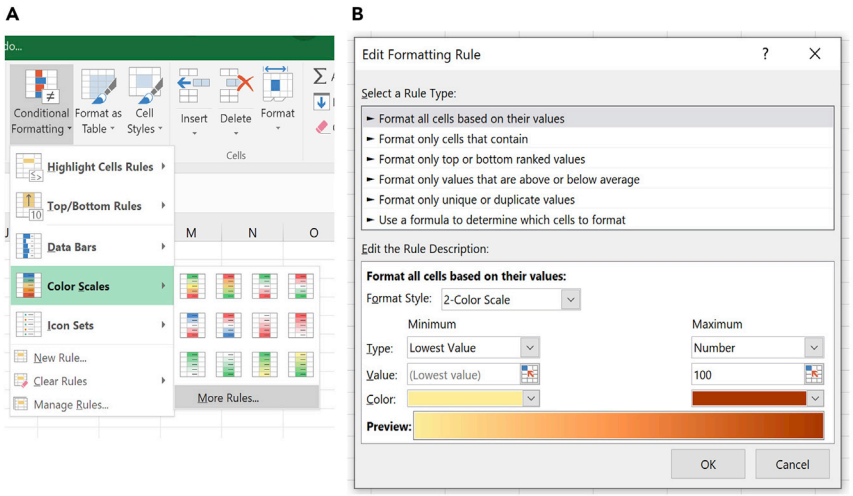

Figure 10. Making an FR score heat map on Microsoft Excel

Generating a heat map of the FR scores provides a visual component of the quantification to compare degradation efficiencies easily.

(A) Select the FR scores and click on Conditional Formatting > Color Scales > More Rules.

(B) Choose 2-Color Scale, set the minimum and maximum values, and set your preferred colors.

case, they are mutants that had FR scores equal to or above 80%. This cut-off value is arbitrary, and so the researcher will need to compare flow cytometry data with other experiments such as microscopy imaging to determine the proper cut-off. The researcher can run biological replicates and calculate the standard deviation values for publication.

LIMITATIONS

This protocol is robust and can be reliably applied to many proteins, but the researchers must consider several critical limitations.

General

While many membrane proteins are degraded via trafficking to the vacuole lumen, other membrane proteins that undergo proteasome-mediated degradation (e.g., substrates for ER-associated degradation) cannot be measured by this assay. In addition, if a protein is degraded by both the vacuole and the proteasome within the same treatment, it may not be compatible with this assay. Since the proteasome degrades the protein as well as the GFP moiety, the signal loss due to the proteasome-dependent degradation will be difficult to determine and so will confound the quantification.

Both protocols rely on the cleavage of the GFP tag and subsequent stability of the GFP tag in the yeast vacuole as a readout for degradation. Therefore, the researchers must use yeast background strains that have a functional lytic vacuole, and so strains with defective vacuolar proteases must be avoided.

As mentioned earlier, the GFP tag should be facing the cytosol. If the GFP tag is inside the lumen, it will be constitutively cleaved off and confound your analysis.

Flow cytometry

For the flow cytometry protocol, fluorescence quenching due to exposure of the GFP tag to the acidic vacuole lumen is used as a readout for degradation. Therefore, background strains that affect the pH of the vacuole (e.g., v-ATPase mutants) may not be compatible with this assay.

	A	B
1		FR score (%)
2	ssh4del + Ypq1-GFP	100
3	6210 + Ypq1-GFP	70.4
4	6210 + Ypq1(W210A)-GFP	91.5
5	6210 + Ypq1(M211A)-GFP	63.2
6	6210 + Ypq1(Q213A)-GFP	89.8
7	6210 + Ypq1(I214A)-GFP	67.9
8	6210 + Ypq1(F215A)-GFP	79.2
9	6210 + Ypq1(G216A)-GFP	74.3
10	6210 + Ypq1(Y217A)-GFP	108
11	6210 + Ypq1(L218A)-GFP	72
12	6210 + Ypq1(S219A)-GFP	76.2
13	6210 + Ypq1(L221A)-GFP	66.4
14	6210 + Ypq1(L222A)-GFP	63.3
15	6210 + Ypq1(Y223A)-GFP	80.6
16	6210 + Ypq1(L224A)-GFP	70.2
17	6210 + Ypq1(G225A)-GFP	62.9
18	6210 + Ypq1(S226A)-GFP	74.8
19	6210 + Ypq1(R227A)-GFP	86.1
20	6210 + Ypq1(I228A)-GFP	97.1
21	6210 + Ypq1(PQ2AA)-GFP	85.7
22	6210 + Ypq1(I231A)-GFP	95.8
23	6210 + Ypq1(L232A)-GFP	80.9
24	6210 + Ypq1(L233A)-GFP	100.9
25	6210 + Ypq1(N234A)-GFP	101.5
26	6210 + Ypq1(F235A)-GFP	57.5

Figure 11. FR score heat map of Ypq1-GFP mutants and controls

Higher FR scores correspond to more Ypq1-GFP stabilized on the vacuole membrane and thus indicate a stronger block in degradation.

Applicability to mammalian cell systems

This protocol has the potential to be applied to mammalian cells based on several papers that used the accumulation of free GFP as a metric for lysosomal degradation following autophagy (Ni et al., 2011), as well as the fluorescence quenching of GFP in the lysosomes (Riccio et al., 2019). However, several steps such as lysate processing and flow cytometry gating will need to be optimized. In addition, we recommend testing proteins expressed at the endogenous level rather than overexpression systems because overexpression could overwhelm the degradation machinery and therefore slow down the degradation kinetics.

TROUBLESHOOTING

Problem 1

Bands are too weak (step 22 of western blot section).

Potential solution

For unoptimized assays, start with an antibody dilution recommended by the manufacturer. Otherwise, start with 1:5,000 dilution for primary antibodies and 1:10,000 for secondary antibodies. Adjust the antibody concentrations and the sample loading amount to improve the signal-to-noise ratio. Alternatively, increase the incubation time.

Problem 2

The membrane has a lot of background signals that interfere with densitometry (step 22 of western blot section).

Potential solution

Increase the number of washes. Use forceps when handling the membrane, as your hand or gloves can introduce stains that can be detected during the blotting. Keep the membrane in a clean box during incubation and washing. Avoid drying out the membrane after the blocking step.

Problem 3

Even after 6 h, the degradation of WT samples in –Lys condition is incomplete, i.e., a significant amount of full-length band remains (step 27 of western blot section).

Potential solution

The lysine concentration in the starter culture will affect the degradation kinetics. In our hands, a starting concentration of 1.6 mM lysine works well. Reduce the lysine concentration in the starting +-Lys media if the degradation kinetics is slow for WT cells.

Problem 4

Cells settle quickly to the bottom of the plate (step 38 of flow cytometry section).

Potential solution

Manually pipet cells up and down with a multi-channel pipette. Using the Shake function of the flow cytometer may not be sufficient to resuspend the cells fully.

Problem 5

There is no significant fluorescence difference between samples that supposedly express Ypq1-GFP and no fluorescence controls (step 52 of flow cytometry section).

Potential solution

Visualize your cells under the microscope to ensure that the GFP-tagged protein is expressed and is localized properly to the vacuole.

Problem 6

There is no significant FR value difference between wild-type and controls (step 52 of flow cytometry section).

Potential solution

It is possible that the background strain may have some genetic modifications that affect the vacuolar pH. For the flow cytometry protocol to work, the vacuole should be acidic (~pH 5.5). Check your background strain to see whether genes related to vacuolar acidification (e.g., v-ATPase genes) or cytosolic acidification (e.g., plasma membrane proton pump genes) have been deleted or modified.

Problem 7

No negative degradation control strain is available for the quantification (steps 29 and 52 of the flow cytometry section).

Potential solution

To calculate the FR score (step 52d), divide the FC value of the samples with that of the wild-type FC value. This will cause wild-type to have an FR score of 100%. Proceed with the next steps, but at step 53c, set the Minimum value Type as 100, and the Maximum value Type as "Highest Value." The heat map should still be able to show which mutants have a darker color (and thus stronger degradation block) compared to the wild-type, but the FR score range will be > 100%.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Ming Li (mlium@umich.edu).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate any new datasets or code.

ACKNOWLEDGMENTS

F.M.A. is supported by the Barbour Scholarship from the University of Michigan. This work is supported by the MCubed 3.0 fund from the University of Michigan, fund from the Protein Folding and Diseases Initiative from the University of Michigan, a Cystinosis Research Foundation grant (CRFF-2018-005), and a National Institutes of Health grant (R01-GM133873) to M.L. The flow cytometry protocol was developed with guidance from the Center for Chemical Genomics (CCG) at the University of Michigan Life Sciences Institute. We would like to thank Aaron Robida and Andrea Hodgins-Davis for the helpful discussions about the flow cytometry protocol.

AUTHOR CONTRIBUTIONS

Conceptualization, F.M.A. and M.L.; investigation, F.M.A.; writing – original draft, F.M.A.; writing – review & editing, F.M.A. and M.L.; funding acquisition, F.M.A. and M.L.; supervision, M.L.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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