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Methods for measuring misfolded protein clearance in the budding yeast *Saccharomyces cerevisiae*

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Abstract

Protein misfolding in the cell is linked to an array of diseases, including cancers, cardiovascular disease, type II diabetes, and numerous neurodegenerative disorders. Therefore, investigating cellular pathways by which misfolded proteins are trafficked and cleared (“protein quality control”) is of both mechanistic and therapeutic importance. The clearance of most misfolded proteins involves the covalent attachment of one or more ubiquitin molecules; however, the precise fate of the ubiquitinated protein varies greatly, depending on the linkages present in the ubiquitin chain. Here, we discuss approaches for quantifying linkage-specific ubiquitination and clearance of misfolded proteins in the budding yeast *Saccharomyces cerevisiae*—a model organism used extensively for interrogation of protein quality control pathways, but which presents its own unique challenges for cell and molecular biology experiments. We present a fluorescence microscopy-based assay for monitoring the clearance of misfolded protein puncta, a cycloheximide-chase assay for calculating misfolded protein half-life, and two antibody-based methods for quantifying specific ubiquitin linkages on tagged misfolded proteins, including a 96-well plate-based ELISA. We hope these methods will be of use to the protein quality control, protein degradation, and ubiquitin biology communities.

1. Introduction

In order for a cell to remain healthy, it must maintain a balanced proteome (“proteostasis”) (Balchin, Hayer-Hartl, & Hartl, 2016; Sontag, Samant, & Frydman, 2017). Proteins that do not attain their native three-dimensional structure—e.g., nascent chains from the ribosome, or correctly folded proteins that denature due to various types of stress—must either be refolded or cleared through a process known as protein quality control (PQC). Like any quality-control system, PQC involves different machineries for detection and disposal of nonnative products. For example, molecular chaperones selectively recognize misfolded proteins and target them to refolding or clearance pathways; E3 ubiquitin ligases tag misfolded proteins with one or more molecules of ubiquitin (a posttranslational modification with many roles, one of which is as a proclearance signal); and the proteasome, which recognizes and degrades ubiquitinated proteins (Fig. 1A).

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The budding yeast *Saccharomyces cerevisiae* has been used extensively for furthering our mechanistic understanding of protein quality control and clearance pathways (Amm, Sommer, & Wolf, 2014; Schneider, Nyström, & Widlund, 2018; Sontag et al., 2017). Not only is its core proteostasis machinery conserved through to humans, but it offers numerous clear advantages over higher eukaryotes, including an unrivaled genetic toolbox (a fully annotated genome, a complete deletion mutant strain collection, widely available GFP- and TAP-tagged libraries covering the majority of its proteome, as well as high-throughput methods to generate libraries of new genetic crosses within weeks), as well as its scale, i.e., the fact that experiments are performed with entire populations, rather than a small number of organisms representative of a population (Botstein & Fink, 2011). With regard to proteostasis specifically, the reduced complexity and redundancy of PQC systems in yeast (e.g., the human genome encodes 300–350 molecular chaperones and over 600 E3 ubiquitin ligases, compared with 69 and ~60, respectively, in budding yeast) have enabled dissection of protein clearance systems in a way that would not have been possible directly using mammalian models.

However, standard cell biology protocols generally do not take into account the intricacies of yeast as a model organism, or of ubiquitin as a covalent yet highly dynamic posttranslational modification. This chapter is aimed at discussing some of the unique considerations that must be addressed when studying ubiquitin-mediated clearance of misfolded proteins in budding yeast. Basic yeast maintenance and growth protocols are provided elsewhere (Bergman, 2001; Curran & Bugeja, 2014). We will also present protocols we have used successfully for quantifying relative levels of ubiquitin linkages.

2. Expression and isolation of misfolded proteins in yeast

2.1 Choice of expression system

We routinely use the Gateway® system (Alberti, Gitler, & Lindquist, 2007), which provides a quick and convenient cloning strategy with its suite of plasmids containing various fluorescent proteins or affinity tags (either N- or C-terminal fusions), promoters (GAL, inducible, or GPD, constitutive), selection markers (*HIS3*, *LEU2*, *TRP1*, or *URA3*), and origins of replication (chromosomally integrating, 2 μ high-copy number, or *CEN* low-copy number).

The *GAL1* promoter is especially useful for inducible expression. Importantly, this system allows us to switch off misfolded protein expression by replacing galactose with glucose in the growth medium. An inducible system is essential for assaying misfolded protein clearance, as we can track the fate of an existing population of our protein of interest without the confounding effect of newly synthesized species (as would be the case with constitutive expression), or of global PQC alterations triggered by translation inhibitors such as cycloheximide. Note that in order to switch on the *GAL1* promoter, the transcriptional repression circuit present during growth in glucose media must first be inactivated through growth on a nonrepressing sugar (e.g., raffinose), rather than transferring directly from glucose to galactose media.

The galactose system is not suitable for certain aspects of PQC research, such as testing the effects of nutrient deprivation and/or chronological aging. For such applications, other expression systems are available, e.g., the *CUP1* copper-inducible promoter (Macreadie, Jagadish, Azad, & Vaughan, 1989), or the doxycycline-controlled Tet-On or Tet-Off systems (Garí, Piedrafitá, Aldea, & Herrero, 1997).

2.2 Choice of misfolded proteins

Studies of PQC in yeast typically involve expression of misfolded proteins from nonnative promoters (see the previous section). The choice of protein will be dictated by the precise question being asked. For the purposes of characterizing general mechanisms of PQC, it is important to include a variety of proteins that misfold for different reasons. In our recent study (Samant, Livingston, Sontag, & Frydman, 2018), we used four different misfolded protein reporters: two thermally unstable proteins (Ubc9^{ts}, luciferase^{ts}); the tumor suppressor VHL, which is terminally misfolded in yeast due to lack of its physiological binding partners Elongin B and Elongin C; and ssCPY*, a mutated secretory enzyme that misfolds in the cytoplasm after removal of its ER-targeting signal sequence (Fig. 1B). In this way, we tried to ensure that the machineries and pathways uncovered were general to PQC and not specific only for the protein reporter being expressed.

Of course, comparing and contrasting the PQC requirements for different misfolded proteins could provide insights into context-dependent proteostasis pathways. This approach led us to the discovery that amyloid proteins (such as polyglutamine-expanded mutant Huntingtin) and prions (such as RNQ1) engage different PQC machineries than soluble misfolded proteins (such as VHL and Ubc9^{ts}) (Escusa-Toret, Vonk, & Frydman, 2013; Kaganovich, Kopito, & Frydman, 2008). As there is little evidence to suggest that this alternative yeast pathway leads to ubiquitination and/or clearance, amyloidogenic misfolded proteins may not be suitable for probing ubiquitin-mediated clearance mechanisms.

2.3 Choice of lysis and solubilization methods

Cell lysis involves the breakdown of a cell using mechanical, enzymatic and/or osmotic methods to release its contents—the “lysate.” Solubilization refers to the extraction of the molecules of interest from the cell into the lysate, usually aided by a detergent or a chaotropic agent. Both processes require methods and conditions that preserve the molecules in a state suitable for, and compatible with, the desired downstream applications. For example, immunoprecipitation techniques for detection of protein–protein interactions must strike a balance between adding enough detergent to solubilize the protein complexes of interest while not adding too much to disrupt the noncovalent interactions in the protein complex itself.

Lysis of higher eukaryote cells is routinely performed by resuspension of cells in a lysis buffer that contains a detergent or urea, which serve both to permeabilize the plasma membrane and to solubilize proteins of interest. In yeast, lysis is complicated by the presence of a cell wall—rendering the aforementioned method unsuitable for releasing the cell’s contents in many cases. Most yeast lysis protocols therefore involve some additional form of disruption before or alongside the detergent or urea. Detailed discussions and

protocols are available on the European Molecular Biology Laboratory's Protein Expression and Purification Core Facility website (<https://www.embl.de/pepcore/>). We have used a number of these methods for different protein clearance assays, with each having distinct advantages and drawbacks.

2.3.1 Boiling in SDS sample buffer—Perhaps the simplest and quickest protein extraction methods are boiling pelleted yeast cells in SDS sample buffer (Horvath & Riezman, 1994), as one would do prior to SDS-PAGE analysis. This technique has the added benefit of immediately denaturing the cell extract, thus minimizing opportunities for nonphysiological postlysis alterations (e.g., protein degradation by proteases). For the same reason, however, SDS boiling is unsuitable for many downstream applications (e.g., protein–protein interaction analysis, *in vitro* activity assays). We have also noticed that SDS is not as effective as 8*M* urea for solubilization of ubiquitinated proteins from the cell extract. We generally use this method for total protein analysis (e.g., cycloheximide chase assays—see Section 3.2).

2.3.2 Bead beating—One of the most popular mechanical lysis methods for yeast is beating the cells with glass beads to physically break apart the cell walls. This is similar to the classical *Escherichia coli* lysis method, except a bead diameter of 0.5 mm (instead of 0.1 mm) is used, to account for the difference in cell size. One caveat of this approach is that some proteins may aggregate during the bead beating process (Papanayotou, Sun, Roth, & Davis, 2010)—particularly problematic for studying misfolded proteins, which are intrinsically aggregation-prone due to the nonnative surface exposure of hydrophobic sequence elements.

Here, we outline some observations that might be useful when considering bead-beating as the lysis method of choice.

- In our experience, the most important factor in determining lysis efficiency is the ratio of beads:lysate—especially when using cells in stationary phase. We found a ratio of 1:1 (v/v) was most effective. Lower bead volumes considerably decrease the extent of lysis.
- One freeze–thaw step (at -80°C) prior to bead-beating improves lysis efficiency.
- Pretreating glass beads with concentrated acid increases lysis efficiency. Although it is possible to perform this in-house, we routinely use acid-washed glass beads from Sigma (catalog number G-8772).
- The presence of detergent in the lysis buffer is often necessary to solubilize the proteins of interest. However, this results in a considerable degree of “foaming” during the bead-beating process—potentially causing proteins to precipitate out of the lysate. One can add antifoaming agents to the lysis buffer (Wheeler, Jain, Khong, & Parker, 2017) or add detergent to the lysis buffer only after bead-beating. However, we generally avoid the use of detergents for bead-beating lysis, instead using 8*M* urea as the solubilizing agent with this method.

- The friction caused by high-frequency homogenization generates heat, which is not desirable for maintaining the proteins in a state suitable for most downstream applications. Therefore, it is necessary to incorporate cooling steps into the lysis protocol. We find that five cycles of bead-beating at 3000 rpm (using a Digital Disruptor Genie from Scientific Industries, catalog number Si-Dd38) for 1 min, with 1 min on ice in between each cycle, is sufficient to lyse and solubilize our model misfolded proteins without overheating the samples. However, this is a common step that could be a source for troubleshooting (e.g., increase time on ice in between cycles). We also perform all steps of this procedure in a cold room set at 4°C.
- We have used various devices for the physical bead-beating process, including different types of vortexer and specialized beading-beating devices—with no clear differences in lysis efficiency.
- Our method of choice for isolating the lysate from the glass beads postbead-beating is to use a 21-gauge needle to punch a hole into the bottom of the 1.5- or 2.0-mL microfuge tube used during the bead-beating, and centrifuging into a new 15-mL conical centrifuge tube (1000 $\times g$ at 4°C for 2 min, or until all of the lysate has collected into a new tube).

2.3.3 Cryogrinding—As discussed earlier, the friction and heat generated during bead-beating are reported to induce aggregation of certain proteins. One of the best solutions to this problem is mechanical disruption of cells at extremely low temperatures. The process of cryogrinding involves freezing cells (suspended in a minimal volume of lysis buffer) as small pellets in liquid nitrogen, followed by grinding them—in liquid nitrogen-cooled equipment—into a fine powder of broken cells. We use a Retsch Mixer Mill MM301 for the grinding process, following a procedure described previously (Ingolia, 2010), but with five cycles at 30 Hz, 3 min each, with 2 min in liquid nitrogen in between cycles. Note that even one cycle at 30 Hz for 3 min is usually sufficient for lysis of >50% of cells.

One of the largest drawbacks of using cryogrinding for the detection of ubiquitinated proteins is that 8M urea—which is typically required to fully solubilize ubiquitin chains—is close to its saturation point even at room temperature. Therefore, freezing samples containing 8M urea will invariably trigger precipitation out of solution. For this reason, we avoid cryogrinding for lysis when detection of ubiquitination is involved in the downstream application. However, if absolutely necessary, we have found some success with cryogrinding in a minimal volume (1:2 volume buffer:cell pellet) of Tris-containing lysis buffer (50mM Tris-HCl pH 7.5, 150mM NaCl, 2mMEDTA, cOmplete EDTA-free protease inhibitor tablet, 1 mM PMSF, 50 mM 2-chloroacetamide, 10μM PR-619 (covalent inhibitor of deubiquitinases, Sigma, catalog number 662141)), followed by solubilization in an equal volume of the same buffer supplemented with 8M urea (resulting in 4M urea final concentration) before clarification of the lysate from the cell debris.

2.3.4 Enzymatic digestion with Zymolyase—We discussed at the start of this section how yeast present an additional challenge over mammalian cells due to the presence of a cell wall. Of course, if the cell wall is removed, standard mammalian cell lysis protocols

are applicable. Zymolyase—an endoglucanase from *Arthrobacter luteus* (Kitamura, Kaneko, & Yamamoto, 1971)—digests the cell wall and exposes the yeast cell membrane for lysis without the need for mechanical bead beating or cryogrinding. If cost were no consideration, lysis with the Zymolyase (Zymo Research), followed by solubilization in 8M urea buffer, would be the method of choice for most assays involving ubiquitin detection. For most labs, however, this is not economically feasible, as protein preparations typically involve purification of multiple milligrams of the protein of interest—requiring hundreds of mLs of yeast culture. We reserve the use of Zymolyase for small volumes of culture (e.g., for immunofluorescence detection, which requires the infiltration of antibodies into the cell—a process that is prohibited by cell walls).

3. Assays of protein clearance

3.1 Clearance of misfolded protein puncta

Our lab and others have shown that proteins that misfold for a variety of reasons are all cleared within 1–2h through the ubiquitin–proteasome system (Escusa-Toret et al., 2013; Kaganovich et al., 2008; Malinowska, Kroschwald, Munder, Richter, & Alberti, 2012; Park et al., 2013). If this process is impaired (e.g., inhibiting the proteasome, or in strains with deletions of genes involved in the clearance pathway), the misfolded proteins accumulate in puncta (Escusa-Toret et al., 2013). One of our standard assays for identifying PQC factors involved in this clearance pathway is expression of the GFP-tagged misfolded protein for 4–6 h, then switching off expression for a set period of time and counting the percentage of cells with puncta at the end of this period using fluorescence microscopy. With this technique, we have identified the role of several molecular chaperones (e.g., Hsp70s, Hsp90, Sse1, Hsp42, Hsp104) and various parts of the ubiquitin–proteasome system (E2 ubiquitin-conjugating enzymes Ubc4 and Ubc5; E3 ubiquitin ligases Ubr1, San1, Doa10, and Hrd1) in misfolded protein clearance pathways (Escusa-Toret et al., 2013; Kaganovich et al., 2008; Samant et al., 2018).

- i. Transform yeast strains of interest with a plasmid containing the GFP-tagged misfolded protein under control of a galactose-inducible promoter (see Sections 2.1 and 2.2). Plate cells on SD-glucose selective agar plates—that allow selective growth only of cells carrying the plasmid—and wait for 2–3 days for colonies to form. Plates can then be stored at 4°C for up to 1 month before restreaking onto fresh selective agar plates is required.
- ii. On the afternoon/evening before the experiment, select single colonies to grow in 5mL SD-raffinose selective medium at 30°C with shaking (300 rpm).
- iii. The following morning, pellet cells (3000 ×g, 2min, room temperature) and resuspend in 2 mL of SD-galactose selective medium to an initial OD₆₀₀ of between 0.05 and 0.1mL⁻¹; galactose will induce expression of the substrate protein. Allow cells to grow (30°C with shaking) to an OD₆₀₀ 0.6–0.8mL⁻¹ (usually 4–6h, depending on the strain).
- iv. Pellet cells (3000 ×g, 2 min, room temperature) and resuspend in 2 mL SD-glucose media, to shut off misfolded protein expression. For the WT +

bortezomib (Bz) positive control, add the proteasome inhibitor Bz (final concentration 50 μ M) to the selective medium 5 min before adding to cells.^a Allow to grow between 1 and 1.5h.^b

- v. After the desired amount of time, take 750 μ L of each sample and add to 250 μ L 16% paraformaldehyde (for final concentration of 4%) in a 1.5-mL microfuge tube.
- vi. Incubate for 15 min at room temperature. Don't leave for longer than 20 min—extended formaldehyde exposure diminishes fluorescent protein signal and deforms nuclei.
- vii. Pellet cells (8000 $\times g$, 0.5 min, 4°C). Resuspend in 1 mL chilled methanol (−20°C) and incubate for 20 min on ice.
- viii. Pellet cells (8000 $\times g$, 0.5 min, 4°C) and wash 2 $\times$ with 1mL phosphate-buffered saline (PBS).
- ix. Resuspend in 1 mL PBS. Mix vigorously to minimize cells clumping together (by sonicating, vortexing, or vigorous pipetting).
- x. Add 50 μ L of cell suspension onto a concanavalin-A-coated coverslip^c (prewashed once with 1 mL PBS, to wash off excess concanavalin-A). Allow cells to adhere for 10 min at room temperature.
- xi. Wash 3 $\times$ with PBS to remove unbound yeast. Any unbound yeast at this stage could interfere with imaging.
- xii. Add one drop (~15 μ L) of yeast mounting medium (ProLongTM Diamond Antifade Mountant with DAPI, Thermo Fisher Scientific, catalog number P36961) onto coverslip and mount onto slide. Leave to cure^d for 24 h in dark at room temperature and then seal edges with nail varnish before imaging.

Our standard assay for testing whether deletion strains affect misfolded protein clearance is by calculating the percentage of cells that have puncta in the strain of interest, and comparing that with the percentage obtained for the wild-type strain. We take representative epifluorescence images of the GFP (for counting of misfolded protein puncta) and DAPI (a DNA-binding dye used for counting total number of cells) channels using a 63 $\times$ oil objective.^{e, f} We then manually count total number of cells (DAPI channel—although the DIC channel is also suitable), and the percentage of those cells with one or more puncta

^aBortezomib has poor solubility in water and will precipitate as soon as it is added. Ensure the precipitate has redissolved (5 min shaking at the shut-off temperature) before adding to cells.

^bThe exact amount of time for clearance varies depending on the misfolded protein substrate. It is important to perform an initial time-course for each misfolded protein in the wild-type strain of choice before proceeding with screens of deletion or overexpression strains. In our experience, the majority (>80%) of a wild-type yeast population from the BY4742 background clears misfolded proteasomal substrates in the range of 1–1.5 h.

^cPoly-lysine coated coverslips are not effective for adherence due to the presence of the yeast cell wall. However, if the yeast cells have been spheroplasted (e.g., to allow penetration of antibodies for immunofluorescence experiments), poly-lysine and not concanavalin-A must be used.

^dProLongTM mountants harden (“cure”) within 24 h, thus forming an optimal optical path for fluorescence microscopy (reflective index = 1.47). For immediate imaging, seal the four corners of the coverslip with nail varnish and allow to dry before microscopy. Leaving part of the coverslip unsealed will still allow the sample to cure. Seal as normal after 24 h.

^eTo obtain a representative sampling of the entire cell population, we ensure at least 300 cells are counted per slide (usually four images from different parts of the slides).

(GFP channel), on ImageJ (<https://imagej.nih.gov/ij/>) using the Cell Counter plug-in. Automated cell counting macros are also a possibility, although this is complicated by the fact that multiple puncta, of varying sizes, can exist in a single cell.

3.2 Cycloheximide chase assay

The puncta-counting assay provides a means of testing whether the clearance of transient inclusions (e.g., Q-bodies, JUNQ, and/or INQ) that contain misfolded proteins is impaired in different genetic backgrounds. However, it is possible that some genetic backgrounds cause puncta to dissolve (or prevent their formation in the first place) without affecting the total level of the misfolded protein. It is difficult to discern cases where the misfolded protein accumulates diffusely in the cytoplasm from those where it is actually cleared. Therefore, it is important to complement the puncta-counting assays with more direct measures of total substrate protein levels.

The classical method for measuring a protein's half-life is through radio-labeling (e.g., with ^{35}S) a population of newly translated proteins for a short period of time (the "pulse") followed by incubation in unlabeled medium for various times (the "chase") (Simon & Kornitzer, 2014). The clearance of the protein of interest can then be determined by lysing cells at specific timepoints, immunoprecipitating the protein, and detecting radioactivity on a gel by autoradiography. However, this technique is cumbersome due to the use of radioactivity and the requirement for immunoprecipitation at each time point. An alternative is the cycloheximide chase assay, where global protein synthesis is blocked using the translation elongation inhibitor cycloheximide. With this method, the clearance of the protein of interest can be ascertained by direct immunoblot—without requiring radioactivity or the immunoprecipitation steps. However, cycloheximide is a cytotoxic agent and would not be suitable to study clearance pathways taking longer than a few hours.

Detailed protocols for cycloheximide chase assays have been provided elsewhere (Buchanan, Lloyd, Engle, & Rubenstein, 2016). Here, we will provide a brief summary of the method we use for detecting clearance of misfolded proteins under control of a *GAL1* promoter.

- i. Grow cells transformed with a plasmid encoding the tagged misfolded protein (as described in Section 3.1) overnight in 5mL SD-raffinose media. The following morning, pellet cells ($3000 \times g$, 2min, room temperature) and resuspend in 100mL SD-galactose media (in a 250-mL conical flask, final OD_{600} 0.05–0.1 mL^{-1}) and grow till OD_{600} 0.6–0.8 mL^{-1} .
- ii. At this stage, split cells into the number of time-points required for the time-course. For our model misfolded proteins, we have 0, 10, 30, 60, and 90 min. Note that it is also helpful to have a positive control with proteasome inhibition

^fWe typically capture images in a single *z*-plane (rather than taking *z*-stacks encompassing the entire cell). Although we may miss some puncta that are out of the focal plane, this is minimized because (1) we are measuring total epifluorescence rather than confocal microscopy; (2) the haploid yeast strains we use are relatively thin ($<0.5 \mu\text{m}$); and (3) the ProLongTM mountant—a hardening mountant—further flattens cells. Furthermore, the effect of "missed" puncta will be averaged out across the strains, especially as we are counting over 300 cells per sample. However, if this is a concern, it is possible to take maximum intensity projections of *z*-stacks.

(e.g., bortezomib or MG132).[§] We typically grow 20 mL of culture (in a 50-mL conical flask) for each time-point.

- iii. Pellet all samples in 50-mL conical centrifuge tubes (3000 $\times g$, 2 min, room temperature). Resuspend the first sample (representing the $t = 0$ time-point) with 2 mL PBS containing 15 mM sodium azide and 1 $\times$ Roche cOmplete[™] EDTA-free protease inhibitor tablet. Pellet again (3000 $\times g$, 2 min, 4°C) and snap-freeze the pellet in liquid nitrogen. Store at -80°C .
- iv. While the cells from the $t = 0$ time-point are being frozen, resuspend the remaining samples into SD-glucose selective media with 50 $\mu\text{g mL}^{-1}$ cycloheximide. For the positive control, add 50 μM bortezomib here.
- v. Collect samples at the desired time-points by pelleting, resuspending in PBS with sodium azide and protease inhibitors, pelleting again, and snap-freezing, as described for the $t = 0$ sample in (iii).
- vi. Once all samples are collected, thaw on ice and extract proteins by boiling in an equal volume of 2 $\times$ SDS sample buffer for 10 min.
- vii. Detect levels of the protein of interest by standard SDS-PAGE and immunoblotting protocols. We use electrochemiluminescence (Pierce ECL Western blotting substrate from Thermo Fisher Scientific) and exposure to GeneMate Blue Ultra Film (BioExpress). Quantitation is performed by densitometry using ImageJ, with values expressed as a percentage of the loading control (e.g., GAPDH, Pgk1) signal for each individual lane.

4. Analysis of ubiquitin linkages

Misfolded protein clearance generally involves the posttranslational attachment of ubiquitin to one or more of the misfolded protein's Lys residues. Although well known as a proteasomal targeting signal, ubiquitination can signal other, diverse fates for the target protein, which affect signal transduction, *endo*-lysosomal trafficking, autophagy, NF κ B activation, and DNA repair, among other processes (Kwon & Ciechanover, 2017; Yau & Rape, 2016). Ubiquitin is often linked to other ubiquitin molecules to form polymers. The precise fate of a ubiquitin-modified protein is dependent on the topology of the ubiquitin chain, with chains linked by any of the seven Lys residues in ubiquitin's 76-residue sequence (as well as the amino-terminal amine to form linear chains) each potentially targeting the modified substrate to a different pathway. Given our evolving appreciation of this diverse role of ubiquitin linkages on critical cellular processes, novel methods and reagents for detecting specific linkages are emerging at a steady pace (e.g., linkage-specific antibodies and affinity reagents; mass spectrometry-based methods for absolute quantification of ubiquitin linkages (Ub-AQUA); in vitro assays making use of the linkage specificity of

[§]In yeast, the highly active efflux pumps make drug treatments challenging. We find that using a high concentration of bortezomib (50 μM) is effective for a 1.5 h chase. However, some clearance of the misfolded protein is still observed at the later time-points. If this is a concern—or if longer chases are required—one can use strains with increased drug permeability or deleted efflux pumps (e.g., *pdv5* or *erg6*, respectively). Alternatively, strains with impaired proteasomal activity (e.g., temperature-sensitive *cim3-1* or *cim5-1*) are more effective than pharmacologic inhibitors—however, these can only be used at nonphysiological temperatures, as well as requiring genetic crosses with the deletion strain of interest.

deubiquitinating enzymes (DUBs) for determination of ubiquitin chain architecture) (Swatek & Komander, 2016). This, in turn, is driving further groundbreaking research. The field of ubiquitin biology is moving past the idea that any ubiquitin “ladder” (by SDS-PAGE and immunoblotting) on a protein of interest must mean that the protein is a proteasomal target.

Here, we outline two antibody-based protocols for quantifying ubiquitin linkages on a tagged misfolded protein of interest.

4.1 Immunoprecipitation of ubiquitinated misfolded proteins

As discussed in Section 2.3, standard immunoprecipitation protocols must also be modified to account for the yeast cell wall. Furthermore, due to the high enzymatic activity of deubiquitinating enzymes (DUBs)—with some residual activity even in 8*M* urea—typical cell lysates that do not contain DUB inhibitors are not suitable for ubiquitination analysis, as most of the proteome is likely to have been deubiquitinated postlysis. For these reasons, preparing cells for ubiquitin detection is not trivial. We supplement all lysis buffers with 50 mM chloroacetamide and 10 μ M PR-619 to minimize DUB activity (Rose et al., 2016).

Even after lysis, ubiquitin analysis on specific proteins of interest introduces challenges different from those posed by standard immunoprecipitation protocols (Bloom & Pagano, 2005). Whereas most protocols involve maintaining noncovalent interactions to identify coprecipitating interaction partners, ubiquitination analysis requires the removal of such coprecipitating partners (whose ubiquitination would be indistinguishable from that of the protein of interest) while not preventing interactions between the antibody and the protein of interest. We typically use the previously published approach where cell lysates are initially denatured in 8*M* urea (to disrupt physiological protein–protein interactions) before diluting the urea in standard Tris-containing immunoprecipitation buffer to enable antibody binding to the protein of interest (Emmerich & Cohen, 2015; Heck, Cheung, & Hampton, 2010; Katzmann & Wendland, 2005). We routinely use Flag-tagged substrate proteins for ubiquitination analysis, for cost and ease of use of the Flag-M2® magnetic beads (Sigma). Note, however, that the same protocol can be used for GFP-tagged proteins, using GFP-Trap nanobodies (ChromoTek) that are resistant to 8*M* urea and 1% SDS—thereby ensuring fully denaturing conditions throughout the immunoprecipitation protocol.

- i. Grow cells transformed with a plasmid encoding the Flag-tagged misfolded protein overnight in 10 mL SD-raffinose selective medium, as described earlier. The following morning, pellet cells in a 15-mL conical centrifuge tube (3000 $\times$ *g*, 2 min, room temperature) and switch to 100 mL SD-galactose selective medium (initial OD₆₀₀ 0.05–0.1) in a 250-mL conical flask. Grow to an OD₆₀₀ of 0.6–0.8 mL^{−1}.
- ii. Pellet each sample in two 50-mL conical centrifuge tubes (3000 $\times$ *g*, 5 min, room temperature) and resuspend in 100 mL SD-glucose selective media with 50 μ M bortezomib.^h Grow at 30°C for 1 h and centrifuge cells as above.

^hNote that bortezomib (or some sort of proteasome inhibition) is required to allow accurate detection of ubiquitination, as otherwise ubiquitinated proteins could be degraded by the proteasome within the 1 h of expression shut-off. If it is possible that the protein of

- iii. Resuspend cell pellet in an equal volume (approximately 0.5mL) of fresh urea lysis buffer (50mM Tris–HCl pH 7.5, 8M urea, 150mM NaCl, 2mM EDTA, 1× cOmplete™ EDTA-free protease inhibitor tablet, 50mM 2-chloroacetamide, 10 μM PR-619, 1 mM phenylmethylsulfonyl fluoride (PMSF)).ⁱ
- iv. Lyse cells by bead-beating (five cycles at 1 min each, with 1 min on ice in between cycles—see Section 2.3.2).
- v. Dilute 10-fold in Triton IP Buffer (same composition as Urea Lysis Buffer, but with 1% Triton X-100 instead of 8 M urea), and clarify lysates (16,000×g for 30min at 4°C). Quantify total protein (e.g., BCA or Bradford assay).
- vi. Incubate 2 mg of lysate with Flag-M2® magnetic beads (Sigma catalog number M8823) for 2h at 4°C to immunoprecipitate the Flag-tagged protein of interest.
- vii. Elute Flag-tagged protein from beads by heating for 30min at 70°C in NuPAGE™ 1× nonreducing LDS sample buffer^j (10% glycerol, 1% lithium dodecyl sulfate, 0.2M triethanolamine–Cl pH 7.6, 1% Ficoll®–400, 0.00625% phenol red, 0.00625% Coomassie G250, 0.5M EDTA disodium; Thermo Fisher Scientific, catalog number NP0007).
- viii. Add DTT (50 mM final concentration) and heat again at 70°C for 10 min, to reduce eluted sample prior to SDS-PAGE analysis.
- ix. Following SDS-PAGE, transfer proteins to methanol-wetted PVDF membrane, according to standard protocols.^k
- x. Denature proteins on PVDF membrane by heating for 10min at 95°C^l.
- xi. Immunoblot with the desired antibodies. We have successfully used: pan-Ubiquitin VU-1 (1/1000; Life Sensors, catalog number VU101), pan-conjugated Ubiquitin FK2 (1/1000; Life Sensors, catalog number AB120) K11-Ub (1/100; EMD Millipore, catalog number MABS107-I), HRP-conjugated K48-Ub (1/1000; Cell Signaling Technology, catalog number 12805), HRP-conjugated K63-Ub (1/1000; Cell Signaling Technology, catalog number 12930), and K11/K48-Ub bispecific antibody (1/500; Genentech).

interest is cleared through another ubiquitin-mediated pathway (e.g., vacuolar targeting through K63 or mono-Ub linkages), inhibitors for the protease in these pathways (e.g., chloroquine, PMSF) may also be added.

ⁱPrepare lysis buffer fresh on day of lysis. For all buffers, add protease inhibitor tablet, 2-chloroacetamide, PR-619 and PMSF immediately before use.

^jThe use of nonreducing LDS sample buffer (without DTT) helps avoid coelution of the Flag-antibody with the protein of interest. This is especially important if the protein of interest runs at a molecular weight similar to the light or heavy immunoglobulin chains. Addition of DTT after this step reduces the eluted samples and helps SDS-PAGE analysis.

^kWe find that PVDF provides much greater sensitivity than nitrocellulose, which is especially important for detection of ubiquitin “ladders.”

^lUbiquitin itself is a very stable protein, and will not fully denature even after SDS-PAGE. This extra denaturation step is required to fully expose the necessary epitopes for antibody binding by immunoblot. Different protocols for denaturation have been described (e.g., 6M guanidium hydrochloride, autoclaving, pouring boiling water). One of the most effective means we have found is to “stamp” a metal heat-block (set at 95°C) for 10 min onto the PVDF-filter paper sandwich (i.e., Just removing the gel after transfer and keeping the rest of the sandwich intact) immersed in water.

4.2 Ubiquitin linkage ELISA

It is often difficult to compare the degree of ubiquitination of proteins across samples (e.g., different deletion strains), as the ubiquitin “ladder” typical of immunoblots is not readily conducive to densitometric quantification. We therefore developed a ubiquitin enzyme-linked immunosorbent assay (ELISA)—using the same antibodies used for immunoblot—to quantify ubiquitin linkages on Flag- or GFP-tagged proteins of interest (Fig. 2). Here, we describe a protocol to compare K11, K48, and K63 ubiquitin linkages on Flag-tagged proteins of interest between different strains. The same protocol can be followed for quantification of linkages on GFP-tagged proteins, except with the use of GFP-multiTrap 96-well plates (ChromoTek) instead of Flag-M2®-coated plates, and using the GFP and Flag signals as positive and negative controls, respectively.

- i. Prepare cell lysates as described for “Immunoprecipitation of ubiquitinated misfolded proteins” (Section 4.1, steps i–v).
- ii. Add 200 µg of lysate to each well of an anti-Flag-M2®-coated 96-well plate (Sigma catalog number P2983).^m Use five wells for each technical replicate.ⁿ Incubate for 2h at room temperature with gentle shaking, to allow binding of the Flag-tagged protein of interest to the antibody-coated plate.
- iii. Wash 4× with Triton IP Buffer to remove unbound protein.
- iv. To each well, add an antibody to one of: GFP-negative control (1/1000; Cell Signaling Technology, catalog number 2956); Flag-positive control (1/1000; Cell Signaling Technology, catalog number 2368); K11-Ub (1/50; EMD Millipore, catalog number MABS107-I); K48-Ub (1/500; Cell Signaling, catalog number 4289); or K63-Ub (1/500; EMD Millipore, catalog number 05–1308).^o Each antibody is diluted in 100µL Triton IP buffer with 0.1% BSA. Incubate for 1 h at room temperature, with gentle shaking.
- v. Wash 4× with Triton IP Buffer to remove unbound primary antibody.
- vi. Incubate for 1h with HRP-conjugated antirabbit secondary antibody (1/2000; Jackson ImmunoResearch, catalog number 711–035-152) diluted in 100 µL Triton IP buffer with 0.1% BSA.
- vii. Wash 4× with Triton IP Buffer to remove unbound secondary antibody.
- viii. Incubate for 30 min with 100 µL Pierce tetramethylbenzidine (TMB) substrate (Thermo Fisher Scientific).
- ix. Stop the reaction by adding 100 µL 0.16M sulfuric acid, and measure absorbance at 450 nm on a plate reader.

^mThe amount of total protein that will saturate the plate needs to be determined prior to ubiquitin analysis. We ensure that the Flag-tagged protein in the lysate is well in excess of the binding capacity of the immobilized antibodies, to ensure that similar amounts of protein are being isolated in all the samples.

ⁿThere is some degree of variability in this assay; therefore, we ensure there are at least two technical replicates per sample.

^oAs the Flag-M2® antibody immobilized to the 96-well plate is isolated from mouse, it is important to use antibodies at this step that are from a different species. As most current ubiquitin linkage-specific antibodies are produced in rabbit, using rabbit antibodies for all conditions ensures that the same secondary antibody can be used for the next step of the ELISA.

- x. Subtract the negative control (GFP) reading from each of the other four readings.
- xi. To calculate the K11, K48, and K63 ubiquitin signals for each strain, normalize to the total protein of interest levels by dividing each reading by the Flag reading for that sample. It is now possible to compare the specific ubiquitin linkage levels from one strain to another.^P

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^PNote that this assay—much like any antibody-based assay—cannot be used to compare relative signals of different Ub linkages, given that antibodies have intrinsically different binding affinities and detection limits. Therefore, we limit this assay to comparison of signals for the same antibodies across different strains (e.g., wild-type vs deletion strains).

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Fig. 1. Misfolded proteins expressed in budding yeast *S. cerevisiae* are cleared through the concerted action of molecular chaperones, E3 ubiquitin ligases, and the proteasome (A). Three model misfolded proteins that misfold for different reasons are shown in (B).

Fig. 2.

Schematic for the ubiquitin linkage ELISA to compare relative levels of K11, K48, or K63 ubiquitin linkages in different strains expressing a Flag-tagged protein.