UNIVERSITY OF CALIFORNIA SAN DIEGO

Discovery and Analysis of Genes Required for Protein Quality Control

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy

in

Biology

by

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2018

The Dissertation of Lilia Ornelas is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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DEDICATION

I dedicate this dissertation to my father and brother, my extended family (of over 200 members), and in honor of David Estrada and Sergio Ortiz who recently passed away. Also, to my cat Sassy who was my loyal, furry friend of 13 years.

EPIGRAPH

"I'm enough of an artist to draw freely on my imagination, which I think is more important than knowledge. Knowledge is limited. Imagination encircles the world."

Albert Einstein

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LIST OF ABBREVIATIONS

Abreviation	Definition
CHX	Cyclohexamide
CQC	Cytoplasmic Quality Control
E1	Ubiquitin activating enzyme
E2	Ubiquitin conjugating enzyme
E2	Ubiquitin ligase
GFP	Green fluorescent protein
TDH3	Glyceraldehydes-3-phosphate dehydrogenase
Ub	Ubiquitination
WT	Wild-type

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ABSTRACT OF THE DISSERTATION

Discovery and Analysis of Genes Required for Protein Quality Control

By

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Doctor of Philosophy in Biology

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Protein-based life faces constant and dynamic stress caused by protein mis-folding. This can cause a variety of potentially lethal alterations in cell physiology that underlie many important clinical maladies. Eukaryotic cells have evolved a variety of quality control mechanisms to ensure toxic proteins are removed. The Ubiquitin Proteasome System (UPS) is the principal mechanism of protein degradation in eukaryotes that utilizes ubiquitin ligases E1, E2 and E3 to tag misfolded proteins with polyubiquitin chains. Of these enzymes, E3 ligases determine the specificity in identifying misfolded substrates. We have discovered that the highly conserved E3 ubiquitin ligase Ubr1 works together with cellular chaperones to detect and destroy misfolded proteins by selectively ubiquitinating them for degradation. Ubr1 is highly conserved in all eukaryotes and well-studied in other capacities, but, little is known about its role in quality control. We have employed the yeast model misfolded substrate ΔssCPY*-GFP in a high throughput array based screen Systematic Genotype-Phenotype Array (SGPA) to uncover other genes involved in cellular quality control, including those that may work in the Ubr1/San1 pathway, and those that work in distinct branches of this process. From our screen, we discovered a set of genes related to translational modification and ubiquitin cleavage that appear to have unanticipated roles in protein quality control. The genes revealed in these studies are, to a large extent, conserved across all eukaryotes, so the knowledge we gain of their roles in quality control will impact future studies and hold clinical relevance.

Chapter 1

Introduction

Introduction

The maintenance of the appropriate folding state of cellular proteins, also known as protein Quality Control (QC), is a universal problem all organisms encounter. Excess misfolded proteins lead to cellular stresses that are harmful at the cellular, organ and whole organism level. A correctly folded protein continuously faces a variety of perturbations caused mutations, heat, chemicals, oxygen radicals and heavy metal ions that can lead to damage and misfolding. This can result in loss of normal function, as well as direct toxicity caused by the misfolded state, referred to as proteotoxicity. [1], [2]. Such proteotoxicity, while still not fully understood, is responsible for a variety of pressing maladies and appears to underlie key aspects of the aging process in all species. Eukaryotes have evolved elegant mechanisms to selectively detect, correct, or and destroy misfolded proteins to diminish the burden of proteotoxicity. These processes are collectively referred to as protein quality control [3].

The Ubiquitin Proteasome System

One of the main strategies of protein quality control involves selective destruction of misfolded proteins, principally by the ubiquitin proteasome system (UPS) [4]. The UPS is highly conserved from yeast to mammals. Many cellular processes utilize the UPS to regulate the degradation of cell-cycle proteins [5], enzymes [6], and misfolded proteins [7] [8]. Proteasomal degradation predominantly depends on ubiquitination of the target protein [4]. Ubiquitin is the targeting molecule used for both normal and aberrant proteins for destruction. It is a small (7.6 kD) protein that is covalently added to targeted proteins to bring about recognition and destruction by the 26S proteasome. In this process, ubiquitin molecules are specifically attached to a degradation substrate

as a polymeric chain to signal proteasomal destruction. Ubiquitin molecules linked to one another at lysine 48 are the principle (but not only) polyubiquitin signal for proteasomal degradation. A linked K48 polyubiquitin chain of 4 or more residues is typically required for proteasomal degradation [9], [10].

Ubiquitination is an ATP dependent process carried out by the sequential action of three classes of enzymes: a ubiquitin activating E1, a ubiquitin conjugating (UBC) enzyme or E2, and the aforementioned E3 ligase. Ubiquitin E1 uses ATP to form a thioester linkage with ubiquitin. The activated ubiquitin is then transferred to a ubiquitin conjugating E2. Finally an E3 ubiquitin ligase catalyzes the transfer of ubiquitin from the E2 to the substrate molecule, forming an isopeptide linkage with a substrate lysine, or a lysine on a previously attached ubiquitin [11]. In this way the multiubiquitin chain is attached to the targeted protein. Thus, the E3 ligases are the key determinants of selectivity in this process for they determine which proteins receive the fatal modification.

Cytoplasmic Quality Control

In the early stage of a protein's life, quality control processes exist to limit the production of misfolded proteins. The ribosome, as the birthplace of every protein, is the first to execute quality control methods during errors in translation. In yeast (*Saccharomyces cerevisiae*), the E3 ligase Ltn1 is part of a surveillance system for nascent proteins stalled by translational arrest caused by defective mRNA [12], [13]. Faulty mRNA can arise from factors such as transcriptional mistakes, premature polyadenylation and DNA mutations. making all organisms susceptible to it. Furthermore, translation of corrupted messages leads to synthesis of non-functional and

potentially toxic proteins. When stalled mRNA's are detected, with polypeptides emerging from the ribosome, they are extracted by Ltn1 machinery, and ubiquitinated for degradation [13]. The ribosome is a prime area in various co-translational quality control pathways to ensure accurate translation or removal of potentially toxic proteins.

The next level of protein quality control functions to degrade cytoplasmic, damaged proteins that have been released from the ribosome, or produced by the variety of insults that can alter folding state in mature proteins. In yeast, the E3 ligase Ubr1, Hsp70 chaperones (Ssa1-4), and Hsp110 (Sse1) co-chaperones are the main players of cytosolic quality control [7]. Ubr1 was first discovered for its role in the N-end rule pathway [14]. It's responsible for ubiquitinating proteins with a degron composed of N-terminal destabilizing residues [14]. It was later discovered in our lab and other groups that Ubr1 acts independently of the N-end rule pathway to regulate cytosolic quality control [7]. Consistent with this idea, various mutant substrates such as ΔssCPY*-GFP have been shown to be unstable in *UBR1* cells but are stabilized in *ubr1Δ* null cells. The E2 enzymes functioning together with Ubr1 are Ubc4 and Rad6.[7], [15]. And as is the case with all yeast UPS pathways, the E1 ubiquitin activating enzyme is Uba1 [16].

Surprisingly a nuclear E3 ligase, San1 also has a role in cytosolic quality control. San1 remains in the nucleus while aberrant proteins are shuttled across the nuclear membrane. It has been observed that Hsp70 and Hsp110 chaperones assist in the localization of substrates to the nucleus [1] [8]. San1 is an intrinsically disordered protein that uses those regions to recognize a large range of misfolded substrates [18]. It is still unknown why San1 participates in cytoplasmic quality control, and some

speculate that the majority of proteasomes reside in the nucleus . Overall, San1 in conjunction with Ubr1 is effective in clearance of cytosolic misfolded proteins. Other cytosolic factors include ERAD E3 ligase Doa10 which monitors the folding state of cytosolic domains on membrane proteins [19]. Overall, different ubiquitin ligase complexes target abnormal proteins based on where the abnormal protein is residing.

Molecular Chaperones

Molecular chaperones are an essential component of protein quality control. They are the first to recognize misfolded proteins by their exposed hydrophobic regions and first to attempt to rescue them [20]. If such an effort fails, chaperones can facilitate the destruction of the protein through the proteasome or autophagy [21]. Toxic proteins are prone to aggregating and this is especially problematic in neurons that do not undergo mitosis. Neurons cannot dilute toxic substrates as efficiently as mitotic cells, making them vulnerable to proteotoxicity, especially during aging [22]. Failure in protein quality control has been linked to neurodegenerative diseases such as Huntington's disease, Alzheimer's disease and Parkinson's disease [23], [24]. Chaperone inducing drugs and anti-aggregation drugs have been used to treat symptoms of disease making them a good target for study. The majority of molecular proteins are called heat shock proteins (HSPs) because they are upregulated during stress such as heat shock, oxidative stress, and toxic stress. They constitute about 10% of the proteome and facilitate in proteostasis during normal conditions and cellular stress responses [25].

The ubiquitination of Ubr1 substrates is dependent on Hsp70 and Hsp110 interaction [7]. Hsp110 regulates Hsp70 affinity for its clients through ATP hydrolysis. These chaperones along with Ubr1 form in a complex to target substrates with ubiquitin

for degradation. Additional quality control chaperones such as Hsp40 (Ydj1) and Hsp90 (Hsc82, Hsp82) fine-tune the quality control cycle. Hsp40 and Hsp70 work in conjunction to de-aggregate substrates [15]. Unlike Hsp70 chaperones that target proteins for degradation, Hsp90s have been proposed to rescue proteins from destruction. It is expressed constitutively in the cell, even in non-stressed conditions to assist in protein folding or re-folding when damaged [26][27]. The coordinated interaction between Hsp70 and Hsp90 have evolved to assist in the renaturation or destruction of damaged proteins that have arisen from stressful conditions. Whether the protein is saved or destroyed is referred to as “protein triage” [28] . Hsp70/Hsp90 serves as the surveillance machinery of cytosolic quality control accompanying proteins to their disposal machinery or saving them from destruction. This serves as the final step of quality control via the ubiquitin-proteasome.

The E3 ligase CHIP is another quality control ligase conserved in some eukaryotes but not yeast. CHIP contains a tetratricopeptide repeat (TPR) motifs for interacting with Hsp70 and Hsp90 which contain similar TPR motifs [28]. Most of CHIP’s clients are chaperone dependent and facilitates the ubiquitination of Hsp70 and Hsp90 substrates. The basic triaging decisions between rescuing or committing a protein for degradation are carried out similarly in yeast as they are in CHIP dependent quality control [29]. The overall mechanisms of CHIP and Ubr1 are still being investigated and more remains to be elucidated.

Approach used for gene discovery of cytoplasmic quality control pathways

To better understand cytosolic quality control, we have developed a high-throughput screen SGPA (Systematic Gene-Phenotype Analsyis) to screen the entire

genome of *Saccharomyces cerevisiae* for quality control factors [30]. We have employed the optically detectable cytosolic misfolded substrate ΔssCPY*-GFP to identify genetic candidates by their fluorescent degradation phenotypes The misfolded substrate was introduced into each null strain by a mating technique adopted from Synthetic Genetic Array Analsyis (SGA) [31]. With our analysis we were able to detect both positive and negative regulators of quality control. With our technique, we comprehensively screened the entire genome of Saccharomyces cerevisiae which included the non-essential gene yeast knockout collection (YKO) and essential gene decreased abundance by mRNA perturbation (DAmP) collection. Surprisingly, we found a subset of genes related to U34 tRNA modification and Urmylation to play a role in pre-translational quality control. In addition, we propose a potential role for the de-ubiquitinases Ubp3-Bre5 in protein triaging decisions. Finally we discovered a surprising high specificity for one of two highly redundant isoforms of Hsp90 in the degradation of sereral misfolded proteins. Taken together, these studies reveal a complex interplay between the established Ubr1/San1 PQC pathways and a number of new players that are woven together to form the dynamic and protective fabric of eukaryotic quality control.

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Chapter 2

Systematic Gene-to-Phenotype Arrays: A High-Throughput Technique for Molecular Phenotyping

Abstract

We have developed a highly parallel strategy, systematic gene-to-phenotype arrays (SGPAs), to comprehensively map the genetic landscape driving molecular phenotypes of interest. By this approach, a complete yeast genetic mutant array is crossed with fluorescent reporters and imaged on membranes at high density and contrast. Importantly, SGPA enables quantification of phenotypes that are not readily detectable in ordinary genetic analysis of cell fitness. We benchmark SGPA by examining two fundamental biological phenotypes: first, we explore glucose repression, in which SGPA identifies a requirement for the Mediator complex and a role for the CDK8/kinase module in regulating transcription. Second, we examine selective protein quality control, in which SGPA identifies most known quality control factors along with U_{34} tRNA modification, which acts independently of proteasomal degradation to limit misfolded protein production. Integration of SGPA with other fluorescent readouts will enable genetic dissection of a wide range of biological pathways and conditions.

Introduction

In yeast [1], [4] and other microbes [5], [6] systematic analysis of large mutant collections has been remarkably successful in mapping the functional genetic architecture of the cell. Such analyses detect alterations in growth caused by genetic mutation, typically by quantifying the sizes of mutant colonies arrayed onto agar [7], [8], or by counting barcode tags within a population of cells after competitive liquid growth [9]. Although colony size and barcode readouts are conducive to screening of cellular fitness, they lack molecular resolution to characterize specific cellular events that fail to induce a growth phenotype. In contrast, optical reporters, including fluorescent probes for pathway activity [10], [11] and tagged proteins [12], [13] can measure a much larger range of phenotypic readouts. Optical readouts are obtained with techniques such as fluorescence-activated flow cytometry [11] or high-content microscopy [14], [15], although they fall short of the throughput of high-density cell colony arrays [16].
We reasoned that combining the advantages of these approaches might dramatically enhance the power of systematic genetic interrogation and thus developed the systematic gene-to-phenotype array (SGPA). SGPA brings together comprehensive mutant arrays with optical phenotype reporters by leveraging ad- vantageous signal-to-noise characteristics of microbial colonies grown on synthetic membranes. This technology allows direct assessment of how each gene contributes to a specific phenotype.

As a specific and biologically relevant test of SGPA, we explored two fundamental cellular processes with different phenotypic markers: first, we tested an inducible, tightly controlled *GAL1* promoter (pGAL1), a classic readout of the so-called

glucose repression pathway [17]. By deploying multiple copies of a pGAL1 fluorescent transcriptional probe per cell, we quantified promoter activation and repression under induced and repressed conditions, respectively, across approximately 6,000 mutant yeast strains. In this context, we found that SGPA enables a broadly useful and sensitive approach to gene discovery, particularly when applied to inherently weak phenotypes such as leaky promoter activity. We identified the highly conserved Mediator complex as a crucial element in transcriptional control from the *GAL1* promoter. Dynamic module changes in Mediator play a central role in controlling eukaryotic transcription and have been the target of intense research efforts [18]. SGPA uncovered a role for the CDK8/kinase module in regulating both promoter repression and induction, depending on environmental context, and identified module interfaces involved in complex function. This enabled us to build a simple model of CDK8/kinase module control of the *GAL1* promoter, advancing our understanding of how this transcriptional element may be regulated over a large dynamic activity range.

In a second set of experiments, we focused on protein quality control (PQC), a basic process in all domains of life that ensures misfolded proteins are diminished to acceptable levels, either by refolding, degradation, or lowered production. One of the most well-studied PQC pathways, the ubiquitin-proteasome system, involves ubiquitin tagging of proteins and subsequent destruction by the proteasome. We probed PQC by deploying a fluorescent, permanently misfolded, but non-toxic protein substrate. Essentially all known PQC components emerged from our SPGA analysis, including the proteasome and the major ubiquitin ligases, and we can show direct contribution of *BRE5*, a ubiquitin protease co-factor, to control of misfolded protein degradation.

Surprisingly, cells deficient in genes underlying the U34 tRNA modification and urmylation pathway also exhibited a clear PQC phenotype. These gene mutants showed selective accumulation of mis-folded proteins, without altering substrate stability or rate of proteasomal degradation, suggesting that selective translational control by modified tRNA serves an underappreciated role in limiting expression of accumulating misfolded proteins.

Design

Genome-wide technologies to quantify the contribution of gene deletion or gene overexpression to a single (growth) phenotype have been used with great success. High-throughput microscopy and flow cytometry-based assay systems measure a wide variety of cellular phenotypes. SGPA now combines efficient high-throughput screening of defined genetic manipulations with the ability to determine a wide range of resulting phenotype changes. Previous attempts at this approach were limited to promoter-driven fluorescent reporters, required the simultaneous expression of a secondary control reporter to overcome noise, or used slow and expensive fluorescent scanners or low colony density, which severely limited throughput [19], [20]. Other genome-wide assays for regulators of protein turnover proved to be extremely data-rich, but required complex tandem degradation assays, followed by scanning or flow cytometry [21], [22] thus exhibiting an analogous throughput bottleneck. Our experience in differential network biology informed core design principles for SGPA: (1) Leverage existing technology platforms to allow for a swift implementation into existing laboratory settings. (2) Rely on a singular fluorescent reporter channel to avoid unintentional phenotype signal bias and utilize independent control screens and population-based normalization instead. (3)

Maximize throughput by optimizing the physical layout of the underlying mutant collections and very fast image acquisition. By adhering to these principles, we could develop a flexible and fast assay system that can be applied broadly to study phenotypes of interest genome-wide.

Results

The Single-Plate ORF Compendium Kit Enables Efficient SGPA

SGPA is built on a super-high-density 6144 yeast colony array format called single-plate ORF (open reading frame) compendium kit (SPOCK). This format unifies the non-essential gene yeast knockout (YKO) [4] and essential gene decreased abundance by mRNA perturbation (DAmP) [23] collections, covering disruptions to >95% of yeast ORFs, and it entails close to 100 wild-type-like controls in the area of a standard 127*3*85 mm microwell plate (Figure 2-1 A). SPOCK ensures efficient and interspersed placement of essential and non-essential deletion strains and resulting in homogeneous growth phenotypes for both collections and well-mixed distribution of mutant chromosome locations.

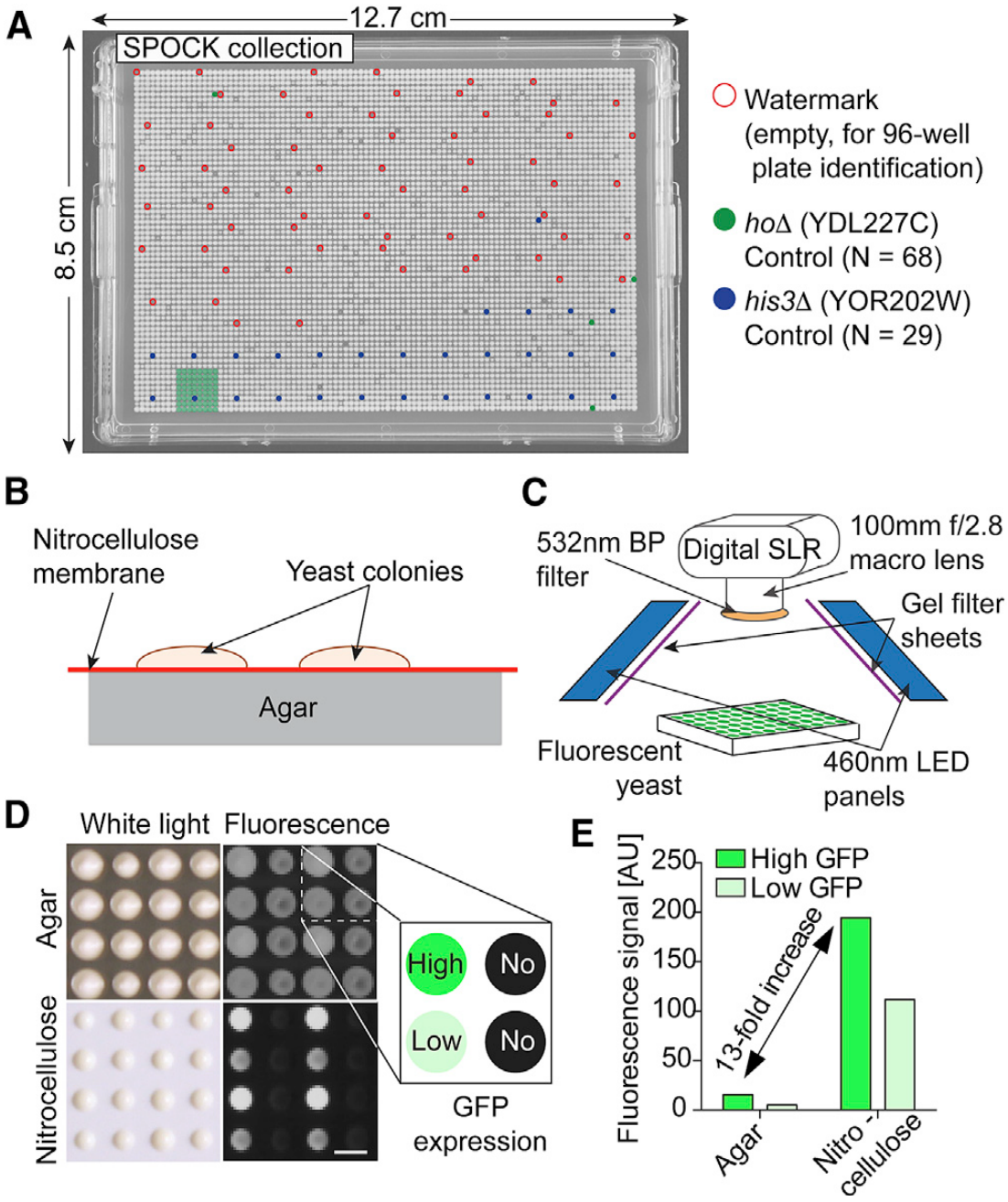

Figure 2-1: Systematic Gene-to-Phenotype Arrays
(A) Overview of SPOCK covering >95% of all yeast ORFs. (B) For SGPA, yeast colonies grow on nitrocellulose instead of agar directly. (C) Imaging setup for the fluorescence screening (BP, band-pass filter; SLR, single-lens reflector camera; LED, light-emitting diode). (D) Comparison of high, low, and no-GFP test strains grown on traditional agar plates (top row) and on nitrocellulose (bottom row). Scale bar, 2 mm. (E) Thirteen-fold increase in signal due to growth on nitrocellulose (signal minus no-GFP background intensity, mean of N = 384 for each, error bars too small to display).

To enable quantitation of molecular phenotypes, the SPOCK library is transformed with a fluorescent molecular reporter using deficient in genes underlying the U34 tRNA modification and urmylation pathway also exhibited a clear PQC phenotype. These gene mutants showed selective accumulation of mis-folded proteins, without altering substrate stability or rate of proteasomal degradation, suggesting that selective translational control by modified tRNA serves an underappreciated role in limiting expression of accumulating misfolded proteins.

We crossed the pGAL1-GFP reporter plasmid into the SPOCK collection and evaluated colony fluorescence under glucose or galactose, on agar or nitrocellulose. As in our initial technical analysis (Figure 2-1 D), nitrocellulose improved fluorescence over agar-grown colonies (Figure 2-1 D) and enhanced our ability to detect GRMs under repressed conditions (Figure 2-1 E). By scattering induced versus repressed conditions, we identified three mutant sets (Figure 2-1 F). The first set we call galactose unresponsive (GU) mutants, which have normal fluorescence under glucose and reduced fluorescence and colony size under galactose conditions. This group is largely overlapping with mutants identified in a traditional fitness-based assay ($p = 3.9\ 3\text{^}10^{42}$ by hypergeometric test; Figure 2-1 F, inset), and the intersection is highly enriched for strains deficient in respiration, mitochondrion function (i.e., ‘‘mitochondrial inner membrane’’; $p = 2.33\ 3\text{^}10^{24}$), and galactose metabolism ($p = 9.99\ 3\text{^}10^{6}$; Data S1). This is expected, as yeast uses simultaneous respiration and fermentation under galactose conditions an effect similar to enhanced oxidative

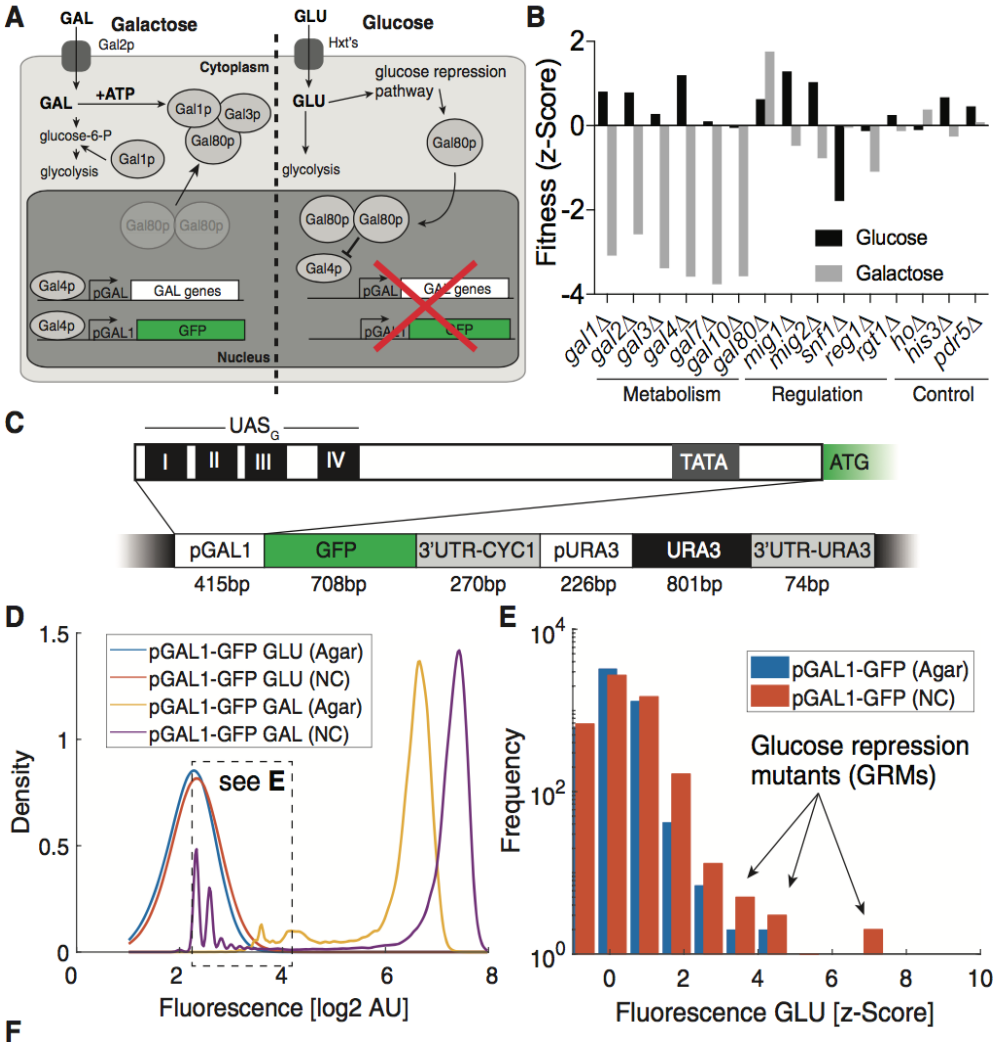

Figure 2-2: Study of Glucose Repression Genes by SGPA
(A) Overview of the galactose pathway; pGAL1- GFP represents our artificial promoter activity sensor on a 2m plasmid. (B) Analysis of fitness defects in galactose pathway mutant strains grown with glucose (black bars) or galactose (gray bars) as sole carbon source (mean of N = 5). (C) Schematic of the reporter cassette: the pGAL1 contains four upstream activating sequences for Gal4p transcription factor Binding (UASG) and the GAL1 TATA box. It also contains selectable auxotrophic marker (URA3) under a sperate promoter, as well as termination sequences (3'UTR). (D) Fluorescence distribution for colonies grown under glucose on agar (blue) or nitrocellulose (red), and colonies grown under galactose (yellow and purple). (E) Distribution of the *Z* scored colonies' fluorescence values for the repressed glucose conditions. (F) Scatter graph showing the pGAL1-GFP fluorescence values under repressed glucose versus induced galactose conditions. Values around zero represent colonies with close-to-population-average intensities under the respective conditions. See text for mutant classifications; selected mutants are named for clarity; red labels are examples of typical, known galactose pathway mutants. Inset shows overlap in hits between a classical, fitness-based assay of glucose-galactose switch and the GU mutants.

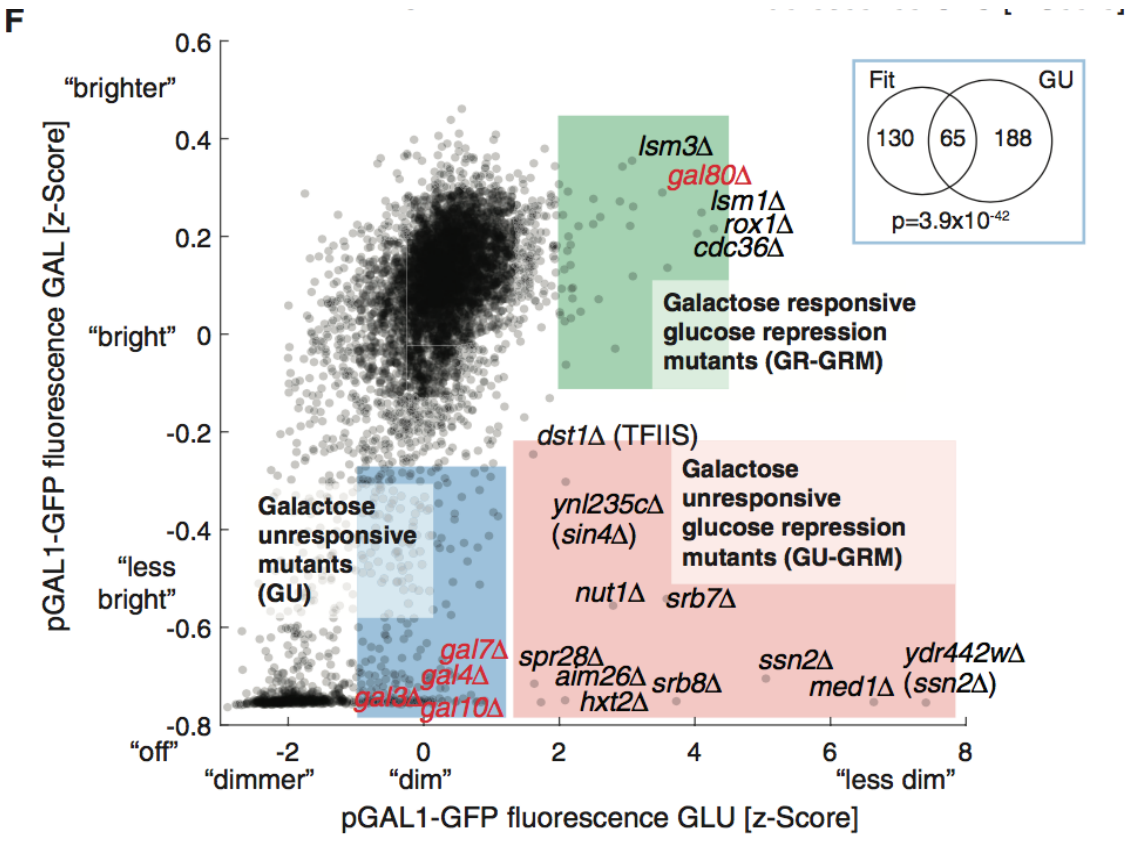

Figure 2-2: Study of Glucose Repression Genes by SGPA continued

metabolism observed in galactose-grown human cells. A second set of mutants we call galactose responsive GRMs (GR-GRMs), which have increased *GAL1* promoter activity under glucose but normal fluorescence under galactose. These genes are necessary for glucose repression, but not for galactose metabolism (i.e., *gal80*Δ; Figure 2-1 F). Third, GU-GRMs are necessary for both glucose repression and growth under galactose. We found that most of these mutations affect the Mediator complex, as discussed below (Figure 2-1 F).

The CDK8/Kinase Mediator Module Acts as a Bimodal Transcriptional Control Unit

Mediator is a modular protein complex that consists of over 20 subunits (Figure 2-2 A) and exists in all eukaryotes [18]. It regulates transcription by RNA polymerase II (RNA Pol II), integrates signals from bound transcription factors, and organizes genomic DNA into topological domains. Mediator's composition and structure are flexible, enabling it to perform diverse roles by exchanging subunits and modules dynamically [18]. Gal4p-Mediator interactions and genome-wide Mediator occupancy have been used to understand eukaryotic transcriptional regulation [24], [26]. Based on these studies and comprehensive chromatin immunoprecipitation sequencing (ChIP-seq) experiments [27], [28] the current model for Mediator function is that a ''Tail'' module interacts with UAS, a ''Head'' module interacts with RNA Pol II, and a ''Middle'' module provides scaffolding and signal transduction. Finally, a ''CDK8/kinase'' module negatively regulates the interactions between the Tail and UAS and needs to be released dynamically before Mediator and RNA Pol II can assemble in the preinitiation complex [27], [28].

In our SGPA assay, we observed enhanced pGAL1 fluorescence in almost all viable mediator mutant strains (Figures 2-2 B and 2-2 C), a phenotype specific to the pGAL1 and entirely undetectable by growth (Figure 2-2 C). The strongest effect was exerted by CDK8/kinase module mutants and the peripheral Middle and Tail subunits *nut1*Δ and *med1*Δ. To compare the transcriptional response between the GAL regulatory element and Mediator, we GAL1 expression is tightly repressed under glucose and exhibits invariance to a wide range of mutations affecting transcription For example, *GAL1* mRNA appeared unchanged in some Mediator mutants (not including the CDK8/ kinase module) in two studies [29], [30] using microarray mRNA quantification highlighting the potential of SGPA in amplifying very weak promoter signal. ChIP-seq data from CDK8/kinase module mutants [27] lends support to the leaky pGAL1 phenotype model (Figure 2-2 F) suggested by SGPA: under glucose-repressed conditions, Mediator binding in the *GAL1* promoter region is virtually absent (Figure S4C; Mediator/wt), while deletion of a CDK8/kinase gene (*ssn2*Δ) increases *GAL11* presence at the UASG (Figure S4C; Gal11/*ssn2*Δ), an effect not observed, for example, at the neighboring gene *FUR4*.

Using SGPA to Examine PQC

As a second case study, we sought to genetically dissect molecular phenotypes related to carboxypeptidase Y (CPY), a well- established substrate for the study of PQC

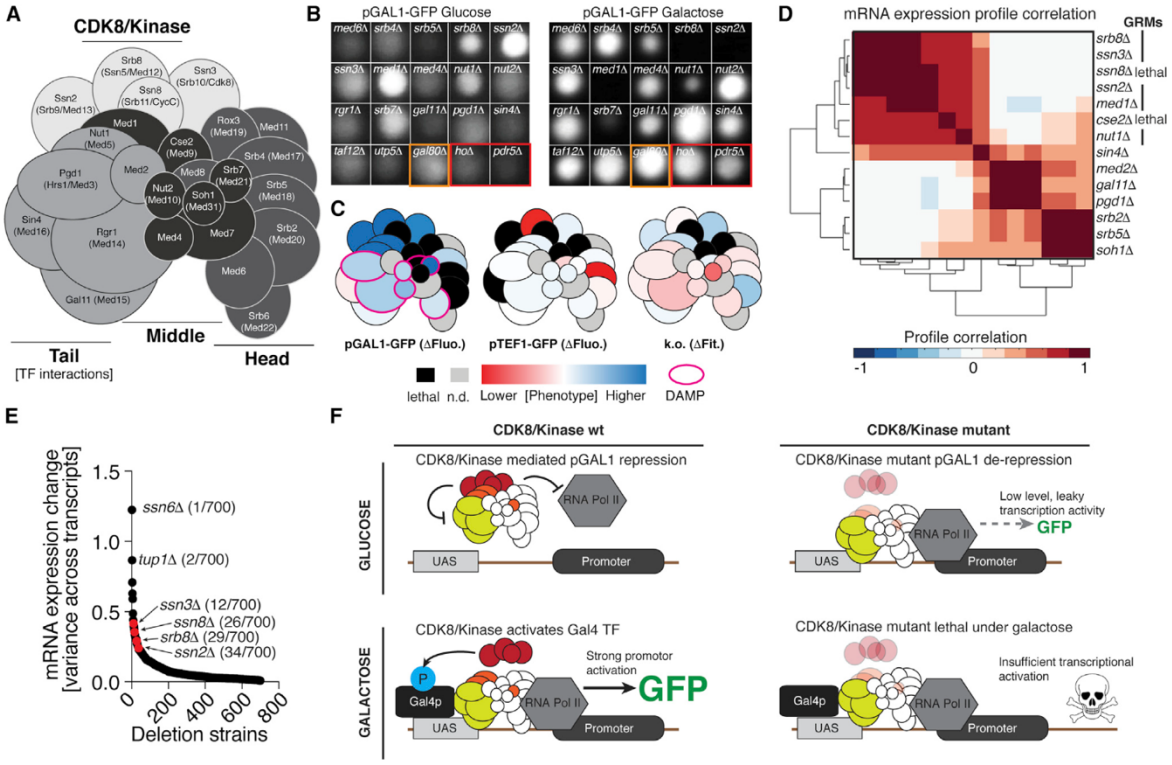

Figure 2-3: A Role for Mediator CDK8/Kinase Module in pGAL1 Repression and Activation

(A) Schematic of the Mediator complex and the four functional modules (Tail, CDK8/kinase, Middle, and Head). (B) Representative examples of Mediator mutant colonies, compared to the most potent mutants from the SAGA complex (*taf2*Δ and *utp5*Δ): *gal80*Δ as positive control (orange box) and *ho*Δ and *his3*Δ as negative controls (red box; box size 2 mm.). Note: the exposure of the glucose mutants has been enhanced (linearly for all mutants) to make the otherwise very faint colonies visible for comparison to galactose-grown colonies. (C) Mapping to the Mediator complex of the corresponding genotype-phenotype changes between glucose and galactose as carbon source for pGAL1-GFP fluorescence, pTEF1-GFP fluorescence (negative control), and colony fitness. Black subunits were lethal in the respective screen, gray subunits were not in SPOCK, and pink outline represents DAMP mutants for essential genes (Phenotype; change in fluorescence or fitness between Glu or Gal). (D) Unsupervised clustering of expression profiles for mediator mutants across ~3,000 transcripts under glucose. GRM bar indicates strongest GRM mutants. (E) Ranked variance for 700 gene deletions across ~3,000 transcripts. Red dots indicate CDK8/kinase mutant strains; value in brackets represents the rank. (F) Proposed model of the bi-modal role of the CDK8/kinase module of Mediator in tight repression under glucose and strong induction under galactose conditions (left side) and the effects of CDK8/kinase module mutants (right side; see text for details).

pathways [31], [33]. A permanently misfolded state in the normal CPY protein is induced by a single amino acid substitution denoted CPY*. Subsequent removal of the endoplasmic reticulum import signal sequence (ss) and addition of GFP result in the model cytoplasmic mis- folded protein ΔssCPY*-GFP (Figure 2-3 A). Normally, this mis-folded protein is rapidly degraded by PQC machinery, whereas disturbances in PQC are identified by accumulation of ΔssCPY*-GFP [33]. Specifically, ΔssCPY*- GFP is marked for degradation by the San1p and Ubr1p ubiquitin ligases in the nucleus versus cytosol, respectively [31], while deubiquitinating enzymes like Ubp3p promote its stabilization (Figure 2-3 B).

We used SGPA to comprehensively evaluate the effect of yeast gene mutations on levels of ΔssCPY*-GFP integrated as a single copy at the *ADE2* locus. To eliminate genes that have general effects on GFP expression or brightness rather than roles in PQC, we assessed the differential fluorescence between each mutant expressing either misfolded ΔssCPY*-GFP or GFP alone (Figure 2-3 C). In a total of 274 gene deletion mutants, we observed significant changes in GFP colony fluorescence relative to control (Figures 2-3 C and S5A; Data S1).

Validation against Known PQC Factors and Robustness to Substrate Location

As a first validation of these results, we scored the extent to which the SGPA gene set recovered known components of PQC, including the established ubiquitinating/deubiquitinating enzymes and the proteasome complex (Data S1). The approach recovered mutant strains for both the ubiquitin ligases (*san1*Δ and *ubr1*Δ) and the deubiquitinating enzyme (*ubp3*Δ), which played opposing roles on the test

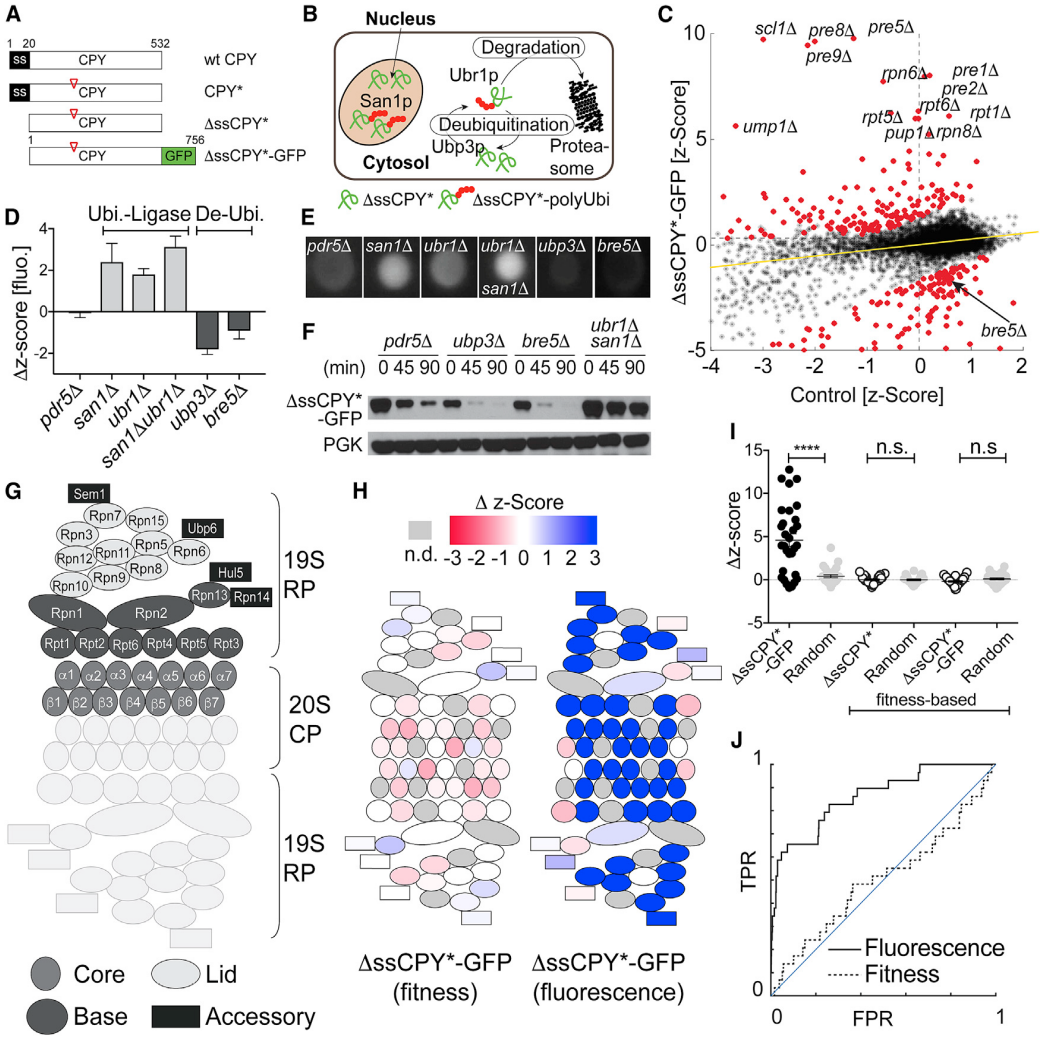

Figure 2-4: Study of PQC Genes by SGPA

(A). Overview of carboxypeptidase Y (CPY) mutants (red triangle denotes point mutation; numberindicate amino acid position). (B) Schematic representation of ΔssCPY*-GFP localization, ubiquitination, deubiquitination, and proteasomal degradation. (C) Mutants of genes involved in PQC (red) were identified based on the differential relative fluorescence (Δ*Z* score) between each mutant expressing either ΔssCPY*-GFP or GFP alone (yellow line, least-squares fit). Mutants of genes normally promoting degradation are above; those of genes normally slowing degradation are below the yellow line. (D) SGPA Δ*Z* scores of known ubiquitinating and deubiquitinating enzymes are shown along with those of BRE5, a previously unappreciated PQC component (*pdr5*Δ serves as wild-type control). (E) Representative colonies for the mutants in (D); box size 2 mm. (F) Western blot analysis of ΔssCPY*-GFP degradation following cycloheximide treatment. (G) Schematic of the 37 subcomponents of the proteasome complex. (H) Fitness (left) and SGPA fluorescence (right) scores for the 30 proteasome mutant ΔssCPY*-GFP strains part of the screen. (I) Comparison between SGPA fluorescence (black) and fitness scores (white) for the 30 proteasome mutants, with and without the GFP fusion or equally sized sets of random control genes (gray; Mann-Whitney U test, ****$p < 0.0001$; n.s., not significant). (J) Receiver operating characteristic (ROC) curve for the successful identification of the 30 proteasome mutants using SGPA versus fitness scores (TPR, true positive rate; FPR, false positive rate).

substrate: loss of the known ligases resulted in elevated GFP levels, while loss of the deubiquitinating enzyme resulted in decreased GFP levels (Figures 2-4 D and 2-4 E) and altered degradation kinetics (Figure 2-4 F; *pdr5*Δ serves as wild-type control). SGPA also recovered 70% (21/30) of essential proteasome complex members based on a strong increase in GFP fluorescence in the hypomorphic mutant strains (Figures 2-4 G and 2-4 J). In contrast, we noted very little change in cellular fitness due to deletion of any of these genes, demonstrating the difficulty in studying a basic biological process such as PQC with a simple assay based only on cellular growth.

We next sought to assess the robustness of these results to defined changes in subcellular location of the misfolded protein. Accordingly, we performed two independent follow-up screens with well-characterized substrate derivatives: first, we used a modified fluorescent substrate predominantly localized in the cytosol (ΔssCPY*-GFP-NES, ΔssCPY*-GFP with a nuclear export signal; [31]. Second, we deleted the nuclear ubiquitin ligase SAN1 across all mutants [31], [34], which is involved in proteasome-dependent degradation of aberrant nuclear proteins (ΔssCPY*-GFP *san1*Δ; Figure 5A). All three screens yielded highly overlapping hits ($p << 10^{-8}$), indicating that misfolded CPY identification and degradation employ

Functional Analysis of PQC Mutants Implicates BRE5 and tRNA Modification Genes

A total of 312 versus 244 mutants were associated with decreased or increased ΔssCPY* fluorescence (Figures 5B and S5A). Functional analysis of the 312 mutants associated with decreased ΔssCPY* levels did not identify any enriched biological processes among the corresponding disrupted genes using Gene Ontology (GO) SLIM

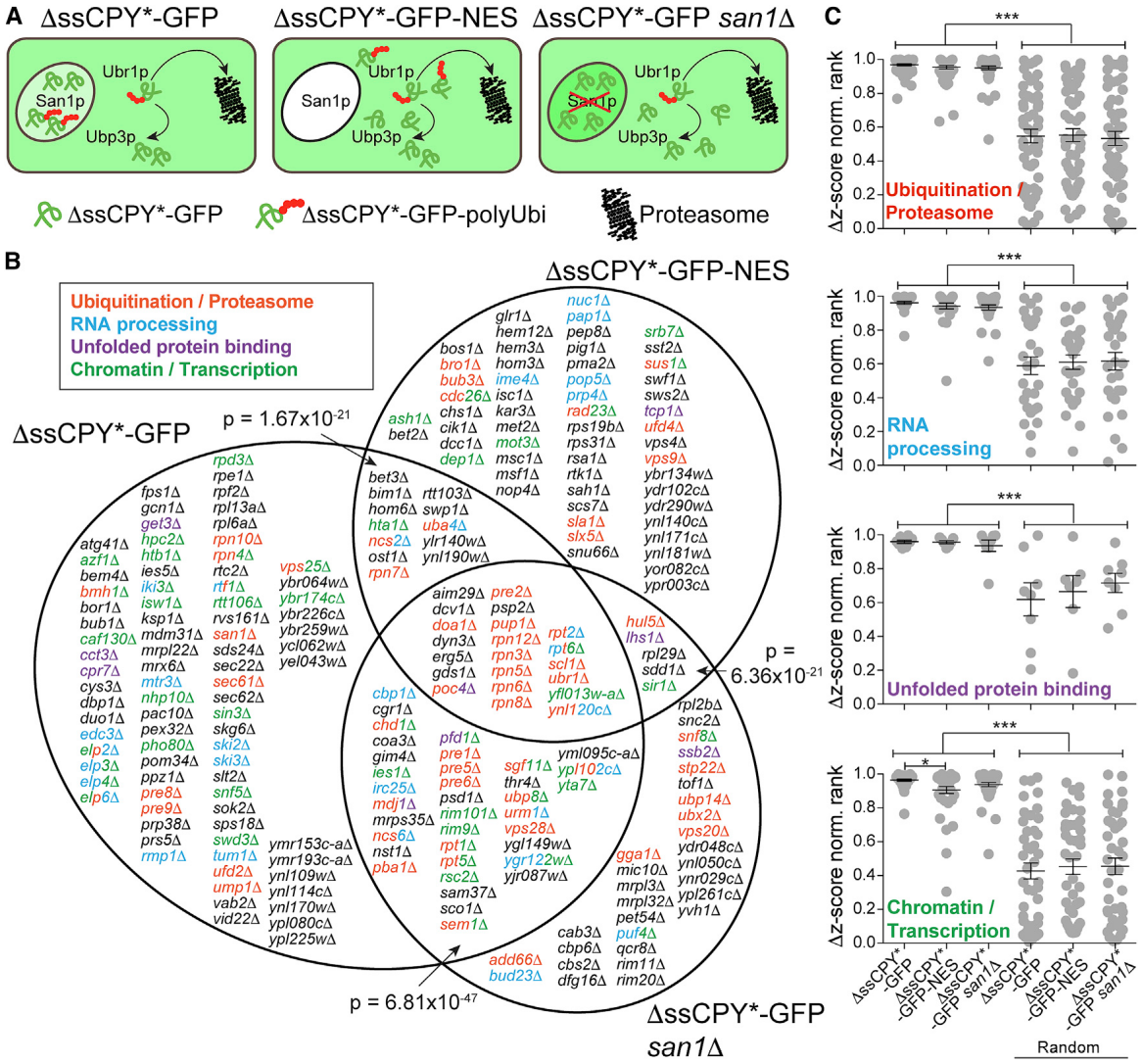

Figure 2-5: Identifying Genes Important for PQC

(A) Schematic of the three sequential screens using different localization of the main ΔssCPY* expression and degradation.

(B) Venn diagram for the 244 genes with elevated fluorescence identified in the three independent screens. p values indicate binary overlap between sets, including the triple hits from the center (Fisher's exact test). Colors indicate high-level functional annotation of enriched groups (Figures S7A and S7B).

(C) Ranked (1 = highest, 0 = lowest score) differential fluorescence scores between hits from the three screens, binned into the four main functional classes, and similarly sized random control groups (ANOVA followed by Tukey's comparison; ***p < 0.0001, *p < 0.05).

[35], [36] (data not shown). Regardless, further investigation of these genes revealed those with functional relevance to PQC (Figure S6A). For instance, lowered ΔssCPY*-GFP levels were observed in the *bre5*Δ mutant, which had not been previously linked to PQC pathways, although Bre5p forms a complex with the Ubp3p ubiquitin-specific protease (Figures 2-4 C–2-4 F and S6A– S6C). This effect was robust and strong enough to be visible to the naked eye (Figure 2-4 E) and was supported by protein degrada- tion pulse-chase experiments, both in western blot (Figure 2-4 F) and fluorescence-activated cell sorting (FACS) experiments (Figure S6C). Analysis of the 244 mutants associated with increased ΔssCPY*-GFP levels was particularly informative, indicating many genes potentially functioning in protein degradation or quality control. The genes were enriched for biological processes (based on GO SLIM enrichment), broadly organized into four superclasses: (1) ubiquitination/proteasome, (2) RNA processing, (3) unfolded protein binding, and (4) chromatin/transcription (Figures 2-5 B and S7A). Mutant fluorescence signatures were robust across superclasses and screens (Figure 2-5 C), further supporting largely location-independent function of the PQC machinery and reliability of the assay. The only significantly different results were obtained for the set of chromatin/transcription mutants in the ΔssCPY*-GFP-NES screen (Figure 2-5 C; ANOVA followed by Dunnett's multiple comparisons test), supportive of the idea that excluding misfolded protein from the nucleus could reduce its direct effect on DNA modifications and transcription. We also performed an enrichment test against known protein complexes. Besides proteasome-related complexes, we observed significant

enrichment for the Elongator holoenzyme complex, the DUBm complex, and the ESCRT complex (Figures 6A and S7B; GO slim terms, Fisher's exact test).

In both types of functional analyses, we observed an overrepresentation of genes involved in U34 tRNA modification (Figures 2-6 A, S7A, and S7B), which included members of the urmylation and elongator complex genes [37]. The urmylation gene (*URM1*) is highly conserved from yeast to humans with a unique dual-function role, acting both as a protein modifier in ubiquitin-like urmylation and as a sulfur donor for tRNA thiolation [38]. Together with the Elongator pathway, the urmylation pathway forms 5-methoxy-carbonyl- methyl-2-thio (mcm5s2) modified wobble uridines (U34) in tRNA anticodons [38], important for structural integrity of the cell, decoding efficiency, and mRNA translation accuracy [38]. Urmylation and elongator complex mutants showed SGPA phenotypes nearly as strong as, and in some cases stronger than, the ubiquitination-deficient *ubr1*Δ and *san1*Δ mutants (Figure 6B), a behavior largely

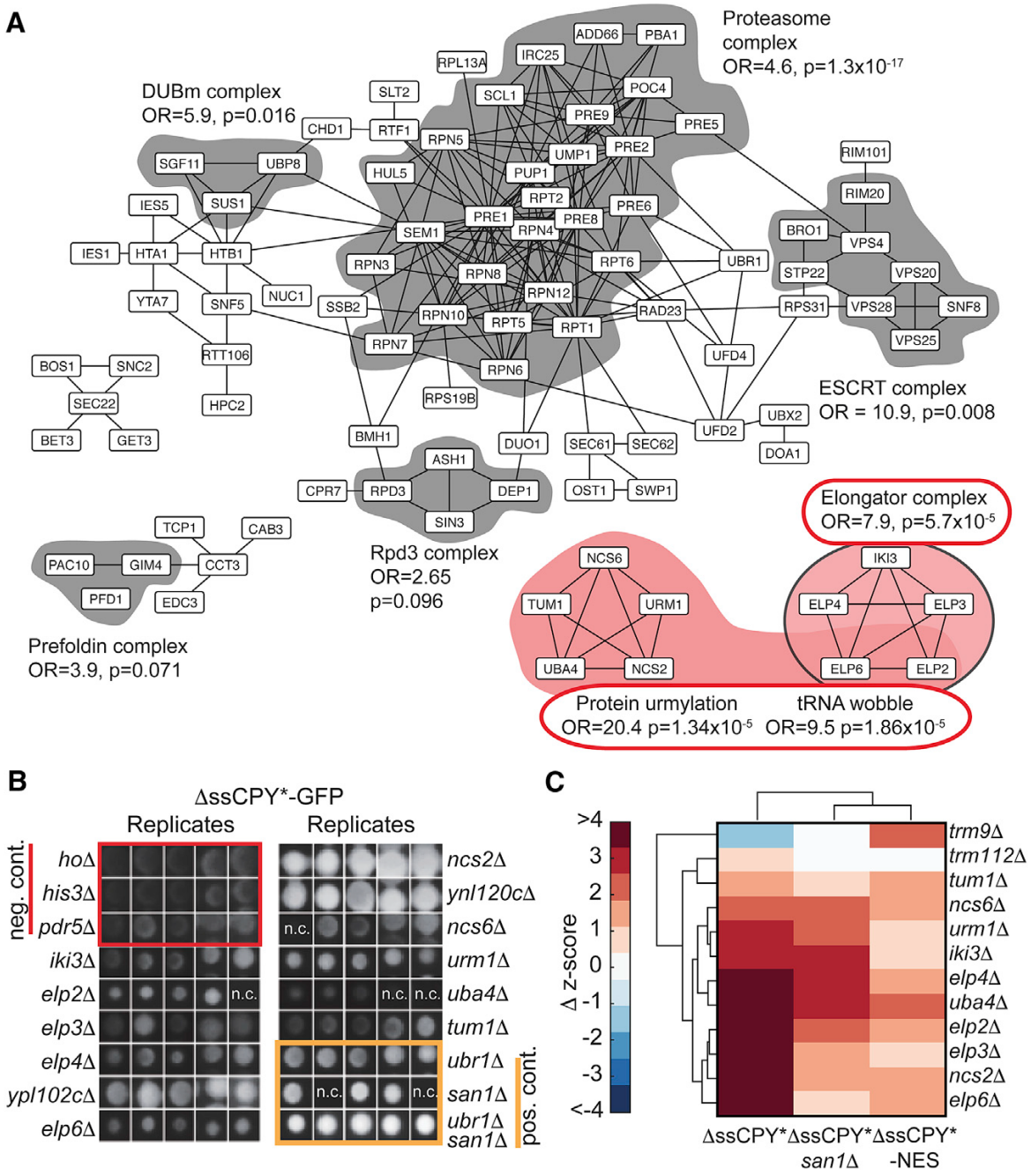

Figure 2-6: Functional and Protein Complex Enrichment Reveals a Role for tRNA Modification in the Process of PQC.

(A) Overlay of the gene hits on a protein-protein interaction network (from BioGRID). Complexes with p < 0.1 (full GO enrichment, Benjamini- Hochberg corrected) are outlined; singlet genes and genes pairs are removed for clarity. Networks highlighted in red relate to U34 tRNA modification and protein urmylation.

(B) Colony view of the ΔssCPY*-GFP mutants relevant to tRNA modification (n.c., no colony growth). (C) Clustering of SGPA scores of the tRNA modification-deficient mutants.

reproducible in all three ΔssCPY* screens (Figure 2-6 C). Two of the tRNA modification mutants (*elp4*Δ and *ncs2*Δ) were independently validated through the existence of ‘‘dubious ORF’’ mutants in the SPOCK collection that overlap partially with the respective gene locus (*ypl102c*Δ and *ynl120c*Δ), causing the same loss of gene product and identical phenotype. We found that temporal expression patterns [39] of tRNA modification genes were very different from those of the proteasome (Figure 2-7 A), and that deletion of tRNA modification or proteasomal genes induced very different expression responses (Figure 2-7 B). Despite their similar effects on ΔssCPY*-GFP fluorescence, these findings suggest that tRNA modification and proteasomal degradation have distinct and non-simultaneous effects on PQC.

Protein Accumulation in U34 tRNA-Deficient Cells Is Not Due to Altered Degradation Rate

To evaluate the importance of U34 tRNA deficiency on ΔssCPY*-GFP degradation, we performed cycloheximide chase experiments on ΔssCPY*-GFP in the candidate mutants, to directly evaluate effects on protein stability (Figure 7C). Remarkably, neither the elongator complex nor urmylation-deficient mutants showed any effects on ΔssCPY*-GFP stability. These behaviors were in striking contrast to the ubiquitin-proteasome mutants detected in the screen, which showed clear changes in substrate degradation (Figure 2-7 C). If misfolded protein degradation is not impaired, we reasoned that the observed increase in ΔssCPY*-GFP in the mutants might be due to increased protein production. To test this hypothesis, we measured the steady-state concentration of ΔssCPY*-GFP via FACS in a set of freshly transformed U34 tRNA modification deficient mutants. To exclude screen-specific artifacts, mutants

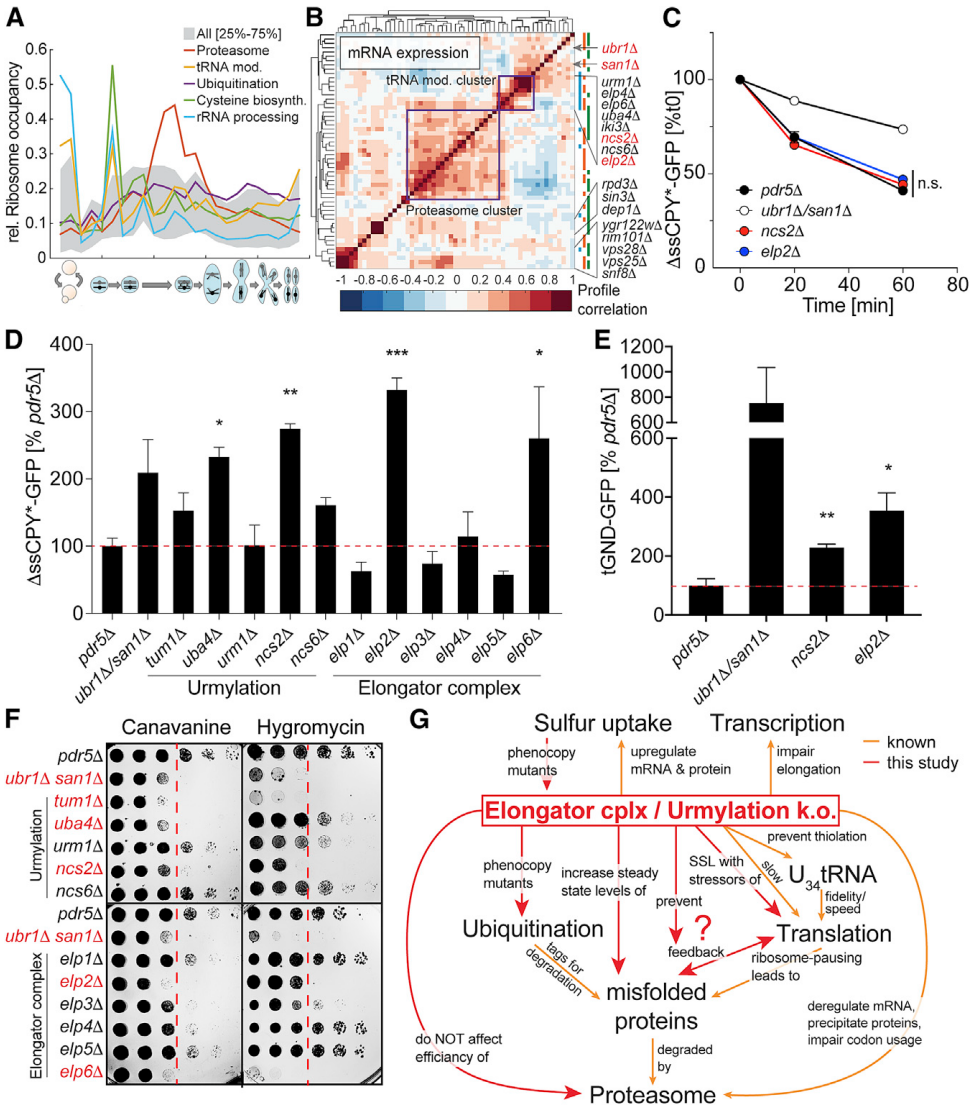

Figure 2-7: Mechanistic Impact of U34 tRNA Modification Deficiency

(A) Expression analysis of protein degradation or tRNA modification genes across yeast cell-cycle stages by ribosome profiling. (B) mRNA expression changes induced by selected gene deletions identified by SGPA as important to PQC. Right hand color stripes indicate superclass annotations (blue, RNA processing; orange, proteasome; green, chromatin/histones). (C) FACS pulse-chase time course of ΔssCPY*- GFP degradation (*pdr5*Δ serves as wild-type control; N = 4 for each mutant and time point). (D) Steady-state concentration of ΔssCPY*-GFP relative to *pdr5*Δ GFP-only values (N = 3 for each mutant, FACS, one-way ANOVA followed by Dunnett's; *p < 0.05, **p < 0.01, ***p < 0.001).

(E) Steady-state concentration of tGND-GFP rela- tive to *pdr5*Δ control (N = 3 for each mutant, FACS, unpaired t test; **p < 0.01, *p < 0.05). (F) Synthetic lethality screen with translation inhibitors (canavanine, 0.25 mg/mL; hygromycin, 62.00 mg/mL). Red line indicates halfway point for control strains without growth defects. Strains that are qualitatively considered synthetic sick/lethal are indicated in red.
(G) Schematic of the proposed effects of U34 tRNA modification deficiency on PQC.

were generated through direct transformation of the ΔssCPY*-GFP expression plasmid (or the analogous plasmid expressing GFP as control) into the respective mutant strains instead of going through the mass-mating and selection process. We observed significantly higher steady-state concentrations of ΔssCPY*-GFP in a wide range of elongator and urmylation-deficient mutants (Figures 2-7 D and S7C), strongly supporting our initial findings with SGPA (Figure 2-6 B). This finding was again confirmed when using a different model protein: a truncated form of the glycolytic enzyme *GND1* (tGnd1), which is a short-lived substrate for the E3 ubiquitin ligases San1p and Ubr1p [31] (Figure 2-7 E). Importantly, the elevation of steady state was specific for the misfolded substrates; no elevation of identically expressed GFP was observed over the wild-type control.

A >3-fold increase in ΔssCPY*-GFP concentration (i.e., as observed with the elongator mutant *elp2*Δ) on the background of normal proteasomal degradation could indicate hyperactive rather than slowed translation, exerting significant pressure on the translational machinery. To test if translation is indeed changed in U34 tRNA modification-deficient cells, we exposed these cells to two different compounds that induce translational stress at sub-toxic concentrations: hygromycin B, which stabilizes the tRNA-ribosomal acceptor site, thereby inhibiting proper ribosome translocation, and canavanine, a non-proteinogenic amino acid that can replace L-arginine during translation, thereby producing structurally aberrant proteins. Remarkably, the same urmylation and elongator complex mutants that exhibit the strongest increase in ΔssCPY*-GFP accumulation are hyper-sensitive to these compounds (Figure 2-7 F),

suggesting that this class of mutants is abnormally affected by increased load of mis-folded proteins.

Discussion

Our first application of SGPA to regulation of *GAL1* promoter activity recovered most of the known biology of galactose metabolism and regulatory elements covering Gal4p-*GAL1* promoter control. The weak signal expected from a repressed promoter represents an ideal test case for the sensitivity of the new mem- brane technology and yielded superior results to agar-based imaging. Functionally, our results support the findings of recent studies suggesting an independent role for the CDK8/kinase Mediator module in repressing Tail interaction with UAS [27], [28]. Our data also highlight a unique, bi-modal role of the CDK8/kinase module in the GAL regulon: since the CDK8/kinase module is necessary for the activation of Gal4p transcription factor activity as well as suppression of the Tail-UASG and Head-RNA Pol II interactions, this Mediator module is ideally suited to exert the extraordinarily tight control of the ‘‘galactose switch.’’ Interference with CDK8/ kinase module function through deletion of any of its members renders the galactose switch both leaky and un-flippable. The glucose repression defect phenotype was extremely weak. This emphasizes that, depending on the magnitude of the expected phenotypic change, it is wise to adapt the reporter construction accordingly: in our *GAL1* regulon case, a high-copy, signal-amplifying 2m plasmid proved beneficial, but in other situations, such as when probing tagged proteins (Using SGPA to Examine PQC) or when the reporter is toxic on its own, low- copy CEN plasmids or chromosomal integration with modestly strong promoters may be better suited to not overload the cell with reporter ‘‘stress.’’

It will be informative to evaluate the role of Nut1p and Med1p in mediating CDK8/kinase module function during glucose repression. While our data show the most comprehensive effects for the CDK8/kinase mutants, most of the Tail module mutants are

DAMP mutants and thus not totally depleted for the respective proteins. It is thus conceivable that complete loss of other Tail subunits could phenocopy CDK8/kinase mutants; however, those strains are non-viable and would need to be constructed in a dynamically inducible fashion. Overall, these data demonstrate the usefulness of SGPA to identify functional complexes that mediate specific roles in transcription and to generate many leads on the organization of eukaryotic transcription control. Given the recent appreciation of Mediator and Mediator mutations in several developmental diseases, it will be interesting to see how far the GAL regulon control model extends into a more general model of gene repression and activation. Intriguingly, MED12, the human homolog of yeast *SRB8*, has recently been identified as a cancer mutation hotspot [40] and has been implicated in affecting the response to multiple cancer drugs [41]. Given that CDK8/kinase mutations have a strongly deregulatory effect on global and de-repressing effect on GAL regulon transcription in yeast, it is possible that similar de-repression of tightly controlled oncogenes could occur in humans. Future molecular work will be needed to better under- stand the functional implications of this effect.

By applying SGPA analysis to misfolded protein phenotypes, we demonstrated two new aspects of this highly conserved process. First, the existence of negative factors *UBP3* and *BRE5* that normally diminish degradation, allowing for a more nuanced approach to triage. Second, and more surprising, a specific involvement of

genes associated with U34 tRNA modification in the accumulation of misfolded proteins, indicating that tRNAs and other ubiquitin-like modifiers could make interesting targets for future therapeutic interventions to combat the numerous proteostasis-related diseases. Previously, deficiency in U34 tRNA modifications had been implicated in slowing translation of certain wild-type proteins, leading to misfolding and proteotoxic stress [38], [42]. This led to the assumption that U34 tRNA modification deficiency exerts predominantly proteotoxic stress via the accumulation of protein aggregates. Here we show instead that U34 tRNA modification mutants have close to normal degradative capacity and proteasome speed when challenged with a single, non-toxic misfolded protein substrate. Rather than slowing translation, accumulation of ΔssCPY*-GFP appears to be driven by increased production in the deficient cells. Consistent with this model, the U34 tRNA modification-deficient cells were sensitive to other translation stressors such as sub-toxic canavanine or hygromycin treatment. This study opens the possibility that U34 tRNA modifications play a previously unappreciated role in controlling production of correctly folded proteins, and thus can act both as accelerators and breaks on protein production, potentially enabling fine-tuning of expression in response to protein levels (Figure 7G). Future, more detailed polysome analysis or ribosomal profiling studies are needed to clarify the exact mechanism and functional relevance underlying this phenomenon.

High-throughput screens of yeast fitness have revolutionized our ability to map the genomic landscape and to identify gene and pathway relationships relevant to cell growth. Recent efforts emphasize the importance of targeted conditional screens to increase hit rate and to build a deeper understanding of genetic dependencies when the

cell faces relevant external stressors [16], [43], [44]. Examples of screens exploring some of these different angles include gene-gene [1], [9], [45] gene-metabolome [6], or triple-genetic interactions. However, fitness-based screening efforts are inherently limited to a single readout—colony growth—restraining the possible richness of the data obtainable, while highly specialized screens (e.g., high-content microscopy, expression profiling, or mass spectroscopy) are extremely slow and cumbersome when applied across thousands of mutant strains. SGPA overcomes these limitations.

Beyond the study of promoter control and protein degradation and folding, other phenotypic markers are readily conceivable: organelle function (e.g., lysosome, autophagosome, and peroxysomes) could be assessed by targeting GFP-tagged proteins to specific compartments and monitoring GFP degradation (or by using any other pH-sensitive marker), expression could be followed by measuring GFP-tagged levels of the protein, protein-protein interactions could be assessed *in vivo* by using bimolecular fluorescence complementation or fluorescent variants of yeast two-hybrid technology, and so on. This versatility has far-reaching implications for the utility of yeast screening in drug discovery, as large-scale discovery datasets can be generated at low cost and in short time and targeted specifically to phenotypes of interest. The SGPA platform is in principle transferable to other species (e.g., *S. pombe*), including to other domains (*C. reinhardtii*) or kingdoms (*E. coli*) of life, since systematic mutant collections are becoming more widespread in those organisms.

Limitations

While the final imaging step is extremely fast and the overall process can be efficiently parallelized, an individual SGPA screen from start to finish can take up to 2

weeks (including growing up the SPOCK collection, crossing in the fluorescent marker(s), followed by the appropriate selection steps). When accounting for growth saturation at each step, this translates into ~100 yeast generations. If a phenotype of interest elicits a strong counter-selective pressure, then this number of generations may be sufficient to give rise to a masking mutation. We describe an effect like that in detail in a companion manuscript [46].

This is, of course, not unique to SGPA, but inherently affects all high-throughput approaches that require a significant number of generations to pass between an event (i.e., a gene suppression experiment) and its readout (i.e., after expansion of the cell line). To some degree this evolutionary adaptation to the phenotype ‘‘fitness’’ has already occurred in the yeast deletion collections that are part of SPOCK [47] and as such should be considered a hidden variable in all derived high-throughput yeast deletions screens. This problem of adaptation could be overcome by designing inducible phenotype reporters for SGPA, controlled, for example, by galactose or tetracycline; however, these ‘‘conditions’’ then in turn require careful additional experiments to control for non-specific inducer effects. We thus always strongly recommend the inclusion of positive controls.

Methods

SPOCK collection and high-throughput yeast screens

Strains from the YKO and DAmP collections (GE Dharmacon, Lafayette, CO) were grown on YPAD medium with 100 mg/ml G418 at 96 colony density and then manually re-arrayed to remove blank spaces, non-growing strains, and duplicates, resulting in the SPOCK collection. A complete strain list and location map can be found in Data S1. The 96 well plates were then re-pinned and condensed to 6144 colony density using the Rotor HAD (Singer Instruments, Taunton, UK). Mating with the CPY or pGAL1 query strains and selection were performed using standard E-MAP procedures [48] except that all incubation steps took place over-night at room temperature to avoid overgrowth. After double mutant selection, strains were pinned onto agar (for fitness measurements) or onto 0.45mm nitrocellulose membrane (BioRad, Hercules, CA; for fluorescence measurements). The membrane was pre-wetted with selection media and rolled onto the agar surface to avoid bubble formation.

Strains and Plasmids

The *Saccharomyces cerevisiae* strains used in this study are listed in Table S1. Media preparation, genetic and molecular biology techniques were carried out using standard methods: Yeast strains were cultured using yeast extract/peptone/dextrose (YPD) at 30 C. Majority of the deletion strains used were in the BY4741 (MATa *ura3*D*0 leu2*D*0 his3*D*1 met15*D*0*) background derived from the Resgen Deletion Collection (GE Dharmacon) except the Y7092 query strain. The Y7092 strains carried the respective insertions for each of the generated screens using standard LiOAc protocols for transformation:

*ade2*Δ::URA3-ADE2

*ade2*Δ::URA3-ADE2-pTDH3-ΔssCPY*

*ade2*Δ::URA3-ADE2-pTDH3-ΔssCPY-GFP

*ade2*Δ::URA3-ADE2-pTDH3-ΔssCPY-NES-GFP

*ade2*Δ::URA3-ADE2-pTDH3-ΔssCPY-GFP*san1*Δ::cNAT

The plasmid cytoplasmic Carboxypeptidase-Y protein ΔssCPY*-GFP (pRH2081) was provided by D. Wolf (University of Stuttgart, Stuttgart, Germany). tGND1 (pRH2476), and ΔssCPY*-GFP-NES (pRH2557) were developed in-house. Plasmids were heat-shock transformed into competent *E. coli* (DH5a), recovered using standard Mini-Prep protocols (Promega), and re-transformed into yeast cells using standard procedures. Competent colonies were selected with the appropriate selection conditions.

Gel preparation, selection markers, and media

Bacto agar (#214040, BD Biosciences, San Jose/CA) was used as the gelling agent. Supplemental reagents and media were Bacto yeast extract (#212720, BD Biosciences), Bacto peptone (#211820, BD Biosciences), Difco Dextrose/Glucose (#215520, BD Biosciences), Difco Yeast nitrogen base without amino acids (#291920, BD Biosciences) and Difco Yeast nitrogen base without amino acids and ammonium sulfate (#233520, BD Biosciences). In case of the galactose experiments, glucose (2%) was replaced with an equal percentage galactose (2%). Synthetic complete (SC) or SC-dropout media were prepared following standard procedures using amino acids from Sigma-Aldrich. If indicated, selective pressure was maintained using geneticin (G418, KSE Scientific, Durham/NC), S-(2-Aminoethyl)-L-cysteine hydrochloride (S-AEC,

A2636, Sigma-Aldrich), or L-(+)-(S)-Canavanine (Can, C9758, Sigma-Aldrich) at the indicated concentrations. Gelling, supplemental, and media reagents were mixed in ddH2O and autoclaved for 15min at 121 C before use; selective drugs were added after the liquid gel solution cooled to below 60 C in a water bath.

White-light imaging station

Images of gels and yeast colonies were acquired using a digital imaging setup described previously [16] with a commercially available SLR camera (18 Mpixel Rebel T3i, Canon USA, Melville/ NY) with an 18–55 mm zoom lens. We used a white diffusor box with bilateral illumination and an overhead mount for the camera in a dark room. Images were taken in highest quality, 8-bit color-depth JPEG.

Fluorescent imaging station

Images of gels were acquired using a custom fluorescent digital imaging setup described previously [49]. We used a commercially available SLR camera (20.2Mpixel EOS 6D, Canon) with a 100mm f/2.8 macro lens (Canon) and a green band-pass filter (BP532, Midwest Optical Systems, Palatine/IL). We used a 460nm LED panels (GreenEnergyStar, Vancouver BC, Canada) with a 1⁄4 white diffusion filter (#251, Lee Filters, Burbank/CA, USA) for 45 bilateral illumination (205560, Kaiser Fototechnik GmbH & Co.KG, Buchen, Germany), and an overhead mount for the camera (205510, Kaiser) in a dark room. Images were taken in highest quality, 8-bit color-depth JPEG.

Image analysis

Colony information was collected after images were normalized, spatially corrected, and quantified using a set of previously published custom algorithms, aka

‘‘The Colony Analyzer Toolkit’’ [16]. Digital images were cropped and assembled in Photoshop and Illustrator (CS6, Adobe, San Jose/CA) for publication.

Western Blot Analysis

Cycloheximide chase degradation assays were performed in a manner previously described [31]. Yeast cells were grown to log-phase cultures and cycloheximide was added to a final concentration of 50 mg/mL. At the indicated time points, cells were collected by centrifugation and lysed with 100 mL of SUME (1% SDS, 8 M UREA, 10mM MOPS, PH 6.8, 10mM EDTA) with pro- tease inhibitors (142 mM TPCK, 100 mM leupeptin, 76 mM pepstatin) and 0.5-mm glass beads, followed by vortexing for 5 min at 4 C and addition of 100 mL of 2 3 USB [75 mM Mops, pH 6.8, 4% SDS, 200 mM DTT, 0.2 mg/mL bromophenol blue, 8 M urea]. The bead slurry was heated to 80 C for 5 min and then clarified by centrifugation before separation by SDS/PAGE and subsequent immuno- blotting with monoclonal anti-GFP (Clontech).

Flow Cytometry Steady State

Cell cultures were grown to low log phase (OD600 = 0.1) in extract/peptone/destrose (YPD) at 30 C. GFP fluorescence levels were measured in living cells (10,000 per sample) with a BD Biosciences flow cytometer and analyzed with Flowjo software.

Phenotyping

To evaluate cell growth, indicated strains were grown at 30°C in YPD medium overnight. Cultures were then diluted, grown to log- phase, and a total of 0.3 OD units were pelleted and resuspended in 250 mL of sterile water. Five-fold dilutions were then performed in a 96-well plate and spotted onto on the indicated media. Studies of

canavanine sensitivity were conducted using minimal media (agarose/yeast nitrogenous bases) with the minimal amino acids (His/Leu/Met/Ura) and 0.2 mg/ml of canavanine (Sigma) grown at 30°C for 3 days. Indicated strains for hygromycin B studies were grown in YPD and 62.5 mg/ml of hygromycin B (Invitrogen) at 30°C for 3 days.

Ribosome occupancy and mRNA expression data analysis

Ribosome occupancy data was available publicly. We computed average ribosome occupancy data for selected ORFs annotated with the specific functions in GO/Yeastmine (Data S1). Expression data for a large set of deletion mutants was available publicly. We extracted the expression profile correlations for mutants that were part of Mediator or our 244 proteasome hits and performed unsupervised clustering.

Quantification and Statistics

Quantification and statistical analysis were performed in MATLAB (MathWorks, Natick/MA). Details of the statistical analysis can be found in the figures, figure legends and the results section of the text. Statistical test and number of samples are indicated whenever appropriate.

Data and Software

All data for the galactose and CPY screens is available in Data S1. Representative images for all screens have been deposited to Mendeley Data and are available at https://doi.org/10.17632/w2rm2fmzz7.1.

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Chapter 3

Further Study of Candidates from the Array Analysis: Negative Regulators of Quality Control Degradation

Abstract

Elimination of damaged proteins is vital to a cell's survival, since misfolded proteins can cause a variety of proteotoxic stresses that drastically impact a variety of cellular systems. Eukaryotes utilize the ubiquitin-proteasome system to detect and remove damaged proteins as a key means to prevent proteotoxicity. The highly selective "quality control" degradation of individual misfolded proteins depends on E3 ligases, molecular chaperones and proteases. Accordingly, our focus in the above studies and many other lines or inquiry in the Hampton laboratory has focused on the identity, function and mechanisms at play in these pathways of misfolded protein destruction. Here we present our findings on two types of factor that function in what could be said to be the "opposite" way, that is, limiting the rapidity and extent of misfolded protein degradation . The first group is the Ubp3-Bre5 ubiquitin proteases that we have found to promote the rescue of misfolded proteins from quality control degradation. Second, we have identified a protective role for one, but only one, of the highly homologous (97% sequence similarity) and functionally redundant isoforms of yeast Hsp90. These studies reveal that protein quality control involves both positive and negative factors that participate in what is clearly a dynamic and highly regulated approach to the management of proteomic health. The "negative factors" are particular intriguing as potential therapeutic targets, since development of inhibitors of each could in principle promote enhanced removal of toxic misfolded proteins in the clinical setting.

Introduction

Proteins are continuously subject to damage due to errors in translation, mutations, stress conditions like heat shock and oxidative stress. Misfolded proteins have intrinsic, poorly understood toxicity, in addition to losing sometimes essential functions, and forming cell-disrupting aggregates. toxic [1], [2]. Taken together, all of these detrimental effects of misfolded proteins are referred to as proteotoxicity. This pathological feature of protein misfolding is thought to be the reason that misfolding underlies many of the most pressing clinical maladies. To minimize the danger of proteotoxicity, protein quality control mechanisms such as the ubiquitin-proteasome system (UPS), heat shock proteins (Hsps) and autophagy exist to selectively remove misfolded proteins to limit deleterious consequences to cell [3], [4]. Molecular chaperones are the first to respond to protein damage by binding on to exposed hydrophobic regions [5]. If the misfolded clients cannot re-fold to their native state, they are targeted to selective degradation by the UPS. The success of protein quality control depends on the recognition of misfolded proteins from the correctly folded ones. E3 ligases such as Ubr1 and Ltn1 function to tag misfolded proteins for destruction, and, chaperones participate in a variety of ways to ensure destruction in the face of recalcitrant or abortive folding. It is now becoming more evident that another layer of protein quality control called “protein triage,” exists to ensure the correct proteins are being degraded [6]. The UPS, proteases and Hsp chaperones triage proteins in a kinetic battle to either rescue or commit a protein to degradation. Under normal quality control conditions, chaperones function to maximize the proper folding of nascent

proteins, and de-ubiquitinases (DUBs) assist to remove poly-ubiquitin chains to dispose of those that cannot be folded [2], [7], [8].

Hsp70 and Hsp90 are conserved molecular chaperones that are constitutively expressed throughout the cell. Both chaperones have ATP binding sites that regulate their conformation. ATP-bound conformation has a low affinity for substrates, and ATP hydrolyzed conformation has a high affinity for substrates [5], [9]. Hsp70 and Hsp90 in some cases share the same co-chaperones that facilitate in nucleotide exchange to regulate their affinity for substrates such as the co-chaperone Hsp110 (Sse1) [5]. It is now becoming more evident that Hsp70 and Hsp90 participate in protein quality triaging decisions in opposing roles. Hsp70 promotes substrate degradation while Hsp90 inhibits the degradation of a substrate [6], [10]. As a cell undergoes proteotoxicity, chaperones like Hsp70s and Hsp90s are upregulated. However, when the protein cannot be re-folded, the chaperone Hsp70, instead of trying to rescue it, favors its degradation. On the other hand, Hsp90s bind to mis-folded proteins, shielding them from ubiquitination. If Hsp90s fail to shield a protein from degradation, it is captured by degrading promoting factors such as Hsp70s and E3 ligases [2], [11]. Thus, Hsp70/Hsp90 molecular chaperones function in “protein triaging” decisions in a variety of ways.

Deubiquitinating enzymes (Dubs) and ubiquitin specific proteases comprise a large family of cysteine proteases that cleave bonds between ubiquitin and covalently tagged substrates, or ubiquitin-ubiquitin bonds [12]. [13]. Yeast have approximately 17 Ubp enzymes with both unique and overlapping qualities. One of the well-known functions is to remove polyubiquitin chains from substrates to prepare them for entry

into the proteasome. It has been reasonably suggested that deubiquitination can also be used to reverse ubiquitination to spare a protein late in the process of ubiquitin-mediated degradation.

The conserved protease Ubp3 has both roles in protein quality control and unrelated processes. Ubp3 with its binding partner Bre5, play are involved in microtubule stabilization [14], autophagy [15], and DNA repair and [16]. During stress, misfolded proteins can accumulate into aggregates called JUNQ (juxta-nuclear quality control). When Ubp3 is over-expressed in Hsp70 deficient cells, it suppresses Hsp70 activity by de-aggregating JUNQ inclusions such as Hsp70 chaperones do [7]. In this case, Ubp3 facilitates the destruction of proteins. Apart from this process, ubps have been show to participate in negative regulation of proteolytic activity. The opposing activities of Hul5 and Ubp6 demonstrate how Ubp6 rescues proteins from degradation at the lid of the proteasome by inhibiting Hul5 activity [17]. Hul5 is a proteasomal ubiquitin ligase, that functions at the lid of the proteasome to further ubiquitinate misfolded substrates thus improving the throughput through proteasomal degradation. Interestingly, Ubp6 has been shown to act as a proteasome inhibitor to prevent substrates from degradation by physically binding to the proteasome [8]. This delay in degradation provides a window of time necessary for refashioning the misfolded protein. Proteasome inhibition by Ubp6 opposes Rpn11 activity preventing it from removing ubiquitin chains of substrates. It is probable that chaperones are nearby to assist substrate folding although further studies are needed to better understand the mechanisms.

Recently in Jaeger & Ornelas et al., a number of genes related to protein quality control emerged from our screen (Chapter 2 above and [18]. We sought to dissect molecular phenotypes related to the cytosolic misfolded substrate ΔssCPY*-GFP to identify protein quality control candidates. Subsequent removal of the endoplasmic reticulum import signal sequence (ss), addition of GFP, and a point mutation to permanently misfold it, enable it to remain in the cytosol as a quality control substrate. Normally, this substrate is quickly degraded by protein quality control machinery, whereas disturbances of protein quality control are identified by accumulation of ΔssCPY*-GFP. This assay was originally designed to characterize degradation defects by stability and hence increase steady-state colony fluorescence in the array candidates with stabilizing mutants. However, we reasoned that the same array system could also be used to identify mutants with increased degradation rates: those candidates would show dimmer steady-state flourescence . The affected genes would be expected to encode factors that normal limit degradation, so that when absent, degradation of the test substrate would be increased. To this end, we quantified the differential expression of GFP in colony fluorescence relative to the control and identified a subset of mutants with significantly decrease in fluorescence. We show Ubp3, its binding partner Bre5, as well as the chaperone Hsc82 (but not its highly redundant isoform) to negatively control misfolded protein degradation . In the absence of any of these genes, ΔssCPY*-GFP degradation is accelerated indicating a role for regulating the degradation kinetics of misfolded substrates.

Results

To find relevant genes related to cytosolic protein quality control, we used SGPA to evaluate the effect of yeast gene mutations on levels of ΔssCPY*-GFP integrated as a single copy in the *ADE2* locus. We scored gene candidates by their fluorescent intensity and found the dequbiquitinating enzymes Ubp3, its co-factor Bre5, and the Hsc82 chaperone dim in fluorescence in comparison to the control [18]. Strains harboring ubiquitin proteasome defects should stabilize the reporters, whereas strains with loses of genes normally involved in rescuing proteins from degradation should accelerate the degradation of the reporter.

Our SGPA analysis predicted accelerated degradation in Ubp3, Bre5 and Hsc82 mutant strains. To test this idea, we used several distinct misfolded test proteins ΔssCPY*-GFP, tGND1-GFP and ΔssCPY*-GFP+NES (cytosolic only substrate) to test the hypothesis that the mutants indeed of accelerated degradation. These substrates are permanently misfolded by point mutations or truncations and appended to GFP to reflect the rate of degradation by fluorescent analysis.

We first examined the effect of freshly generated *ubp3Δ* and *bre5Δ* nulls on the rates of turnover of several mis-folded proteins. Specifically we conducted cycloheximide chase experiments, and observed enhanced degradation of the reporter ΔssCPY*-GFP, with half-lives reduced up to 50% (Figure 3-1). This effect of accelerated degradation was observed in by each mutant, indicating a strong role

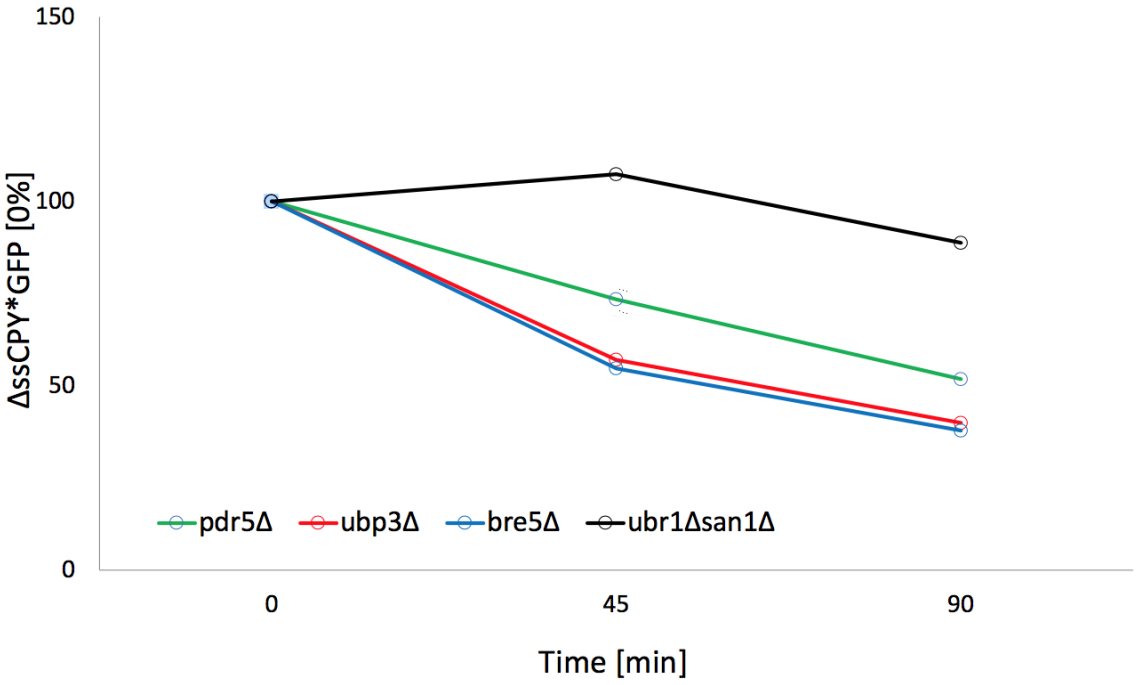

Figure 3-1: Ubp3 and its co-factor Bre5 delay the degradation of ΔssCPY*-GFP.
Cyclohexamide chase of ΔssCPY*-GFP shows accelerated degradation in *ubp3*Δ and *bre5*Δ mutants. The isogenic wild-type control *pdr5*Δ, which plays no role in quality control, indicates the wild-type degradation rate. As expected, the double E3 ligase mutants *ubr1*Δ*san1*Δ strongly stabilized the substrate. Measured by flow cytometry. Mean of 10000 cells per point.

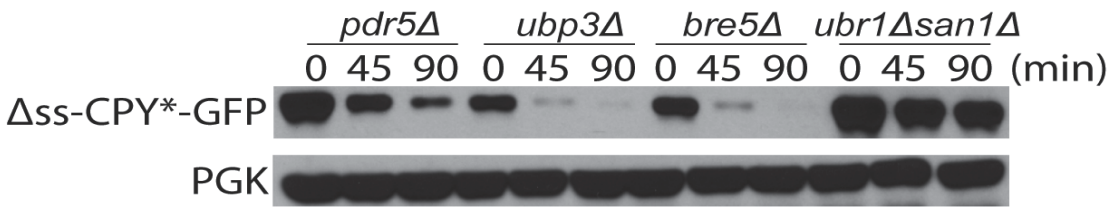

Figure 3-2: Ubp3 and its co-factor Bre5 delay the degradation kinetics of substrate ΔssCPY*GFP. Cyclohexamide chase evaluated by anti-GFP immunblotting of ΔssCPY*-GFP in *pdr5*Δ (isogenic wild-type control), *ubp3*Δ, *bre5*Δ and *ubr1*Δ*san1*Δ strains.

degradation. In contrast, ΔssCPY*- GFP was highly stabilized in *ubr1Δsan1Δ* and cleared at normal levels in the isogenic wild-type control *pdr5Δ* . The acceleration of degradation caused by loss of UBP3 or BRE5 was observed in both flow cytometry of the optical degradation reporter (Fig 3-1) or by immunoblotting of the substrate after SDS-PAGE electrophoresis.

We were curious whether the same type of clearance could be observed with the Ubr1 only substrate *Δ*ssCPYGFP+NES. This substrate is identical to the original substrate but has a Nuclear Export Signal (NES) fused to it to ensure it remains cytosol and is thus only subject to Ubr1-mediated degradation. In a *ubr1*Δ strain, ΔssCPYGFP+NES is strongly stabilized, but not in a *san1*Δ strain as expected (citations). When we tested ΔssCPYGFP+NES, its degradation was similarly hastened by either of the Ubr3, or Bre5 null mutants. This suggests that Ubp3-Bre5 interact with Ubr1-mediated quality control pathways in the cytosol.

We then wanted to test how robust the effect of degradation was when tested with an unrelated misfolded substrate. Truncated phosphogluconate dehydrogenase (tGnd1) is a cytosolic misfolded substrate of Ubr1 and San1 that is strongly stabilized in a *ubr1*Δ*san1*Δ mutant [19]. We set out to further characterize the role of ubiquitin proteases in degradation of tGnd1-GFP and found the same effect of accelerated degradation in *ubp3*Δ and *bre5*Δ strains. The levels of substrate in *ubr1*Δ*san1*Δ remained stable, indicating the specificity of these effects (Figure 3-4). We propose that Ubp3 with its co-factor Bre5 slow Ubr1-mediated degradation by promoting removal of ubiquitin chains from substrates, possibly to gain more time to be re-folded by

chaperones. Overzealous protein quality control may have detrimental effects on fitness therefore ubiquitin proteases have an important role in balancing such control.

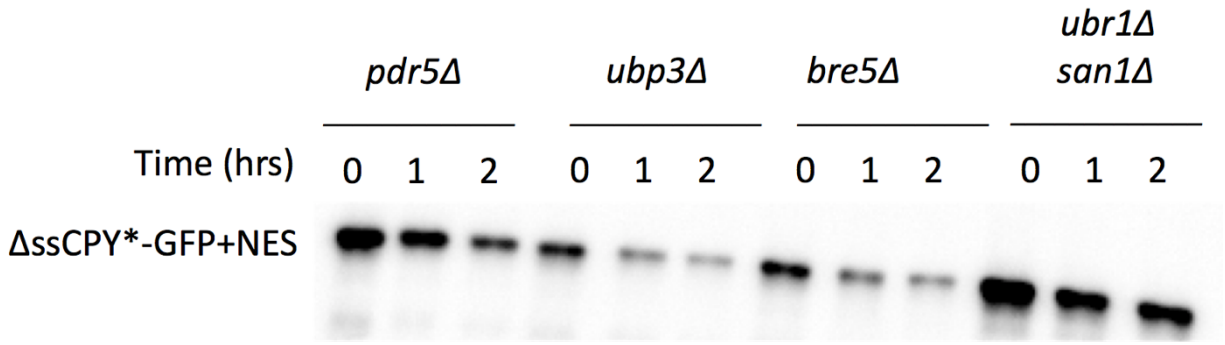

Figure 3-3: Ubp3 and its co-factor Bre5 regulate a range of misfolded substrates. To separate pathways of nuclear and cytosolic quality control, ΔssCPY*-GFP+NES was appended with a nuclear export signal to prevent nuclear localization. Cyclohexamide chase of ΔssCPY*-GFP+NES is shown in *pdr5*Δ (isogenic wild-type control), *ubp3*Δ*, bre5*Δ and *ubr1*Δ*san1*Δ strains.

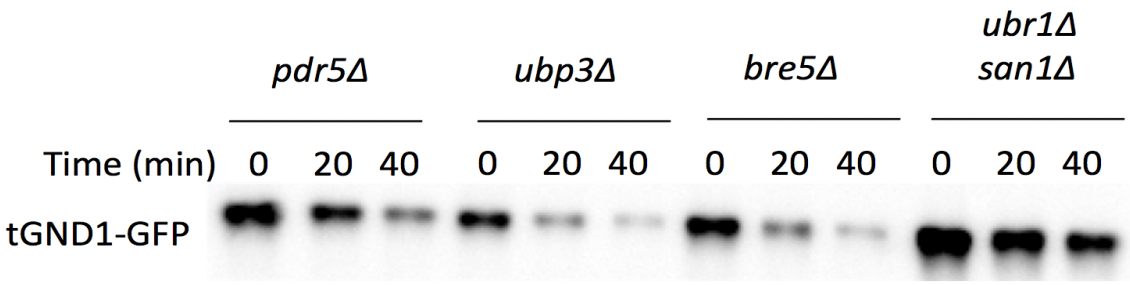

Figure 3-4: Ubp3 and its co-factor Bre5 regulate the kinetics of a nuclear-cytosolic substrate tGND1-GFP. Cyclohexamide chase of tGND1-GFP in *pdr5*Δ (isogenic wild-type control), *ubp3*Δ, *bre5*Δ and *ubr1*Δ*san1*Δ strains.

Hsp90 chaperones are a highly conserved class of chaperones among all species. In yeast, there are two cytosolic Hsp90 homologues, Hsp82 and Hsc82 that are usually considered highly redundant in function; which is not surprising considering they have 97% sequence similarity. Hsp90 chaperone activity is essential; loss of both Hsp90 isoforms results in cell death. Usually the effects of Hsp90 loss of function are studied by preparing a double null of the Hsp90 isoforms and then covering the essential function with a ts version of one of the versions on a plasmid. In this way the Hsp90 activity can be "shut off" by elevating the growth temperature of the test strain. When we studied the requirement for Hsp90 in this manner, , substrates such as ΔssCPY*-GFP, and tGND1 were *stabilized* [19], implying that Hsp90 activity is required for degradation. Surprisingly, Hsc82 appeared in our screen with a null phenotype of rapid ΔssCPY*-GFP degradation, . Furthermore, only the *hsc82*Δnull showed the accelerated degradation phenotype; a null of *hsp82*Δ showed normal kinetics. Despite the surprising different between the single null and the earlier results, it is clear that this hsp80 isoform specific null stabilization is highly reproducible and completely specific for that version of the Hsp90 pair. (Figure 3-5). Indeed, we observed rapid ΔssCPY*-GFP clearance in an *hsc82*Δ null strain and not hsp82Δ. Thus, even though both chaperones are highly similar in sequence, and usually serve redundant functions, in this case interestingly they appear to be playing different roles in quality control.

We then tested the distinct quality control substrate tGND1-GFP. We hypothesized tGND1-GFP would also clear rapidly in an *hsc82*Δ null background since this protein has similar E3 and Hsp70 chaperone requirements . Surprisingly, tGND1-GFP was cleared quickly in both *hsp82*Δ and *hsc82*Δ null strains. It appears as though

both chaperones interact with this substrate. Unlike previous data that show substrate stabilization with conditional alleles of Hsc82 and Hsp82, our data suggest that Hsc82 and Hsp82 can serve to stabilize misfolded proteins, which adds a protective layer to quality control.

Figure 3-5: Hsc82 but not Hsp82 regulates degradation of the substrate ssCPY*-GFP.
Cyclohexamide chase of ΔssCPY*-GFP in *pdr5*Δ (isogenic wild-type control), *hsc82*Δ, *hsp82*Δ and *ubr1*Δ*san1*Δ strains.

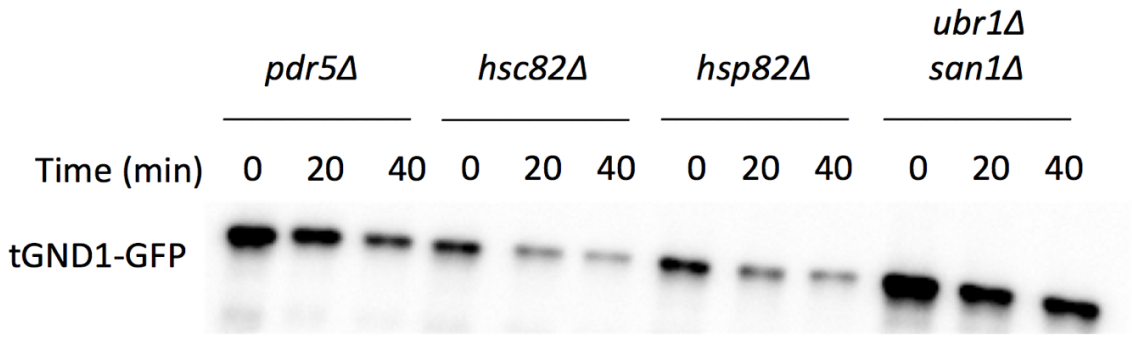

Figure 3-6: Hsc82 and Hsp82 substrate alter the kinetics of tGND1-GFP. Cyclohexamide chase of tGND1-GFP in *pdr5*Δ (isogenic wild-type control), *hsc82*Δ, *hsp82*Δ and *ubr1*Δ*san1*Δ strains.

Discussion

Our high-throughput screen, SGPA has revolutionized our ability to map the genomic landscape for protein quality control factors in yeast, *Saccharomyces cerevisiae*. By applying SGPA analysis to misfolded protein phenotypes, we demonstrated the reliability of finding targeted hits in a model system such as protein quality control. Second, and more surprising, we were able to identify negative regulators of quality control with an unconventional phenotype in which mutant strains appear to clear protein quicker indicated by its dim fluorescence. It seems as if a specific involvement of the proteases Ubp3, Bre5 and chaperones Hsc82, and Hsp82 favor substrate salvaging as opposed to substrate degradation. The interplay between the ubiquitin proteasome system, chaperones, and deubiquitinating enzymes in protein quality control is only beginning to be understood. Cells have evolved layers of quality control to assist in renaturation or destruction of damaged proteins. The partitioning of proteins to either be saved or destroyed has been referred to as "protein triage [6]." With our work we show Ubp3, Bre5, Hsc82 and Hsp82 demonstrate a protective influence from protein destruction.

We have further characterized an role for Ubp3, Bre5, Hsc82, and Hsp82 in protein triaging decisions. The model substrates ΔssCPY*-GFP, ΔssCPY*-GFP+NES, and tGND1-GFP were dramatically degraded when either protease Ubp3 or Bre5 was deleted. This raises the question of how a ubiquitin protease can either save or destroy a damaged protein. The process may be explained by deubiquitination occurring at different stages of protein quality control. The proteases remove ubiquitin tags to give

mis-folded proteins a second change to be recaptured by chaperones that assist in re-folding.

Our data also suggests that Hsc82 may be a chaperone for re-capturing or substrates to assist in re-folding, or protecting them from degradation based on determinants of misfolding. Both ΔssCPY*-GFP, and tGND1-GFP were cleared rapidly when Hsc82 is deleted. The role of Hsp82 remains unclear since it only seemed to participate in the partial clearance of tGND1-GFP. Traditionally, when Hsc82 and Hsp82 temperature sensitive mutants stop their activity at high temperatures, substrates are stabilized suggesting it is required in degradation. Here we show the redundant and highly similar in sequence Hsc82 and Hsp82 with different roles in protein quality control. A possible explanation for the difference in activity is due to the promoters. Hc82 is expressed constitutively 10-fold higher at basal levels than Hsp82 and 2-3 fold more inducible in proteotoxic conditions.

Taken together, the data obtained for Ubp3, Bre5, and Hsc82 are consistent with the hypothesis that these factors limit or provide a governor on E3-mediated quality control, since each appears to function normally to slow degradation of several substrates. Follow-up studies could help determine where in the degradation pathway(s) these factors function. Our model is that Ubp3-Bre5 form a deubiquitination complex that harnesses the Ubp3 enzymatic activity to remove ligase-added ubiquitin, whereas the Hsp90 isoform(s) probably function to limit access to the tagging machinery in the first place. Direct studies on the ubiquitination state of the various substrates in the presence and absence of these nulls would be an import launch point to discern where these factors operation in limiting QC degradation.

The difference in phenotypes between the nulls of Hsc82 and Hsp82 observed with one of our substrates could tested by swapping promoters with the prediction that the HSP82 gene is more important in some cases because it is expressed to a much greater extent. To further understand the generality of these new degradation limiting functions, a survey of substrates that reside in different cellular compartments, and undergo degradation by distinct QC pathways (e.g. Hrd1, Doa10, San1 alone, Ltn1, etc) can be tested for similarly modulation by these nulls in this chapter. It is hoped that these approaches will help us understand how quality control is regulated, and serve as an intellectual and conceptual launch pad for translation approaches in the therapy of clinical proteotoxopathology.

Materials and Methods

Yeast Strains and Plasmids. The Saccharomyces cerevisiae strains used in this study are listed in Table 3-1. Media preparation, genetic and molecular biology techniques were carried out using standard methods: Yeast strains were cultured using yeast extract/peptone/dextrose (YPD) at 30°C. All deletion strains used were in the BY4741 (MATa *ura3Δ0 leu2Δ0 his3Δ1 met15Δ0*) background derived from the Resgen Deletion collection. The plasmid cytoplasmic Carboxypeptidase-Y protein ΔssCPY*-GFP (pRH2081) was provided by D. Wolf (University of Stuttgart, Stuttgart, Germany). tGND1 (pRH2476), and ΔssCPY*-GFP-NES (pRH2557) was provided by D. Hampton (University of California San Diego, La Jolla, CA).

Degradation Assays. Cycloheximide chase degradation assays were performed in a manner previously described. Yeast cells were grown to log-phase cultures and cycloheximide was added to a final concentration of 50 µg/mL. At the indicated time points, cells were collected by centrifugation and lysed with 100 µl of SUME [1% SDS, 8 M UREA, 10mM MOPS, PH 6.8, 10mM EDTA)] with protease inhibitors (142 µM TPCK, 100 µM leupeptin, 76 µM pepstatin) and 0.5-mm glass beads, followed by vortexing for 5 min at 4°C and addition of 100µl of 2× USB [75 mM Mops, pH 6.8, 4% SDS, 200 mM DTT, 0.2 mg/mL bromophenol blue, 8 M urea]. The bead slurry was heated to 80°C for 5 min and then clarified by centrifugation before separation by SDS/PAGE and subsequent immunoblotting with monoclonal anti-GFP (Clontech). With a flow cytometer (BD Biosciences), GFP levels were measured in living cells (10,000 per sample) by flow cytometry of log-phase cultures and analyzed by Flowjo software. Cycloheximide was added to the cultures and measured at the indicated time points. Flow Cytometry Steady

State Fluoresence at steady state for GFP-tagged substrates was performed as described. Cell cultures were grown to low log phase (OD600 = 0.1) Samples were then measured for fluorescence with a BD Biosciences flow cytometer and analyzed with Flowjo software.

Table 3-1: List of yeast strains used in chapter 3

Name	Genotype	Source
BY4741 Library	MATa ura3Δ0 leu2Δ0 his3Δ1 met15Δ0	Resgen Deletion Collection
RHY10417	BY4741 pdr5Δ::KanMX	Resgen Deletion Collection
RHYxxx	BY4741 ubr1Δsan1Δ ubr1Δ::KanMX san1Δ::NatMX	This Study
RHYxxx	BY4741 ubp3Δ::KanMX	Resgen Deletion Collection
RHYxxx	BY4741 bre5Δ::KanMX	Resgen Deletion Collection
RHYxxx	BY4741 hsp82Δ::KanMX	Resgen Deletion Collection
RHYxxx	BY4741 hsc82Δ::KanMX	Resgen Deletion Collection
RHY10868	BY4741 pdr5Δ::KanMX pRH2081 (PTDH3-ΔssCPY*-GFP, ADE2 URA3)	This Study
RHY10869	BY4741 ubr1Δ::KanMX san1Δ::NatMX pRH2081 (PTDH3-ΔssCPY*-GFP, ADE2 URA3)	This Study
RHYxxx	BY4741 ubp3Δ::KanMX pRH2081 (PTDH3-ΔssCPY*-GFP, ADE2 URA3)	This Study
RHYxxx	BY4741 bre5Δ::KanMX pRH2081 (PTDH3-ΔssCPY*-GFP, ADE2 URA3)	This Study
RHYxxx	BY4741 hsc82Δ::KanMX pRH2081 (PTDH3-ΔssCPY*-GFP, ADE2 URA3)	This Study
RHYxxx	BY4741 hsp82Δ::KanMX pRH2081 (PTDH3-ΔssCPY*-GFP, ADE2 URA3)	This Study
RHY10868	BY4741 pdr5Δ::KanMX pRH2081 (PTDH3-ΔssCPY*-GFP+NES, ADE2 URA3)	This Study
RHY10869	BY4741 ubr1Δ::KanMX san1Δ::NatMX pRH2081 (PTDH3-ΔssCPY*-GFP+NES, ADE2 URA3)	This Study
RHYxxx	BY4741 ubp3Δ::KanMX pRH2081 (PTDH3-ΔssCPY*-GFP+NES, ADE2 URA3)	This Study
RHYxxx	BY4741 bre5Δ::KanMX pRH2081 (PTDH3-ΔssCPY*-GFP+NES, ADE2 URA3)	This Study
RHY10985	RHY10412 pdr5Δ::KanMX pRH2476 (PTDH3-3HA-tGND1-GFP, ADE2 URA3)	This Study
RHY10989	RHY10521 ubr1Δ::KanMX san1Δ::NatMXpRH2476 (PTDH3-3HA-tGND1-GFP, ADE2 URA3)	This Study
RHYxxx	RHY10412 ubp3Δ::KanMX pRH2476 (PTDH3-3HA-tGND1-GFP, ADE2 URA3)	This Study
RHYxxx	RHY10412 bre5Δ::KanMX pRH2476 (PTDH3-3HA-tGND1-GFP, ADE2 URA3)	This Study

Table 3-1: List of yeast strains used in chapter 3 continued…

RHYxxx	RHY10412 hsc82Δ::KanMX pRH2476 (PTDH3-3HA-tGND1-GFP, ADE2 URA3)	This Study
RHYxxx	RHY10412 hsp82Δ::KanMX pRH2476 (PTDH3-3HA-tGND1-GFP, ADE2 URA3)	This Study

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