UNIVERSITY OF CALIFORNIA, SAN DIEGO

Discovery of Ubiquitin Ligases Involved in Cytoplasmic Quality Control

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

in

Biology

by

Jarrod Wesley Heck

Committee in charge:

Professor Randolph Hampton, Chair
Professor Andrew Dillin
Professor Jeff Hasty
Professor Gentry Patrick
Professor Jim Wilhelm

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The Dissertation of Jarrod Wesley Heck is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

Chair

University of California, San Diego

2010

DEDICATION

I dedicate this dissertation to my mom, who picked out my name because it sounded good with Dr. at the beginning.

EPIGRAPH

Empty your mind; be formless, shapeless - like water. Now you put water into a cup, it becomes the cup, you put water into a bottle, it becomes the bottle, you put it in a teapot, it becomes the teapot. Now water can flow or it can crash. Be water, my friend.

Bruce Lee

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LIST OF ABBREVIATIONS

Abbreviation	Definition
ATP	Adenine triphosphate
CHX	Cycloheximide
CQC	Cytoplasmic quality control
E1	Ubiquitin activating enzyme
E2	Ubiquitin conjugating enzyme
E3	Ubiquitin ligase
GFP	Green fluorescent protein
HA	Hemaglutinin
Hsp	Heat shock protein
OD	Optical density
PCR	Polymerase chain reaction
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TDH3	Glyceraldehydes-3-phosphate dehydrogenase
TPR	Tetratricopeptide repeat
Ub	Ubiquitination
WT	Wild-type

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the primary experimenter. S.K. Cheung assisted with experiments. Randy Hampton and I wrote the manuscript.

VITA

Education

2010	Ph.D., Biology, University of California, San Diego
2003	B.A., Biology, Biochemistry and Biotechnology Emphasis, Minnesota State University, Moorhead B.A., Chemistry, Minnesota State University, Moorhead

Awards/Honors

2009	Summer Graduate Teaching Award Fellow
2008	Excellence in Teaching Award
2006-2008	UCSD Genetics Training Grant Recipient
2005-2006	National Research Service Award, UCSD
2003-2004	National Institutes of Health Pre-doctoral Intramural Research Training Award
2003	John C. Johnson award for excellence in undergraduate research

Publications

Heck, J.W., S.K. Cheung, and R.Y. Hampton, *Cytoplasmic protein quality control degradation mediated by parallel actions of the E3 ubiquitin ligases Ubr1 and San1.* Proceedings of the National Academy of Sciences, 2010. **107**(3): p. 1106-1111.

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Teaching Experience

University of California, San Diego

Lecturer – BIMM100 (Molecular Biology) – Summer Session 1 2009

Head Teaching Assistant - BIMM100 (Molecular Biology) – Winter 2008
Teaching Assistant – BIMM110 (Molecular Basis of Disease) – Spring 2007
Teaching Assistant – BIMM101 (Recombinant DNA Techniques) – Fall 2005

ABSTRACT OF THE DISSERTATION

Discovery of Ubiquitin Ligases Involved in Cytoplasmic Quality Control

by

Jarrod Wesley Heck

Doctor of Philosophy in Biology

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Professor Randolph Hampton, Chair

Selective degradation of non-native proteins by cytoplasmic quality control (CQC) mechanisms is at the heart of many misfolded protein disorders. The first line of defense is the molecular chaperone network that binds to misfolded proteins and prevents them from engaging in deleterious

interactions with the surrounding environment. Degradation by the ubiquitin proteasome system is a main avenue for eliminating these misfolded proteins. How the chaperone network and the ubiquitination machinery are working together in the cytoplasm to accomplish this feat is only beginning to be understood. These studies have discovered two ubiquitin ligases that are responsible for ubiquitinating and promoting the degradation of cytoplasmic misfolded proteins. These two ligases are Ubr1 and San1. Ubr1 is the first cytoplasmic ligase to be discovered to carry out quality control in this compartment in *S. cerevisiae*. San1 is a nuclear quality control ligase, and its discovered role in CQC highlights a previously unknown pathway for degrading cytoplasmic misfolded proteins. Both ubiquitin ligases function in a chaperone dependent manner. San1 requires chaperones for transport of misfolded proteins to the nucleus where it is believed it then ubiquitinates its substrates directly. Ubr1 more directly utilizes the chaperones for to facilitate ubiquitination. The chaperone networks of Hsp70 and Hsp90 were required for degradation and ubiquitination of CQC substrates. The Hsp70 cochaperones, Ydj1 and Sse1, were found to also be required for elimination of CQC substrates; however, other cochaperones previously found to be involved in CQC were not. This suggests that the framework for CQC is built upon the Hsp70 and Hsp90 machines, and the cochaperones modulate their activity by providing substrate specificity and recruitment of certain UPS factors to promote degradation. Additionally, Ubr1 is able to ubiquitinate insoluble CQC substrates, but requires the activity of Hsp70, Ydj1 and Sse1

to do so; indicating that chaperone function is not merely relegated to keeping misfolded proteins soluble, but is needed for presentation of misfolded substrate to the ubiquitin ligase. The E3 ligase Ubr1 is highly conserved across eukaryotes, from yeast to humans, and the knowledge gained of its role in chaperone dependent CQC will impact future studies of misfolded protein diseases.

Chapter 1

Introduction

Introduction

Protein misfolding and aggregation is an ongoing problem that cellular systems are required to ameliorate to maintain protein homeostasis. Cells have developed general quality control (QC) systems that are highly conserved among eukaryotes to recognize and deal with these misfolded proteins. Two such systems are the molecular chaperone network and the ubiquitin proteasome system (UPS). Defects in either of these two systems have been implicated in a number of disorders, to include Alzheimer's, Parkinson's, Huntington's and prion diseases. Understanding how chaperones and the UPS recognize aberrant proteins and eliminate them from the milieu is extremely important for understanding normal and disease physiology, and will open new avenues for therapeutic intervention[1].

The Ubiquitin Proteosome System

The UPS is the major system for degradation of short-lived proteins in eukaryotes [2]. Many cellular processes utilize degradation by the proteasome; from regulated degradation of biosynthetic enzymes [3] and cell-cycle proteins [4], to selective degradation of misfolded proteins in the cytosol [5] and the endoplasmic reticulum [6]. Proteosomal degradation predominantly depends on ubiquitination of the target protein [2]. Ubiquitination is the process by which ubiquitin is covalently attached to a protein, often by formation of an isopeptide bond between ubiquitin's C-terminal glycine residue and a lysine on the target protein. Ubiquitin is a

small 7.6 kDa protein that is highly conserved, and attachment to another protein can occur with a single monomer (mono-ubiquitination) or in a chain of multiple ubiquitin molecules linked to one another (poly-ubiquitination) [7]. Ubiquitination is used as a signaling event in a diverse set of functions from DNA repair, signal transduction, endocytosis, autophagy, and protein degradation[8]. It is the type of ubiquitination, mono- vs. poly-, and how the ubiquitin molecules are linked to one another, at lysine 48 (K-48) or lysine 63 (K-63) of the ubiquitin molecule, that ultimately determine how the ubiquitination signal is interpreted by the cellular machinery. A K-48 linked poly-ubiquitin chain of 4 or more residues is a signal for degradation in a proteasome dependent manner [9].

A cascade of 3 enzymes carries out ubiquitination. The first is an ubiquitin-activating enzyme (E1) that charges the free ubiquitin by forming a high-energy thioester bond and then subsequently transferring the ubiquitin to one of a small number of ubiquitin conjugating enzymes (E2 or UBCs). In most cases, the E2 then transfers the ubiquitin molecule onto the target substrate via an isopeptide linkage that is facilitated by the third enzyme in the cascade, a ubiquitin ligase (E3) [10]. It should be noted that there is a small family of E3 ligases that participate in an additional intermediate step whereby the ubiquitin is transferred briefly to the E3 and then attached to the target substrate [10]. Whichever the case, the E3 ligase provides the specificity for the reaction by binding both the target substrate and the E2, and facilitating transfer of the ubiquitin molecule.

Ubiquitination is a tightly controlled process; too little or too much can both lead to disease. A mechanism by which ubiquitination of quality control substrates are regulated is by use of the molecular chaperone network. An increasingly pervasive theme in the study of quality control degradation is chaperone action being required for E3 ligase ubiquitination.

Molecular Chaperones

Molecular chaperones participate in a wide range of cellular functions, all of which coalesce around the ability to protect the interactive surfaces of a protein from non-productive interactions [11]. In this capacity, chaperones are involved in most cellular processes to include assisting in the folding of nascent proteins, signal transduction, vesicular trafficking and degradation [12, 13]. The ability of chaperones to selectively recognize non-native proteins makes them the first line of defense in maintaining protein homeostasis. By binding to short stretches of hydrophobic residues that are normally buried within correctly folded proteins, chaperones provide a protective environment in which a series of actions can then be undertaken. The first is that cellular machinery can attempt to (re)fold the misfolded protein to a functional native state. Second, chaperones can sequester the misfolded protein to prevent deleterious interactions. Finally, proteins that cannot achieve a correctly folded state are eliminated by the UPS.

Sequestration of non-native proteins by chaperones has been shown to suppress their toxicity[14, 15]. Overexpression of heat shock protein (Hsp) 70 suppresses the toxicity associated with α-synuclein in Parkinson's,

amyloid-β and tau in Alzheimer's disease, superoxide dismutase in amyotrophic lateral sclerosis (ALS), and polyQ expanded proteins in Huntington's and other ataxias [15]. Hsp70 action has been demonstrated to alter the conformations of these proteins preventing the formation of specific intermediate fibril states [14]. Sequestration is not a permanent solution however and elimination of these toxic proteins must be undertaken.

Chaperones have been shown to target misfolded proteins for degradation in a number of instances. The overexpression of Hsp70 and its cochaperone Hsp40 increase degradation of α-synuclein and polyQ-expanded proteins by the UPS [15]. Hsp70 and Hsp90 networks are required for proteosomal degradation of misfolded Von-Hippel Lindau (VHL) and CFTR, as well [16-18]. However, the precise role of chaperones in promoting degradation is only beginning to emerge. On one hand, chaperones are intimately involved in protein folding, and keeping non-native proteins soluble. Thus, promoting degradation may just be an extension of solubilizing non-native proteins so that the UPS machinery can interact with them. Solubilization is most likely only part of the story of chaperone action, though; as there are E3 ligases that specifically bind to chaperones before ubiqutinating the chaperone bound substrate[19, 20]. The cochaperone HSJ1, a modulator of Hsp70 function, has been found to promote the ubiquitination of Hsp70 bound clients via its ubiquitin interaction motif (UIM). Also, Proteomic analysis of the proteasome interacting proteins has demonstrated that Hsp90 associates with it [21] and may be providing some ATPase

function to the proteosome. These observations point to a close relationship between the chaperone and UPS machinery in the degradation of quality control substrates.

Quality Control: linking chaperones to degradation

The archetype of the interplay between chaperones and the UPS is found in the activity of C-terminal Hsp70 Interacting Protein (CHIP). CHIP is an E3 ligase that contains tetratricopeptide repeat (TPR) motifs for interacting with chaperones. Both Hsp70 and Hsp90 contain TPR domains, and CHIP has been shown to ubiquitinate Hsp70 and Hsp90 clients. CHIPs role in the interaction with each of these chaperone networks is dependent on the substrate. Most CHIP substrates are clients of Hsp90, e.g. CFTR, ERBB2, and steroid receptors. The inability to fold those substrates would suggest that Hsp90 recruits CHIP to triage the substrate down a proteolytic pathway [12]. CHIPs role with Hsp70 clients is different in that CHIP inhibits the J protein stimulated ATPase activity of Hsp70 [22]. CHIP is also known to possess its own chaperone activity [23], bind misfolded proteins directly, and initiate folding [24], or degradation [23] in a Hsp70 dependent manner. The overall mechanisms of CHIP function are still actively being debated and investigated but it is clear from its example that the chaperone and UPS networks are closely cooperating in quality control functions.

While CHIP is an excellent example of the interplay of chaperones and UPS it was the only ligase known to carryout such a function in cytoplasmic quality control (CQC). It is the goal of this dissertation to discover more

broadly conserved ubiquitin ligases involved in CQC. In yeast, quality control ligases have been described in the nucleus [25, 26] and the endoplasmic reticulum [27-29]. However, no ligases have been identified in the cytoplasm that carryout CQC. While CHIP is conserved among some eukaryotes, it is not found in yeast. This work has discovered two distinct ligases and pathways by which CQC is carried out in yeast. Those two ligases are San1 and Ubr1. San1 degrades CQC substrates via chaperone dependent transport into the nucleus, where it is believed to interact directly with the misfolded substrates. Ubr1 resides in the cytoplasm where it ubiquitinates unfolded proteins with the help of chaperones. Ubr1 was found to be the more physiologically important ligase in its ability to alleviate global protein misfolding stress, and appears to act in a “CHIP-like” manner in that it requires an intimate interaction with chaperones and substrate beyond the previously discussed solubilization-only role of chaperones. Furthermore, Ubr1 is conserved from yeast to humans, so it is possible that Ubr1 undertakes a CQC role across species.

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Chapter 2

Cytoplasmic protein quality control degradation mediated by parallel actions of the E3 ubiquitin ligases Ubr1 and San1

Abstract

Eukaryotic cells maintain proteostasis by quality control degradation. These pathways can specifically target a wide variety of distinct misfolded proteins, and so are important for management of cellular stress. Although a number of conserved quality control pathways have been described in yeast, the E3 ligases responsible for cytoplasmic quality control are unknown. We now show that Ubr1 and San1 mediate chaperone-dependent ubiquitination of numerous misfolded cytoplasmic proteins. This action of Ubr1 is distinct from its role in the "N-end rule". In this new capacity, Ubr1 functions to protect cells from proteotoxic stresses. Our phenotypic and biochemical studies of Ubr1 and San1 indicate two strategies are employed for cytoplasmic quality control: chaperone-assisted ubiquitination by Ubr1, and chaperone-dependent delivery to nuclear San1. The broad conservation of Ubr ligases and the relevant chaperones indicates that these mechanisms will be important in understanding both basic and biomedical aspects of cellular proteostasis.

Introduction

Protein quality control (QC) functions to ensure that damaged and misfolded proteins are maintained at acceptable levels to limit their stress-causing, or proteotoxic, effects. One strategy of protein quality control is the selective degradation of misfolded proteins. In order for degradative quality control pathways to be effective, they must be specific for aberrant proteins, be sufficiently general to selectively recognize common structural hallmarks shared by numerous unrelated proteins, and be physiologically important, allowing the cell to better survive a proteotoxic stress. Because protein QC underlies many pressing maladies, such as Parkinsonism, cystic fibrosis, and aging, discovery of the rules of substrate selectivity and destruction are key steps in understanding these conditions and designing appropriate therapeutic interventions to combat them.

In eukaryotes, the ubiquitin proteasome system is employed in the selective degradation of many proteins [1]. A substrate protein is marked for degradation by assembly of a polyubiquitin chain, initiated by covalent addition of the small (7.6 kD) protein ubiquitin to a lysine in an isopeptide bond, followed by iterative addition of the next ubiquitin to the previous added one to create a polyubiquitin chain, that is uniquely recognized by the 26S proteasome. Protein ubiquitination is catalyzed by a three enzyme cascade. The single E1 ubiquitin-activating enzyme hydrolyzes ATP to acquire ubiquitin in labile thioester linkage, which is then transferred in thioester linkage to one of a small group of E2s or ubiquitin conjugating enzymes (UBC). E2-bound

ubiquitin is finally transferred to an isopeptide linkage on the target protein or the growing polyubiquitin chain by the action of the E3 ubiquitin ligase. It is the E3 ubiquitin ligase that determines the specificity of a given ubiquitination process; identifying and understanding the E3s involved in a degradative pathway is thus a key part of understanding the mechanisms of substrate selection and modification.

E3s for several quality control pathways have been discovered and include the ER-associated ligases Hrd1 and Doa10 involved in ER-associated degradation (ERAD), and the San1 ubiquitin ligase that mediates destruction of misfolded nuclear proteins [2-4]. The mechanism used by the QC ligases to detect misfolded substrates can vary with some employing chaperones [5], and others not. The details of substrate recognition are key to understand the envelope of structures subject to destruction by a given pathway.

So far, no widely conserved ubiquitination pathway has been described for cytoplasmic quality control. Metazoans express the CHIP ubiquitin ligase that mediates cytoplasmic QC, using Hsp70 chaperones to detect misfolded proteins [6, 7]. However, CHIP is not conserved in all eukaryotes. For example, no CHIP is encoded in yeast. Nevertheless, chaperone-dependent ubiquitination of misfolded proteins has been observed in yeast [8], indicating that new, and probably highly conserved, cytoplasmic QC pathways remain to be discovered.

To that end, we have investigated the E3 ligases involved in ubiquitination of misfolded cytoplasmic proteins in yeast. We have discovered that two E3 ligases collaborate in ubiquitination of a diverse set of misfolded proteins including full-length substrates with point mutations and truncated proteins. The two E3s are the nuclear E3 San1 [9, 10], and Ubr1, best known in the N-end rule pathway [11, 12]. A variety of misfolded substrates undergo selective, chaperone-dependent ubiquitination by either ligase. In this function Ubr1 and San1 appear to function independently. In vitro experiments indicate that the Ubr1 ligase directly employs chaperones in substrate ubiquitination, while the San1 E3 may require chaperones for delivery to the nucleus. Our phenotypic studies show that Ubr1 had the principle role in mediating cytoplasmic proteotoxic stress imposed by model substrates or chemical stressors. This new quality control function of Ubr1 was independent of its function in the well-described N-end rule, and so represents a novel, physiologically important role for this molecule. Our demonstration of parallel pathways indicates the importance and complexity of cytoplasmic proteostasis. Understanding them will provide novel opportunities for management of damaged proteins in the clinical setting.

Results

We launched a genetic study of CPY‡, a model cytoplasmic substrate derived from the misfolded vacuolar protease CPY*, in which the signal sequence is removed to restrict it to the cytosol [13]. CPY‡ was fused to GFP, yielding the optically detectable misfolded cytoplasmic protein CPY‡-GFP [13, 14] (Fig. 2-1A). We expressed CPY‡-GFP from the constitutive *pTDH3* promoter, and confirmed that the strongly expressed protein showed similar degradative behavior to that previously reported for this substrate (Fig. 2-1 and 2-2): CPY‡-GFP degradation was mediated by the ubiquitin proteasome system, and required Hsp70. In addition, we tested other chaperones and cochaperones and observed that Ydj1 (Hsp40) and Sse1 (Hsp110) were required, with Sse1 playing a major role in substrate ubiquitination, while Hsp104 and Sti1 did not play a role.

To find the relevant ligases we screened an inclusive collection of yeast nulls in genes relevant to the ubiquitin pathway provided by the Hochstrasser laboratory. A single genomically integrated CPY‡-GFP expression plasmid was introduced into each null strain by array mating [15], and CPY‡-GFP stability was assayed by cycloheximide chase and flow cytometry. Surprisingly, the *san1Δ* null stabilized CPY‡-GFP, despite the San1 E3 being involved in nuclear quality control [9], showing stabilization (Fig. 2-3A), and decreased ubiquitination of CPY‡-GFP (Fig. 2-3B, C). We

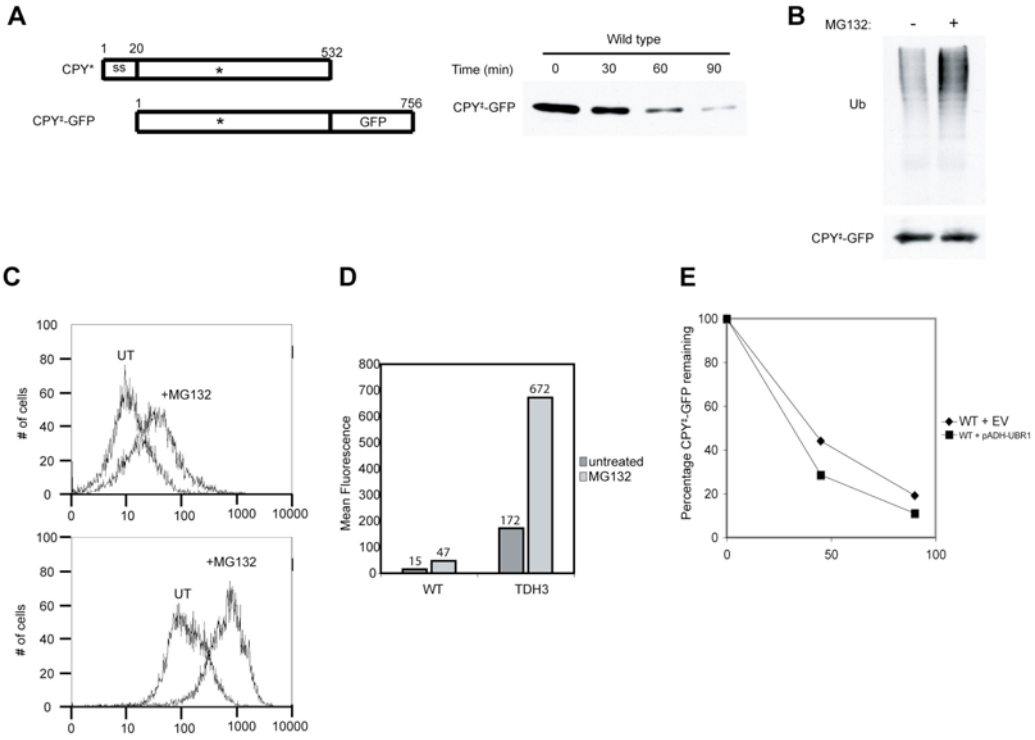

Figure 2-1: Characterization of P_{TDH3}- CPY‡-GFP degradation.

(A) Graphic representation of CPY* and CPY‡-GFP (Δss-CPY*-GFP). ss denotes the ER localization signal sequence, and cycloheximide chase of CPY‡-GFP expressed in wild type cells. Anti-GFP antibodies were used to detect CPY‡-GFP. (B) Effect of proteasome inhibitor (MG132; 1hr) on in vivo CPY‡-GFP ubiquitination, assayed by anti-GFP immunoprecipitation (IP) followed by anti ubiquitin or anti-GFP immunoblotting. (C) Effect of MG132 on CPY‡-GFP steady-state levels when expressed from native promoter (top panel), or strong *TDH3* promoter (bottom), as measured by flow cytometery for GFP fluorescence. (D) Mean fluorescence for the histograms in (C) are plotted for each strain, using arbitrary fluorescence units. Magnitudes are written above each bar. (E) Overexpression of Ubr1 results in increased degradation rate of CPY‡-GFP. WT cells with empty vector plasmid (EV), or highly expressing ADH promoter driven *UBR1* plasmid.

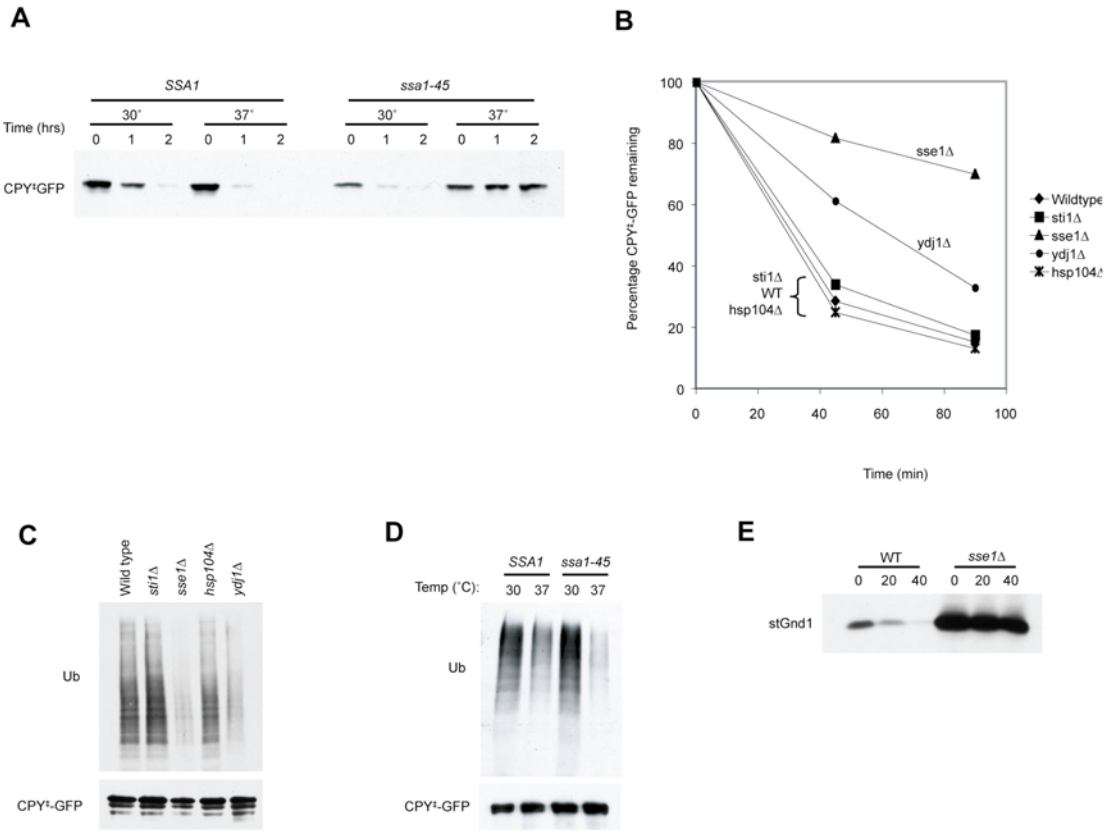

Figure 2-2: Chaperone dependent degradation of QC substrates.
(A) CPY‡-GFP degradation in *ssa2Δssa3Δssa4Δ* nulls with either WT *SSA1* or temperature sensitive *ssa1-45* present, evaluated by cycloheximide chase at 30°C or 37°C, followed by anti-GFP immunoblotting. (B) Flow cytometry analysis of cycloheximide chase of CPY‡-GFP in chaperone nulls in WT, *sti1Δ*, *sse1Δ*, *ydj1Δ*, and *hsp104Δ*. Mean fluorescence of CPY‡-GFP at each time point was normalized to the steady state at time zero and graphed as percentage remaining. (C) In vivo ubiquitination of CPY‡-GFP expressed in chaperone nulls used in (B), IP with anti-GFP and immunoblot with anti-ubiquitin or anti-GFP. (D) Ubiquitination of CPY‡-GFP in WT or *ssa1-45* strains used in (A), at 30°C or 37°C. Cells were preincubated at the indicated temp for 1 hour prior to lysis and IP. Western blots were probed with anti-ubiquitin or anti-GFP. (E) Cycloheximide chase of stGnd1 in WT and *sse1*Δ null.

tested if San1 could function in the cytoplasm to ubiquitinate CPY‡-GFP with San1 missing its nuclear localization signal (NLS) [9]. San1-NLS was also fully competent for CPY‡-GFP degradation and ubiquitination (Fig. 2-3C, D).

In the *san1*Δ null there remained measurable CPY‡-GFP degradation and ubiquitination. To discover the remaining E3, we again employed the null collection, this time crossing a *san1Δ* strain expressing CPY‡-GFP to each null, and selecting for haploids that bore the CPY‡-GFP plasmid, the *san1Δ* null, and each test null. The *UBR1* gene accounted for the residual degradation [11] [16]: the *ubr1*Δ*san1*Δ double null showed nearly complete stabilization of the test substrate. We turned our attention to evaluating the participation of these two ligases in the destruction of misfolded cytoplasmic proteins.

A suite of strains including wt, *san1Δ*, *ubr1Δ* and *san1Δubr1Δ* was prepared to directly assess the contribution of each ligase in cytoplasmic QC. The stability of CPY‡-GFP was affected by either single null, with *san1Δ* showing the larger effect (Fig. 1A). Each null decreased CPY‡-GFP ubiquitination to a similar extent, while the *san1Δubr1Δ* showed a stronger, additive, decrement in ubiquitination (Fig. 2-3B, C). By comparing the two *san1*Δ lanes, it is clear that the Ubr1 ligase alone could mediate CPY‡-GFP ubiquitination. This was confirmed by adding a Ubr1-expressing plasmid to the *san1Δubr1Δ* double null. Ubr1 enhanced degradation and ubiquitination of

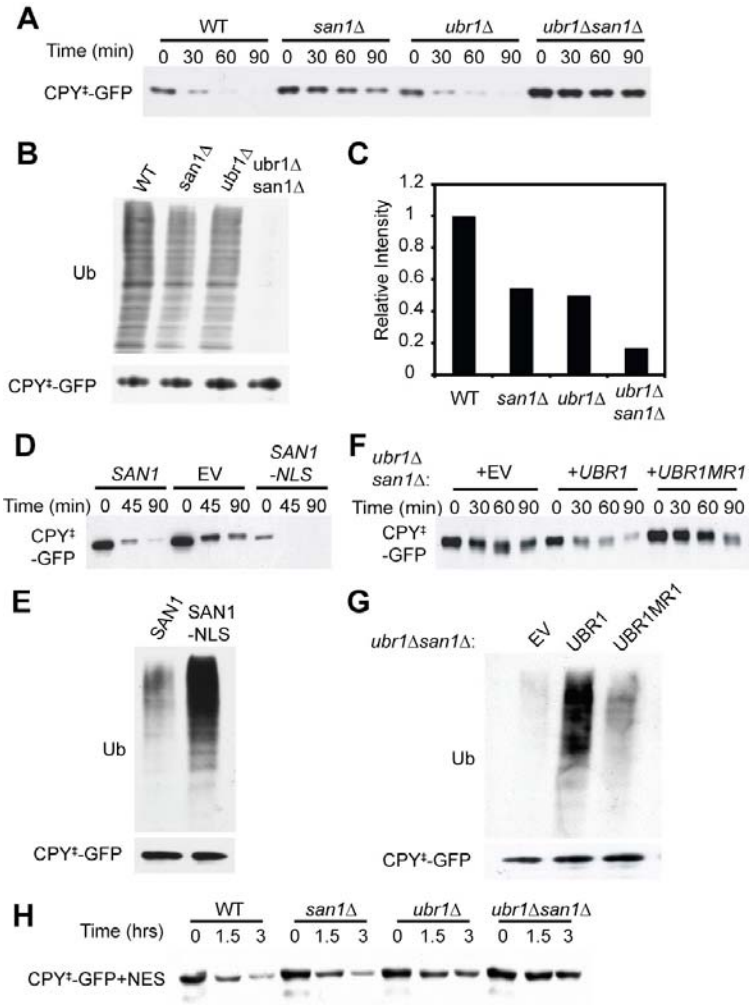

Figure 2-3: Ubr1 and San1 mediate CPY‡-GFP degradation and ubiquitination.

(A) Cycloheximide chase of CPY‡-GFP in the suite of WT, *san1Δ*, *ubr1Δ*, and *ubr1Δsan1Δ* strains. Incubation time following cycloheximide addition is indicated in minutes. Anti-GFP immunoblotting. (B) CPY‡-GFP ubiquitination assayed in the suite of strains in A and IP with anti-GFP followed by immunoblotting for ubiquitin (Ub) or GFP. (C) Ubiquitination immunoblotting intensities normalized to the total precipitated CPY‡-GFP for each assay, as determined using ImageQuant TL. Results are graphed as a fraction of WT, which was set to 1.0. (D) Cycloheximide chase of CPY‡-GFP in san1Δ background with either SAN1, empty vector (EV), or SAN1-NLS expressed from plasmids. (E) Ubiquitination of CPY‡-GFP in the *SAN1* or *SAN1-NLS* strain in D. (F) Cycloheximide chase of CPY‡-GFP from *ubr1Δsan1Δ* strains expressing UBR1 or the UBR1MR1 ring mutant compared with the EV strain. (G) Ubiquitination of CPY‡-GFP from the Ubr1-expressing strains in F. (H) Cycloheximide chase of cytoplasm-restricted CPY‡-GFP+NES in the strains used in A.

CPY‡-GFP (Fig. 2-3F, G) while the non-functional C1220S RING mutant of Ubr1 did not (“UBR1MR1” in Fig. 2-3F,G), indicating that Ubr1 ubiquitin ligase activity of the protein was required for this effect. Similarly, overexpression of Ubr1 caused increased degradation of CPY‡-GFP (Fig. 2-1E). To elucidate the roles of Ubr1 and San1 in degradation of the reporter, a nuclear export signal (NES) was placed at the C-terminus of CPY‡-GFP (CPY‡-GFP+NES). Degradation of this cytoplasmically restricted substrate was now only dependent on Ubr1. The *san1*Δ null had no effect on degradation of the cytoplasmically restricted substrate, either alone or in combination with *ubr1*Δ (Fig. 2-3H).

The generality of Ubr1 and San1 in cytoplasmic quality control was revealed in a dosage suppression screen. We screened a yeast high-copy genomic library (2m plasmids; ~ 20 copies per cell) for plasmids that inhibited CPY‡-GFP degradation in a wild-type strain, using colony GFP fluorescence [17], and cycloheximide chase. There was a striking uniformity in the resulting coding regions. Each stabilizing plasmid included a truncated coding region that expressed a C-terminally truncated protein. In the cases examined, the truncated coding regions were verified to cause CPY‡-GFP stabilization. None of the candidate coding regions had any functional connection to protein degradation or misfolded proteins. Because truncations frequently result in proteins that fold incorrectly, we posited that the plasmids encode competing QC substrates. We tested this possibility with three truncated coding regions from the screen (the % indicate the fraction of the coding region present):

FAS1 (48%), *YOR296w* (39%), and *GND1* (75%) encoding the truncated proteins tFas1, tYor296w, tGnd1 respectively. *FAS1* encodes the cytoplasmic multi-domain fatty acid synthetase, *YOR296w* encodes an unknown protein predicted to reside in the cytosol, and *GND1* encodes cytoplasmic phosphogluconate dehydrogenase. Epitope-tagged versions of each truncated protein were degraded in a Ubr1- and San1- dependent manner, and the three showed a range of degradation rates. Each truncated protein was stabilized by each null and strongly stabilized in the *ubr1Δsan1Δ* double null (Fig. 2-4A, B). In order to study tGnd1 in more depth, it was expressed from a plasmid that added 3HA tags to the N-terminus and GFP to the C-terminus, producing 3HA-tGnd1-GFP, which was similarly degraded and ubiquitinated (Fig. 2-4C, D). Importantly, the addition of GFP to tGnd1 created a full-length fusion protein with a folded C-terminal domain. Thus, the pathway defined by the CPY‡-GFP reporter is involved in the degradation of a variety of unrelated cytoplasmic proteins, including both truncated and misfolded full-length proteins.

Quality control pathways selectively degrade misfolded proteins, sparing the fully folded forms, e.g., [9]. We evaluated this specificity with Gnd1. The original CPY‡-GFP reporter cannot be used in this test because secretory proteins such as BSA [7], and non-mutant CPY-GFP do not correctly fold in the cytosol (J.H. and R.H. unpublished observation). Conversely, the full length cytoplasmic Gnd1 is normally folded in the cytosol. 3HA-Gnd1-GFP, with full-length, normal Gnd1 in the fusion was quite stable

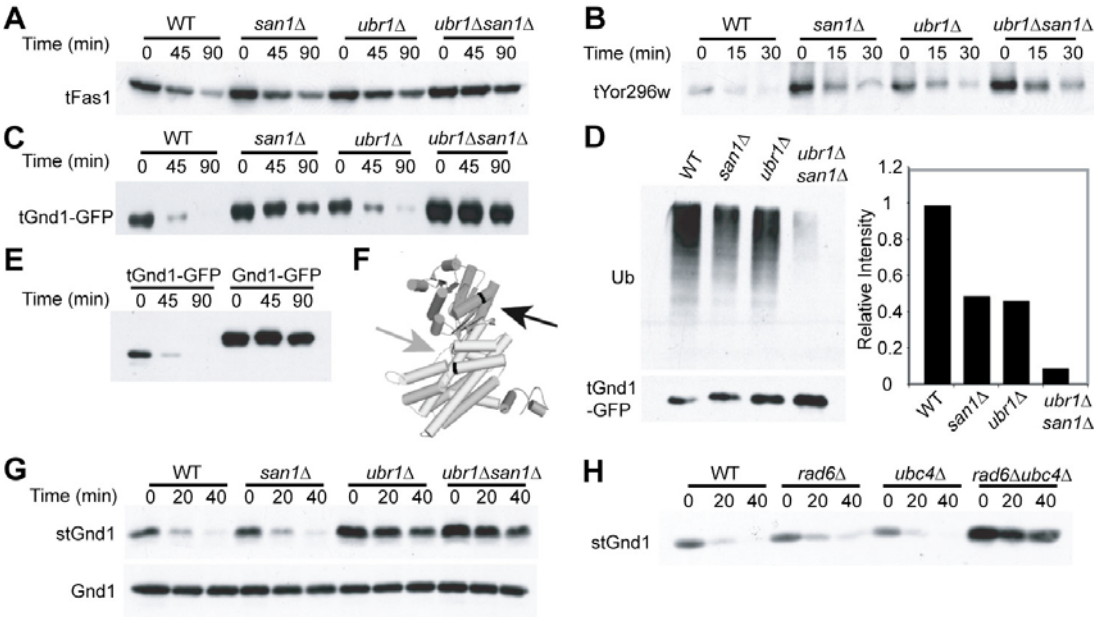

Figure 2-4: Ubr1 and San1 mediate the degradation of multiple cytoplasmic QC substrates.

Ubr1 and San1 mediate the degradation of multiple cytoplasmic QC substrates. Cycloheximide chase and immunoblot of HA-tagged truncated Fas1 (48% total, tFas1) (A), Yor296w (39% total, tYor296w) (B), and Gnd1 (75% total, tGnd1-GFP) (C) in the indicated strains. (D) San1 and Ubr1 dependence of tGnd1-GFP ubiquitination measured by anti-GFP IP, followed by immunoblotting with anti-GFP or anti-ubiquitin (Ub) as indicated (Left). (Right) Ubiquitination immunoblotting intensities were normalized to the total precipitated tGnd1-GFP for each strain, as determined using ImageQuant TL. Results are graphed as a fraction of WT, which was set to 1.0. (E) Cycloheximide chase and anti-HA immunoblotting of 3HA-tGnd1-GFP or full-length Gnd1 fused to GFP, 3HA-Gnd1-GFP. (F) Crystal structure of full-length yeast Gnd1 created using PyMOL (19). The black arrow indicates the stGnd1 truncation point. The gray arrow indicates the tGnd1 truncation point. (G) Cycloheximide chase of 3HA-stGnd1 (Upper) or 3HA-full-length Gnd1 (Lower) in the indicated strains. Detection was with anti-HA antibodies. (H) Cycloheximide chase of stGnd1 in E2 UBC strains *rad6Δ* and *ubc4Δ* as indicated.

in cells compared to the identically expressed 3HA-tGnd1-GFP (Fig. 2-4E). QC pathways are able to selectively degrade a large variety of misfolded proteins. This flexibility includes diverse substrates, or distinct misfolded versions of the same protein, as seen with the many unstable “type II” CFTR mutants [18]. As a separate test of the generality of this cytoplasmic QC pathway, we used the X-ray structure of yeast Gnd1 protein to generate a new QC substrate [19]. The original tGnd1 substrate is truncated in the middle of the second folded domain (Fig. 2-4F grey arrow, residues 1-368, 75%). The first domain is a widely observed structure that allows NADP+ binding with a Rossman fold. We tested a version of Gnd1p with a truncation in the first domain (Fig. 2-4F black arrow, 1-150, 30%), calling the new substrate stGnd1 (“short truncated Gnd1”). Indeed, 3HA-stGnd1 was rapidly degraded, (Fig. 2-4G), showing a 10 minute half-life in the presence of Ubr1 and strong stabilization in the *ubr1Δ* null. Interestingly, this substrate was entirely dependent on Ubr1: the presence of the *san1Δ* alone or in combination with *ubr1Δ* had no effect on the stability of stGnd1. Identically tagged, full-length Gnd1 was completely stable (Fig. 2-4G, bottom row). Thus, Ubr-mediated quality control is specific for misfolded proteins, but is broadly inclusive in selection of substrates.

We employed the Ubr1-specificty of stGnd1 degradation to evaluate the E2 enzymes involved in Ubr1-mediated quality control. As observed in

other actions of this E3 [20], both Rad6/Ubc2 and Ubc4 could mediate degradation, and the double E2 null phenocopied the ubr1Δ strain (Fig. 2-4H).

The above studies show that both Ubr1 and San1 are required for degradation of a wide variety of misfolded proteins that originate in the cytoplasm. We next tested each ligase for roles in management of proteotoxic stress. We first tested a stress caused by overexpression of CPY‡-GFP. Although this protein can be expressed in any of the nulls without consequence from the TDH3 promoter in glucose medium, expression of CPY‡-GFP from the stronger galactose promoter caused growth sensitivity that was exacerbated by either the *ubr1Δ* or the *san1Δ*, and greatly worsened in the double null (Fig. 2-5A, top four rows). Thus, as expected from the CPY‡-GFP degradation data, both Ubr1 and San1 can lessen the stress caused by strong expression of this model substrate. Identical expression of the cytoplasm-restricted CPY‡-GFP+NES resulted in a stress that was only affected by the ubr1Δ; the presence of san1Δ alone or in combination with ubr1Δ had no additional effect on growth (Fig. 2-5A, bottom four rows). This result highlights Ubr1's direct role in survival of cytoplasmic proteotoxic stress.

We tested a variety of growth conditions to evaluate physiological roles for these ligases, including ethanol, which has been employed as a proteotoxic stress [21]. We found that 8% ethanol in solid medium caused a significant cold-sensitive growth phenotype in the *ubr1Δ* null. *ubr1Δ* strains

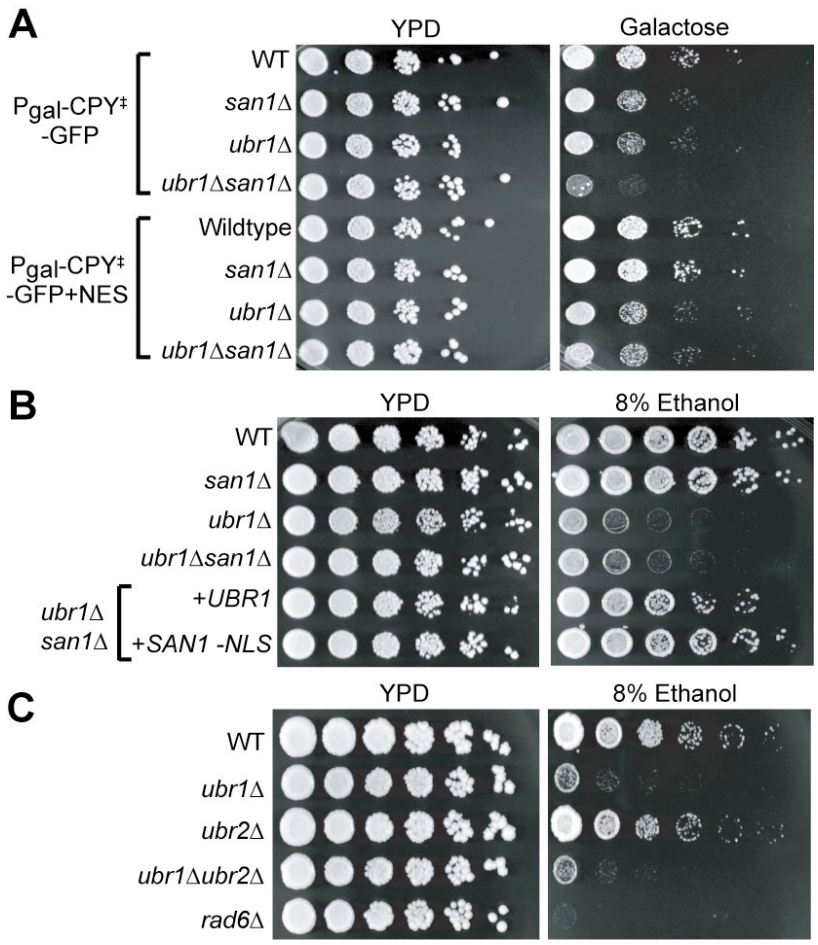

Figure 2-5: Involvement of Ubr1 and San1 in managing proteotoxic stresses.

Involvement of Ubr1 and San1 in managing proteotoxic stresses. (A) Cultures of strains expressing CPY‡-GFP (Top four rows) or cytoplasmic CPY‡-GFP +NES (Bottom four rows) under the control of the galactose promoter were spotted in 10-fold dilutions onto plates with either dextrose [yeast peptone dextrose (YPD)] or galactose as the carbon source and grown at 23°C. (B) Test of ethanol stress. WT, *san1Δ, ubr1Δ*, or *ubr1Δsan1Δ* null strains were spotted onto media containing 8% (vol/vol) ethanol in 5-fold serial dilutions and grown at 23°C as indicated. (Bottom two rows) Effect of expressing UBR1 or cytoplasmic SAN1-NLS in the ubr1Δsan1Δ strains is shown. (C) Same test of effect of 8% (vol/vol) ethanol on *ubr1Δ*, *ubr2Δ*, *ubr1Δubr2Δ*, or *rad6Δ* as indicated.

showed a strong growth defect on the ethanol plates at 23˚C (~ 600 fold; Fig. 2-5B). In contrast, the *san1Δ* null showed no defect of growth, nor did it enhance the sensitivity of the *ubr1Δ* (Fig. 2-5B, *san1Δ* vs. wt; *san1Δubr1Δ* vs. *ubr1Δ*). Thus, full length San1 was not involved in this *ubr1Δ*-sensitive stress. However, the growth phenotype of the *ubr1Δsan1Δ* double null *was* suppressed by cytoplasmic SAN1-NLS expressed in single copy from its native promoter (Fig. 2-5B "+SAN1-NLS"). This strongly implied that the ethanol stress imposed in this test occurred in the cytosol, and that it was remediated by destruction of misfolded proteins, either by normally present Ubr1, or by the cytoplasmic –NLS version of San1.

The EtOH growth phenotype was also observed in the absence of the E2 Rad6 (Fig. 2-5C, "*rad6Δ*"), implicated in many actions of Ubr1, including QC (Fig. 2-5G). The decreased survival in a rad6Δ was more pronounced than the ubr1Δ, which may be due to Rad6 having numerous roles in cell function. Alternatively, the Ubr1 homologue Ubr2 (~50% identical to Ubr1) also uses Rad6 [22], and could also have been involved in cytoplasmic QC. However, the *ubr2Δ* null showed no growth defect in the 8% EtOH test (Fig. 2-5C), nor did the presence of the *ubr2Δ* null make the *ubr1Δ* sensitivity any greater ("*ubr1Δubr2Δ*"). Furthermore, the *ubr2Δ* null had no effect on degradation of our substrates, and overexpression of Ubr2 did not increase

the degradation rate of any tested QC substrates. Thus, quality control function is unique to the Ubr1 isozyme.

The observation that San1 can recognize a variety of misfolded substrates is consistent with its known role as a quality control E3 [9]. However, a role for Ubr1 in quality control was novel and unexplored. Accordingly, we next examined mechanistic features of Ubr1-mediated QC in greater detail. We used the substrate tGnd1p-GFP to more fully test the idea that Ubr1-mediated QC was distinct from its well-studied role in the N-end rule, in which the N-terminus of a protein determines its rate of Ubr1-mediated degradation [11, 23, 24]. Ubr1 is the E3 ligase, or “N-recognin” of the N-end rule pathway, binding to and catalyzing ubiquitination of proteins with appropriate N-terminal amino acids present by cleavage or enzymatic addition [16]. It was formally possible that the Ubr1-dependent ubiquitination of our misfolded substrates was due to cleavage of a small amount of the protein to reveal a fast-degradation N-terminal residue, followed by traditional N-end recognition by Ubr1. We addressed this issue in several ways. We expressed and immunoprecipitated FLAG-tGnd1-GFP, bearing a single N-terminal tag, with anti-FLAG antibody to evaluate the ability of Ubr1 to ubiquitinate tGnd1-GFP with an intact N-terminus. The resulting ubiquitination in the suite of four strains was identical to that seen above with anti-GFP antibodies (Fig. 2-6A, 2D), with a similarly strong Ubr1-dependent component of ubiquitination, indicating that cleavage was not required for this

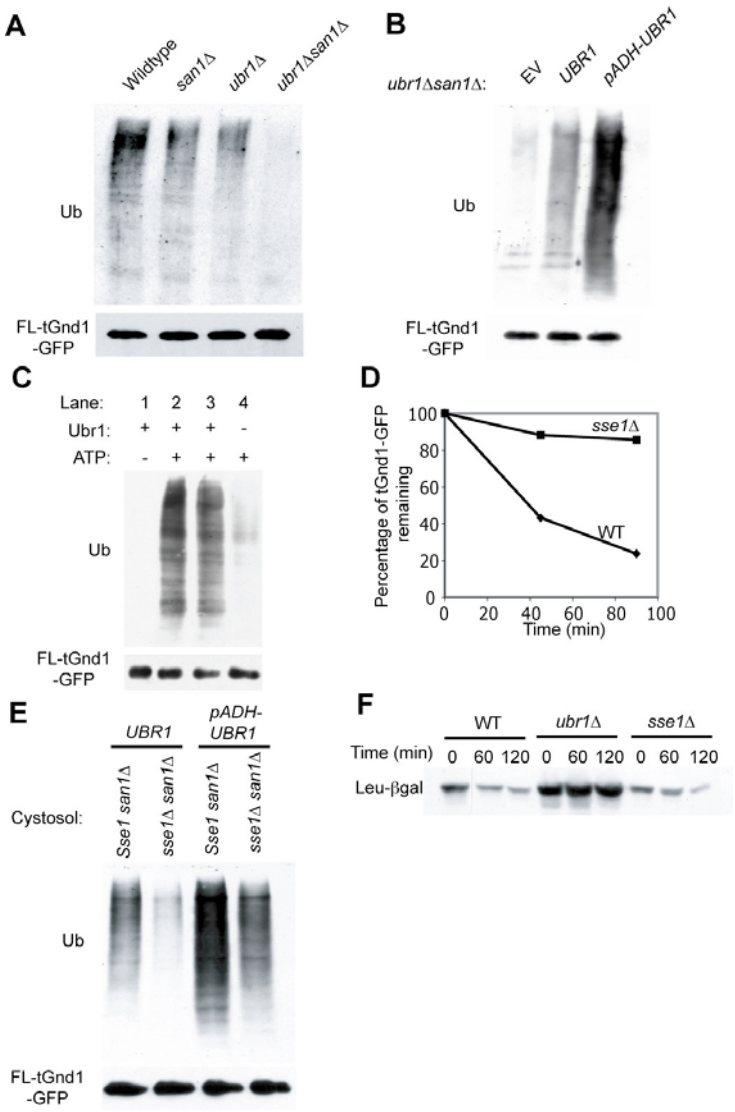

Figure 2-6: Ubr1 acts independent of the N-end rule in cytoplasmic QC.

Ubr1 acts independent of the N-end rule in cytoplasmic QC. (A) IP of FLAG-tGnd1-GFP with anti-FLAG antibody from the indicated strains, followed by immunoblotting with antiubiquitin (Ub) or anti-GFP. (B) Ubr1 dependence of in vitro tGnd1-GFP ubiquitination. Immunopurified FLAG-tGnd1-GFP was incubated with cytosol from *ubr1Δsan1Δ* strains expressing empty vector (EV), native promoter-driven UBR1, or pADH-UBR1 as indicated and was incubated for 1 h at 30°C and evaluated for tGnd1-GFP ubiquitination. (C) In vitro ubiquitination of bead-bound tGnd1-GFP after depletion of FLAG-UBR1 from *san1Δ* cytosol. Reaction cytosols were either untreated (lanes 1 and 2) or preincubated with IgG agarose beads as a control (lane 3) or with anti-FLAG beads (lane 4). “Depleted” cytosols were then added to substrate-bound beads, with or without ATP, and ubiquitination was assayed as above. (D) In vivo degradation of FLAG-tGnd1-GFP in WT or sse1Δ by flow cytometry, normalized to the mean fluorescence at time 0. (E) Sse1 requirement for Ubr1-dependent in vitro ubiquitination of tGnd1-GFP. Bead-bound tGnd1 was incubated in *san1Δ* null cytosols with or without Sse1. Left-pair strains expressed Ubr1 at native levels, and right-pair strains expressed Ubr1p from the strong ADH promoter. (F) Cycloheximide chase of N-end rule substrate Leu-β-gal in WT, *ubr1Δ*, or *sse1Δ* strains, immunoblotted with anti-β-gal antibodies.

ubiquitination. We developed an in vitro ubiquitination assay to directly test the ability of Ubr1 to intact FLAG-tGnd1-GFP. The substrate was first immunoprecipitated from a *ubr1Δsan1Δ* strain with anti-FLAG antibody beads. The beads were washed several times and then incubated with wt cytosol and added ATP. The beads were then washed and the bound protein was evaluated for ubiquitination by immunoblotting as above (Fig. 2-7A). A strong ubiquitination signal was observed only when the beads with bound substrate were incubated in cytosol and added ATP (Fig. 2-7B). We used this assay to directly test Ubr1-mediated ubiquitination of full-length FLAG-tGnd1-GFP. Anti-FLAG bead-bound substrate, immunoprecipitated from a *ubr1Δsan1Δ* strain, was subjected to in vitro ubiquitination by cytosol from *ubr1Δsan1Δ* strains with no Ubr1, ("EV"), plasmid expressing native promoter Ubr1 ("UBR1"), or overexpressed Ubr1 ("pADH-UBR1). The FLAG-tGnd1-GFP bound by its intact N-terminus was ubiquitinated in vitro in a Ubr1-dependent manner, and Ubr1 was rate-limiting (Fig. 2-6B). To ascertain whether Ubr1 was directly involved in tGnd1-GFP ubiquitination, we examined the effects of immunodepletion from the reaction cytosol using FLAG-Ubr1 as the E3. Anti-FLAG precipitation of Ubr1 from cytosol immediately prior to the ubiquitination assay resulted in complete inhibition of ubiquitination, while no effect was observed with non-specific control beads (Fig. 2-6C). Taken together, these results demonstrated that N-terminal cleavage was not required for Ubr1-dependent ubiquitination of tGnd1-GFP, and that Ubr1-mediated ubiquitination of QC substrates is directly mediated by this ligase.

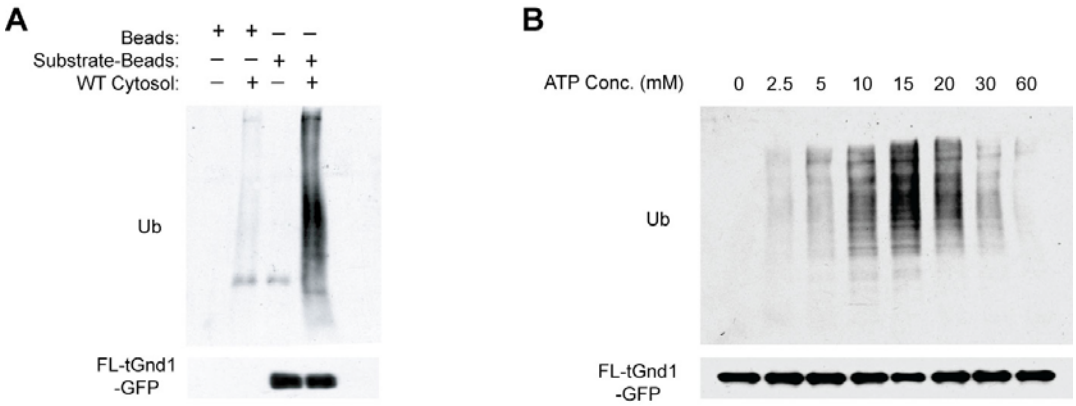

Figure 2-7: In vitro ubiquitination assay characterization.

(A) In vitro ubiquitination of FLAG-tGnd1-GFP. Anti FLAG agarose beds with bound FLAG-tGnd1-GFP ("Substrate-Beads") or untreated anti-FLAG agarose beads ("Beads") were incubated with wild type cytosol ("+") or buffer ("-") for 1 hour at 30˚C. The beads were then washed and resuspended in sample buffer prior to SDS-PAGE and immunoblotting for ubiquitin or GFP. (B) ATP dependent In vitro ubiquitination. Indicated ATP concentrations were added to 150mg of total cytosol protein and 10ul of bead-bound tGnd1-GFP, incubated at 30˚C for 1 hr. Immunoblotted for ubiquitin or GFP.

The quality control function of Ubr1 can be further distinguished from its role in the N-end rule through its clear chaperone dependence. A defining feature of Ubr1-mediated quality control in our studies is dependence on molecular chaperones for both degradation and ubiquitination (see below). In particular, all of our QC substrates reveal a strong dependence on the Sse1 chaperone (Fig. 2-6D, 2-1B-C, E). This was contrary to earlier published work concerning Sse1 and CPY‡-GFP [14]. Using the in vitro assay, we directly tested the role of Sse1 in Ubr1-mediated ubiquitination of tGnd1. Bead bound FLAG-tGnd1-GFP was incubated in *san1Δ* cytosol with wild-type or over-expressed Ubr1, with or without Sse1 (Fig. 2-6E). Ubr1-dependent ubiquitination of FLAG-Gnd1p-GFP was strongly decreased in the *sse1Δ* cytosol, at both levels of Ubr1. Thus, in vivo and in vitro Ubr1 QC function is dependent on Sse1. This is another criterion that distinguishes Ubr1-mediated QC from the N-end rule: no chaperone dependence of the classic N-end rule pathway has been reported, implying that the chaperone dependence is a unique feature of Ubr1-mediated QC. We confirmed this by testing the effect of the *sse1Δ* null on the classic N-end rule substrate, Leu-bgal [23]. This substrate showed identical degradation in wt or *sse1Δ* strains, but the expected strong stabilization in an isogenic *ubr1Δ* strain was observed (Fig. 2-6F).

N-end rule dependent degradation can also occur when a “fast” N-terminus is added to a stable N-terminus by the action Ate1 arginyl transferase [25]. Accordingly we tested Ubr1-dependent degradation in ate1Δ

and ate1Δsan1Δ nulls (Fig. 2-8). No effect was observed in ate1Δ strains, indicating that the Ubr1-dependent degradation of these misfolded proteins did not require addition of arginine at the N-terminus. Taken together, the above experiments demonstrated that Ubr1-mediated recognition of QC substrates was independent of its well-described function in the N-end rule.

We used the in vitro assay to evaluate the role of chaperones in Ubr1 and San1 mediated QC. In vivo, loss of either Sse1 or Hsp70 caused a strong defect in ubiquitination implying that they were involved in the action of each ligase. However, our in vitro analysis indicated that there are clear differences in the role of chaperones with each ligase. In vivo, FLAG-tGnd1-GFP ubiquitination was mediated by either Ubr1 or San1 (Fig. 2-6A). However, in vitro, the same substrate was ubiquitinated almost entirely by Ubr1. A *ubr1Δ* null cytosol was incapable of supporting tGnd1-GFP ubiquitination, while the reaction in a *san1Δ* cytosol was identical to wild-type (Fig. 2-9A, lanes 1-3). What is the cause of this difference in vitro? We discovered that the strong in vitro bias towards Ubr1-dependent tGnd1 ubiquitination was due to a surprising inhibition of San1 by Sse1. An *sse1Δ* cytosol supported robust ubiquitination of the substrate (Fig. 2-9A, lane 4). This sse1Δ-stimulated ubiquitination was strongly dependent on San1 (Fig. 2-9A, lane 6), showing a large decrease in the sse1Δsan1Δ double null. This is in striking contrast to the case in the intact cell, in which the San1-dependent component of tGnd1-

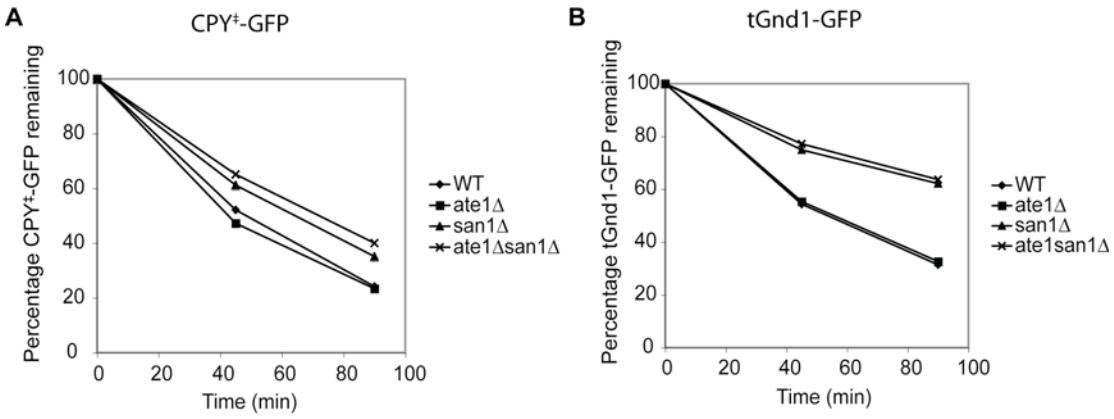

Figure 2-8: No effect of ate1Δ on CPY‡-GFP nor tGnd1-GFP degradation.

(A) Flow cytometry analysis of CPY‡-GFP degradation in WT, *ate1*Δ, *san1*Δ, *ate1*Δ*san1*Δ nulls. Mean fluorescence of CPY‡-GFP at each time point was normalized to the steady state at time zero and graphed as percentage remaining. (B) Flow cytometry analysis of tGnd1-GFP degradation in strains used in (A).

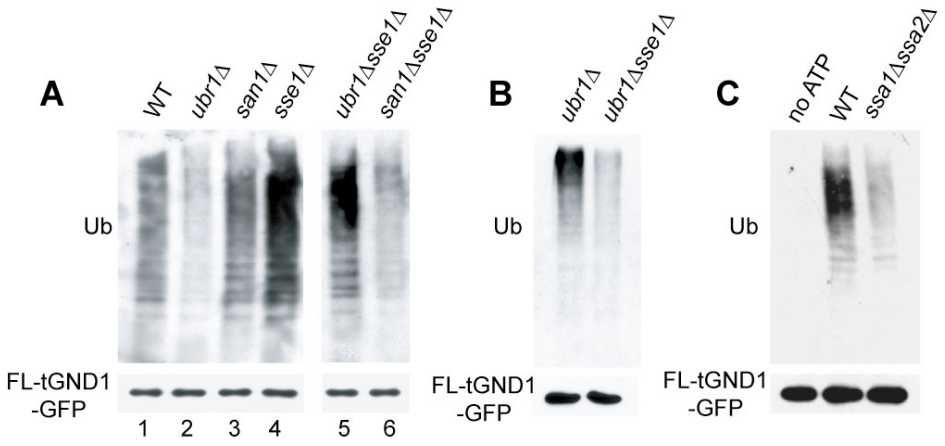

Figure 2-9: Differential chaperone requirement for Ubr1- and San1-mediated ubiquitination.

Differential chaperone requirement for Ubr1- and San1-mediated ubiquitination. (A) In vitro ubiquitination of tGnd1-GFP bound to anti-FLAG beads after incubation with cytosol from WT, *ubr1Δ, san1Δ, sse1Δ, ubr1Δsse1Δ*, or *san1Δsse1Δ* strains. Ub, ubiquitin. (B) In vivo ubiquitination of FLAG-tGnd1-GFP in ubr1Δ or ubr1Δsse1Δ strains. FLAG-tGnd1-GFP was immunoprecipitated from cell lysates with anti-FLAG beads and immunoblotted for GFP or Ub. (C) In vitro ubiquitination of FLAG-tGnd1-GFP. tGnd1-GFP bound to anti-FLAG beads was incubated with cytosol from WT or *ssa1Δssa2Δ* strains for 1 h at 30°C and assayed for ubiquitination by immunoblotting for GFP or Ub.

GFP ubiquitination (measured in a *ubr1Δ* null) was strongly *inhibited* by the presence of the *sse1Δ* null (Fig. 2-9B).

Our in vivo studies indicate that Ubr1-mediated QC is Hsp70 dependent. The in vitro assays above (Fig. 2-9E) show that Ubr1-mediated ubiquitination depends on the Sse1 cochaperone. Because the in vitro assay is almost entirely Ubr1 dependent in these conditions, we used this assay to directly examine the role of Hsp70 in Ubr1 QC. Indeed, loss of two of the four redundant Hsp70 genes (*ssa1Δssa2Δ)* resulted in a strong decrease in Ubr1-mediated tGnd1-GFP ubiquitination in vitro (Fig. 2-9C), indicating a direct role for Hsp70 in this novel function of Ubr1. Taken together, these studies indicate that Ubr1 directly employs chaperones for ubiquitination of substrates, while San1 has a more complex relationship with chaperones that appears to operate separately from substrate ubiquitination (see below).

Discussion

These studies have defined Ubr1 and San1 as E3 ligases involved in chaperone-dependent cytoplasmic quality control. Degradation mediated by these E3s meets the criteria for physiologically relevant PQC: ubiquitination is selective for misfolded proteins, a broad range of misfolded substrates are recognized by either ligase, and both participate in management of proteotoxic stress. Each ligase has been studied in other capacities. Ubr1 is known for degradation of N-end rule substrates, as well as numerous proteins recognized by distinct mechanisms [11, 12]. San1 is the first described nuclear quality control E3 [9]. Our work thus significantly extends the functional range of each ligase.

The new quality control function for Ubr1 was distinct from its elegantly described action in the N-end rule, and was restricted to the Ubr1 isozyme. Both E2s reported to work with Ubr1 could support ubiquitination of a Ubr1-selective QC substrate. Intriguingly, Ubr1 has a number of reported substrates that are not recognized by the N-end rule, including some that bear degradation determinants also found in misfolded proteins [26-28]. It will be important to re-evaluate these proteins as possible QC substrates. In addition, a variety of phenotypes have been observed in murine nulls of several Ubr isoforms [29-32]; it may be that these effects are the result of deficient quality control in the affected tissues.

Although the San1 ubiquitin ligase was originally reported as a quality control E3 [9], our studies significantly expand the range of substrates to included misfolded proteins of cytoplasmic origin. Since San1 originates in the cytosol, it was possible that the cytoplasmic QC function was due to the subset of molecules present in that compartment at steady state. Indeed, San1 restricted to the cytosol was fully capable of mediating degradation of CPY‡-GFP. However, the other studies in this work imply that San1 and Ubr1 function in distinct compartments to mediate destruction of misfolded cytoplasmic proteins (see below).

It has been previously reported that degradation of cytoplasmic proteins requires chaperones [5, 14], and the Ubr1/San1 dependent substrates appropriately showed a strong dependence on Hsp70 and the Sse1 (Hsp110) co-chaperone. However, the role of chaperones in the action of each was distinguishable. Ubr1 appeared to directly use both Sse1 and Hsp70, as indicated by the in vitro assays. In contrast, while San1-dependent degradation (observed in a ubr1Δ null) was strongly Sse1 dependent in vivo, its action was *inhibited* by Sse1 in vitro.

Taken together, our data suggest a two-compartment model for the roles of Ubr1 and San1 in cytoplasmic quality control. The degradation and phenotypic studies with the cytoplasm-restricted CPY‡-GFP+NES were consistent with distinct actions of these ligases. Ubr1 maintained its ability to degrade this purely cytoplasmic substrate, while San1 had no role in its

degradation. Similarly, the stress phenotype caused by this cytoplasmic substrate was only sensitive to the ubr1Δ, but not san1Δ, again indicating a direct cytoplasmic role for Ubr1 but not San1. Taken together these data suggest the following model. A misfolded cytoplasmic protein can undergo ubiquitination by either Ubr1 or San1. The Ubr1-dependent branch occurs in the cytosol, using chaperones in conjunction with Ubr1 to recognize and ubiquitinate a substrate. In contrast, San1-dependent ubiquitination of the same substrate requires chaperone-dependent transport into the nucleus, where San1 ubiquitinates the substrate. The dichotomous requirements for Sse1 observed for San1 in vivo and in vitro are consistent with this model: in vivo, Sse1, in conjunction with Hsp70, promotes delivery of misfolded substrate to the nuclear pool of San1. In vitro, Sse1 gains access to San1 and inhibits it, perhaps by interacting with San1 features that interact with misfolded proteins. In support of this idea, we have observed that a fraction of CPY‡-GFP builds up in the nucleus in a san1Δ null, and this build up is dependent on Sse1 (Fig. 2-10): in an sse1Δ san1Δ double null CPY‡-GFP accumulates in the cytoplasm only. This dependency appeared to be strong since addition of the NES construct to the CPY‡-GFP had almost the same effect as sse1Δ in this optical experiment (Fig. 2-10). Direct tests of this model will be an important avenue of future inquiry.

Our studies with nulls indicate that the two pathways function independently: each E3 appears to contribute a component of ubiquitination

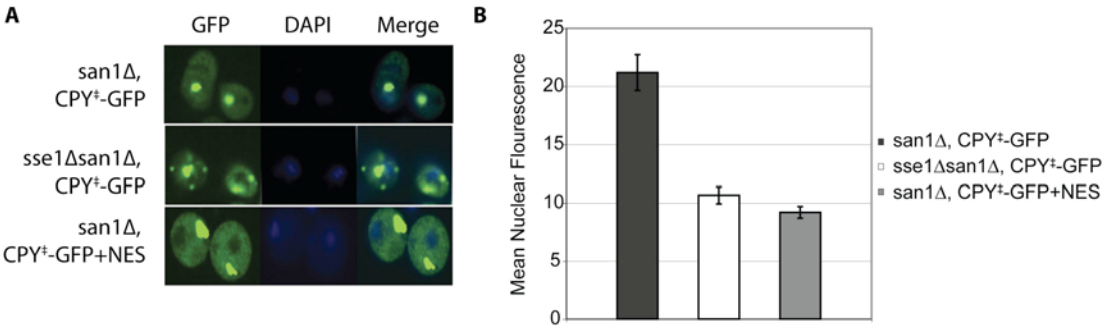

Figure 2-10: Sse1 mediated nuclear localization of CPY‡-GFP. (A) Representative images of fluorescence microscopy carried out on san1Δ and sse1Δsan1Δ cells expressing CPY‡-GFP, as well as san1Δ cells expressing CPY‡-GFP+NES. GFP and DAPI staining was captured to demonstrate the change in localization of CPY‡-GFP within the cell in the absence of *sse1*Δ. CPY‡-GFP accumulates in the nucleus of san1Δ cells, but is restricted to the cytoplasm in the sse1Δsan1Δ. B) Quantitation of the mean nuclear signal intensity of GFP fluorescence in the nucleus in *san1*Δ and *sse1*Δ*san1*Δ using ImageJ software [1], computed as the ratio of DAPI colocalized GFP signal divided by the total GFP signal in the cell. CPY‡-GFP+NES localization in a san1Δ was used to gauge the lower limits of detection. Error bars are S.E.M. (n = 25 in each condition).

that is independent of the presence of the other ligase. However, there was phenotypic synergy in the stress experiments involving CPY‡-GFP, since loss of both E3s had a much more drastic effect than that of either single null (Fig. 3A). Thus, the issue of regulatory or mechanistic crosstalk between these two modes of cytoplasmic QC is an open and interesting one. It is clear that Ubr1 plays the principle role in both degradation of cytoplasmic substrates, and management of cytoplasmic stress. Another open question is the role of chaperones in the QC action of Ubr1. This includes discerning the full panoply of chaperones and co-chaperones involved, and understanding the mechanism by which they assist Ubr1 in substrate ubiquitination. It could be that they are required to produce substrate forms that are recognizable by Ubr1. Alternatively, it may be that Hsp70 and Sse1 from a substrate recognition module that works directly with Ubr1, in a manner analogous to CHIP [7]. Finally, we have observed that some substrates that undergo chaperone-dependent degradation are not subject to Ubr1/San1 ubiquitination [5], indicating other E3 QC ligases remain to be discovered. In addition, it is clear that the Doa10 ERAD ligase can also participate in recognition of some cytoplasmic QC substrates [2]. Thus, the full picture of cytoplasmic quality control will involve a network of interacting options.

A large number of clinically pressing maladies have etiologies that pertain to protein misfolding, including aging, Parkinsonism, Huntington's, and a variety of somatic illnesses [33]. The identification of the relevant QC ligases opens numerous doors to understanding pathologies of proteostasis.

Detailed knowledge of these quality control pathways will allow their manipulation for basic and clinical purposes.

Materials and Methods

Yeast Strains and Plasmids. The *S. cerevisiae* strains used in this work are listed in Table S1. All strains and plasmids were constructed with standard molecular biology techniques, as described in [34, 35]. Yeast strains were cultured as described (Gardner et al., 1998), in minimal media with 2% dextrose and appropriate amino acid supplements, at 30°C unless otherwise indicated. The majority of strains used were in the BY4741 background (*MATa ura3Δ0 leu2Δ0 his3Δ1 met15Δ0*) with the exception of: *ubc4Δubc5Δ* and WT, MHY508 (*ubc4Δ::HIS3 ubc5Δ::LEU2*), and MHY501 (MATα his3-Δ200 leu2-3,112 ura3-52 lys2-801 trp1-1). SSA1 (JN516; MATα ura3-52 leu2-3 his3-11, 15 trp1-D1 lys2 SSA1 ssa2::LEU2 ssa3::TRP1 ssa4::LYS2) and ssa1-45 (JB67; MATα ura3-52 leu2-3 his3-11, trp1-D1 lys2 ssa1::ssa1-45 ssa2::LEU2 ssa3::TRP1 ssa4::LYS2). Null alleles with coding regions replaced were constructed in the BY4741 background by transforming yeast using the LiOAc method with a PCR product encoding the indicated selection marker and 50bp flanks homologous to the gene to be disrupted (Baudin et al., 1993) or using knockout cassettes in the lab collection. Oligo sequences are available on request.

The *UBR1* (pRH2444), *UBR1MR1* (pRH2445), and *PADH-UBR1* (pRH2471) plasmids were a gift from A. Varshavsky (California Institute of Technology, CA). The orignal *–CPY*‡*-GFP* expression plasmid was provided by D. Wolf (U. of Stuttgart; DE). The *SAN1* (pRH2475), *SAN1-NLS*

(pRH2439), *san1Δ::NatMX* (pRH2376), *PGAL-CPY*‡*-GFP* (pRH2533), and *PGALCPY*‡*- GFP-NES* (pRH2534) plasmids were a gift from R. Gardner (University of Washington, WA)

In vitro ubiquitination assay. In vitro ubiquitination assays were adapted from [36]. Briefly, Bead-bound substrate was mixed with cytoplasm isolated from the indicated genetic background. 150mg of total protein was mixed with 15mM ATP and 10ul of substrate-bound beads. The reaction was incubated at 30˚C for 1 hr. Bead-bound substrate was then washed several times with IP buffer, protease inhibitors, and N-ethylmaleimide, then aspirated to dryness and subjected to electrophoretic sample buffer and immunoblotting.

In vivo ubiquitination assay. In vivo ubiquitination of substrates was evaluated by immunoprecipitation (IP) followed by ubiquitin immunoblotting as described in [37]. Cells were lysed in the presence of protease inhibitors and N-ethylmaleimide, followed by IP of the target substrate with anti-GFP, anti-HA (Covance), or anti-FLAG M2 beads (Sigma-Aldrich). After several washes, electrophoretic sample buffer was added, followed by immunoblot analysis.

Degradation assays. Cycloheximide chase degradation assays were performed as previously described (Gardner et al., 1998). Briefly, yeast cells were grown to log phase (approx. OD600< 0.5) and cycloheximide was added to a final concentration of 50ug/ml. At the indicated time points cells were

collected by centrifugation and lysed with 0.1ml SUME (1% SDS, 8 M urea, 10 mM MOPS, pH 6.8, 10mM EDTA) with protease inhibitors (260uM ABESF, 142uM TPCK, 100uM Leupeptin, 76uM Pepstatin) and 0.5 mm glass beads followed by vortexing for 2 minutes, followed by addition of 100ul 2x USB (75mM 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 3 min, and then clarified by centrifugation before separation by SDS-PAGE and subsequent immunoblotting with appropriate antibodies.

Flow Cytometry Anaysis. Flow cytometry for GFP tagged substrates was performed as described (Cronin and Hampton, 1999). Cell cultures were grown in minimal medium to low log phase (OD600=0.1) before addition of 50ug/ml cycloheximide for the indicated times. Samples were measured for fluorescence with a BD Biosciences FACScalibur instrument and statistical analysis was conducted with CellQuest flow cytometry software. Histograms represent 10,000 individual cells.

Cytoplasm preparation. Cytoplasm for in vitro assays was prepared from the respective genetic backgrounds using an approach modified from our in vitro ERAD assay (Garza et al., 2009): Cells were grown in YPD to an OD600 of 0.8-1.0, and 100 ODs of cells were pelleted. The pellet was washed 2x H2O, once with cold B88 buffer (20 mM HEPES–KOH, pH 7.4, 150 mM KOAc, 250 mM sorbitol, and 5 mM Mg(OAc)2) with protease inhibitors (260uM ABESF, 142uM TPCK, 100uM leupeptin, 76uM pepstatin) and DTT,

and resuspension in 100 ul B88 buffer with PI and DTT for lysis by grinding in a mortar and pestle. The mortar and pestle were pre-cooled with liquid nitrogen before addition of the cells. The cells were added to the mortar with a 5 ml of liquid nitrogen. The frozen cells were ground by hand with the pestle. The cells were kept frozen during the process by addition of liquid nitrogen as needed. The ground cells were then placed in a 2 ml tube on ice, and allowed to thaw back to liquid. The resulting cytoplasm was clarified by centrifugation at 5000 g at 4°C for 5 min. The supernatant was then transferred to a new tube and centrifuged again at 20000 g at 4°C for 15min. A final ultracentrifugation was carried out at 100000 g at 4°C for 60 min. Protein concentration of individual cytoplasms was determined using Bradford Reagent (Sigma-Aldrich). Cytoplasms were kept on ice until use.

In vitro ubiquitination assay. Bead-bound immunoprecipitated substrate was mixed with the isolated cytoplasm (see supplemental methods) from the indicated genetic background in the following way: All cytoplasmic reactions took place in a final volume of 30 ul and were prepared on ice. 150 mg of total protein from the respective cytoplasmic preparation was mixed with 15mM ATP, and 10 ul of FLAG beads bound to pre-IP substrate. The reactions were incubated in a 30°C water bath for 1 hour with periodic agitation. The reaction was terminated by adding 800 ul of IP buffer (15 mM sodium phosphate, 150 mM NaCL, 10 mM EDTA, 2% Trition X-100, 0.1% SDS, 0.5% deoxycholate) with protease inhibitors and 5mM N-ethylmaleimide. The FLAG beads were washed 3x with 1 ml IP wash buffer

(50 mM NaCl, 10 mM Tris, pH 7.5), aspirated to dryness, and heated in the presence of sample buffer to 100°C for 3 min before SDS-PAGE and immunoblotting. Immunoprecipitation of substrate prior to in vitro ubiquitination experiments was conducted in the following manner: Strains lacking San1 and Ubr1 and contained FLAG-tGND1-GFP, were grown as described for cytoplasmic preparation above. 10ul of anti-FLAG M2 beads (Sigma Aldrich) were added per 150mg of cytosol, and allowed to nutate overnight at 4°C. The beads were then pelleted at 1000 rpm for 1min. 3 washes with IP buffer were conducted before final resuspension in B88 reaction buffer.

In vivo ubiquitination assay. Cells were grown and lysed as outlined above. To assess in vivo ubiquitination: After vortexing in the presence of beads and SUME, 1 ml of IP buffer with protease inhibitors and N-ethylmaleimide was added. The lysate was clarified by centrifugation at 14000 rpm for 5 min. The supernatant was transferred to a new tube and either polyclonal anti-GFP, anti-HA (Covance), or monoclonal anti-FLAG M2 beads (Sigma-Aldrich) were added depending on the substrate. The lysates were nutated overnight at 4°C. In the case of anti-GFP and anti-HA pulldowns, 100ul of protein A sepharose beads were then added and allowed to nutate for an additional 2 hours at 4°C. The beads were then spun down at 1000 rpm, and washed three times with IP wash buffer (50 mM NaCl, 10 mM Tris, pH 7.5), and aspirated to dryness before addition of electrophoretic sample buffer.

Phenotyping. Plate dilution assays, to evaluate cell growth, were carried out by growing all strains in supplemented minimal medium overnight. A total of 0.35 OD units were centrifuged and resuspended in 1 ml sterile water. Five fold or ten fold dilutions were then performed in a 96-well plate and spotted onto on the indicated media. Studies of ethanol sensitivity were conducted using YPD plates with the appropriate ethanol concentration. EtOH plates, as well as the YPD control plates were then wrapped in parafilm to prevent ethanol evaporation and grown for 5-9 days at various temperatures.

Table 2-1: List of Yeast Strains Used in Chapter 2

Name	Genotype	Source
BY4741	*MATa ura3Δ0 leu2Δ0 his3Δ1 met15Δ0*	Resgen Deletion Collection
RHY4622	JN516; *MATα ura3-52 leu2-3 his3-11, 15 trp1-D1 lys2 SSA1 ssa2::LEU2 ssa3::TRP1 ssa4::LYS2*	Jeff Brodsky
RHY4623	JB67; *MATα ura3-52 leu2-3 his3-11, 15 trp1-D1 lys2 ssa1::ssa1-45 ssa2::LEU2 ssa3::TRP1 ssa4::LYS2*	Jeff Brodsky
RHY6336	BY4741 pRH2081 *(P_{TDH3}-CPY‡-GFP, URA3)*	this study
RHY6337	BY4741 *sti1Δ::KanMX* pRH2081 *(P_{TDH3}-CPY‡-GFP, ADE2 URA3)*	this study
RHY6338	BY4741 *sse1Δ::KanMX* pRH2081 *(P_{TDH3}-CPY‡-GFP, ADE2 URA3)*	this study
RHY6364	*MATα ade2-101 met2 lys2-801 ura3-52 trp1::hisG leu2Δ his2Δ200 CDC34::cdc34-2* pRH2047 *(P_{TDH3}-CPY‡-GFP, URA3)*	
RHY7135	BY4741 ubr1Δ::KanMX pRH2081 (P_{TDH3}-CPY‡-GFP, *ADE2 URA3*)	this study
RHY7136	BY4741 *san1Δ::NatMX ubr1Δ::KanMX* pRH2081 *(P_{TDH3}-CPY‡-GFP, ADE2 URA3)*	this study
RHY7157	BY4741 san1Δ::NatMX pRH2081 (P_{TDH3}-CPY‡-GFP, *ADE2 URA3*)	this study
RHY7161	BY4741 *san1Δ::NatMX ubr1Δ::KanMX* pRH2081 *(P_{TDH3}-CPY‡-GFP, ADE2 URA3)* pRH2439 *(San1(-NLS)-3HSV)*	this study
RHY7165	RHY7136 pRH2444 *(UBR1, LEU2, YCp)*	this study
RHY7169	RHY7136 pRH2445 *(UBR1MR1, LEU2, YCp)*	this study
RHY7447	BY4741	Resgen Deletion Collection
RHY7448	BY4741 *san1Δ::NatMX*	this study
RHY7449	BY4741 *ubr1Δ::KanMX*	Resgen Deletion Collection
RHY7450	BY4741 *san1Δ::NatMX ubr1Δ::KanMX*	this study
RHY7616	RHY7447 pRH2460 (*tFAS1-3HA*, *URA3,* 2μ*)*	this study
RHY7617	RHY7448 pRH2460 (*tFAS1-3HA*, *URA3,* 2μ)	this study

Table 2-1: continued

RHY7617	RHY7449 pRH2460 (*tFAS1-3HA*, *URA3,* 2μ)	this study
RHY7618	RHY7450 pRH2460 (*tFAS1-3HA*, *URA3,* 2μ)	this study
RHY7620	RHY7447 pRH2476 *(*P_{TDH3}*-3HA-tGnd1-GFP ADE2 URA3)*	this study
RHY7621	RHY7448 pRH2476 (P_{TDH3}*-3HA-tGnd1-GFP ADE2 URA3)*	this study
RHY7622	RHY7449 pRH2476 *(*P_{TDH3}*-3HA-tGnd1-GFP ADE2 URA3)*	this study
RHY7623	RHY7450 pRH2476 *(*P_{TDH3}*-3HA-tGnd1-GFP ADE2 URA3)*	this study
RHY7630	BY4741 *pdr5Δ::KanMX* pRH2081 *(*P_{TDH3}*-CPY*‡*-GFP, ADE2 URA3)*	this study
RHY7782	RHY7447 pRH2486 (P_{TDH3}*-FLAG-tGnd1-GFP*, *ADE2 URA3*)	this study
RHY7783	RHY7448 pRH2486 (P_{TDH3}*-FLAG-tGnd1-GFP*, *ADE2 URA3*)	this study
RHY7784	RHY7449 pRH2486 (P_{TDH3}*-FLAG-tGnd1-GFP*, *ADE2 URA3*)	this study
RHY7785	RHY7450 pRH2486 (P_{TDH3}*-FLAG-tGnd1-GFP*, *ADE2 URA3*)	this study
RHY7873	RHY7447 pRH2491 (P_{TDH3}*-3HAtYor296w*, *ADE2 URA3*)	this study
RHY7874	RHY7448 pRH2491 (P_{TDH3}*-3HAtYor296w*, *ADE2 URA3*)	this study
RHY7875	RHY7449 pRH2491 (P_{TDH3}*-3HAtYor296w*, *ADE2 URA3*)	this study
RHY7876	RHY7450 pRH2491 (P_{TDH3}*-3HAtYor296w*, *ADE2 URA3*)	this study
RHY7878	BY4741 *sse1Δ::KanMX san1Δ::NatMx*	this study
RHY7983	RHY7878 pRH2474 (P_{ADH1}, *LEU2,* 2μ)	this study
RHY7984	RHY7878 pRH2471 (P_{ADH1}*-UBR1, LEU2*, 2μ)	this study
RHY7987	RHY7447 pRH2516 *(*P_{TDH3}*-3HA-stGnd1 ADE2 URA3)*	this study
RHY7988	RHY7448 pRH2516 *(*P_{TDH3}*-3HA-stGnd1 ADE2 URA3)*	this study
RHY7989	RHY7449 pRH2516 (P_{TDH3}*-3HA-stGnd1 ADE2 URA3)*	this study

Table 2-1: continued

RHY7990	RHY7450 pRH2516 *(P$_{TDH3}$-3HA-stGnd1 ADE2 URA3)*	this study
RHY7993	BY4741 *ydj1Δ::Leu2* pRH2081 *(P$_{TDH3}$-CPY‡-GFP, ADE2 URA3)*	this study
RHY7994	BY4741 *hsp104Δ::Leu2* pRH2081 *(P$_{TDH3}$-CPY‡-GFP, ADE2 URA3)*	this study
RHY8075	BY4741 *sse1Δ::KanMX*	Resgen Deletion Collection
RHY8198	RHY7447 pRH2531 *(P$_{TDH3}$-3HA-Gnd1-GFP ADE2 URA3)*	this study
RHY8199	RHY7448 pRH2531 *(P$_{TDH3}$-3HA-Gnd1-GFP ADE2 URA3)*	this study
RHY8200	RHY7449 pRH2531 *(P$_{TDH3}$-3HA-Gnd1-GFP ADE2 URA3)*	this study
RHY8201	RHY7450 pRH2531 *(P$_{TDH3}$-3HA-Gnd1-GFP ADE2 URA3)*	this study
RHY8308	RHY7450 pRH2471 (P$_{ADH1}$-*UBR1*, *LEU2*, 2μ)	this study
RHY8309	RHY7450 pRH2439 (*SAN1-NLS*, *LEU2*)	this study
RHY8368	BY4741 *sse1Δ::KanMX ubr1Δ::LEU2*	this study

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Chapter 3

The Characterization of molecular chaperone function in yeast cytoplasmic quality control degradation.

Abstract

Molecular chaperones are at the crossroads between protein folding and degradation by the ubiquitin proteasome system. Chaperones triage misfolded proteins for (re)folding, sequestration, or degradation. Many protein misfolding disorders, like Alzheimer's, Parkinsons, and Huntingtons disease, share hallmarks of an overloaded quality control network, which can often be ameliorated by increasing chaperone capacity. Discovering the mechanisms that participate in cytoplasmic quality control and determine how chaperones triage misfolded proteins to the degradative machinery of the UPS is of growing importance. To investigate the role of chaperones in cytoplasmic quality control we undertook studies of the model substrates of the Ubr1 quality control pathway in Yeast. We found that the Hsp70/90 chaperone network was required for degradation of multiple Ubr1-CQC substrates. Specific cochaperones, Ydj1 and Sse1, that modulate Hsp70 and Hsp90 activity were also required for degradation, however, others, Sti1, were not. Efficient ubiquitination of CQC substrates required both Hsp70 and Hsp90 activity. This ubiquitination also required a chaperone-substrate interaction beyond merely keeping the misfolded protein soluble, but rather an intimate interplay between substrate, chaperone, and ubiquitin ligase for efficiency.

Introduction

The intracellular environment is extremely crowded, which makes proper protein folding a complicated task. Nascent and mature polypeptides need to fold and maintain the correct conformation in an environment that is extremely concentrated with protein and other macromolecules, to the tune of 300-400 g l^{-1} [1]. A polypeptide that cannot fold correctly can lead to non-productive interactions with other polypeptides, thus interfering with their function as well. Molecular chaperones play a role in this process by interacting with these polypeptides to stabilize them and isolate them from the surrounding milieu. A hallmark of misfolded proteins is the presentation of short stretches of hydrophobic residues, which are typically buried in the interior of correctly folded proteins. Chaperones bind to these hydrophobic patches and initiate a sequence of events that include folding and refolding of these intermediate states. This process occurs for newly translated polypeptides as they emerge from the ribosome, during transport of proteins across membranes, in oligomeric assembly, in stabilizing protein-protein interactions by controlling conformational changes, and in response to cellular stress, such as heat shock[2]. Often times, the proper fold is not achieved, and in these cases the chaperones facilitate degradation[3].

The triaging of misfolded proteins in the above-mentioned processes is accomplished in large part to the Heat shock protein 70 (Hsp70) class of

chaperones. The Hsp70 family is ubiquitous in nature and encompasses many isoforms with general and specific cellular roles and targets. All Hsp70 molecules share a similar architecture in that they have an N-terminal adenine nucleotide binding domain (NBD) and a carboxy-terminal peptide binding domain (PBD) [4]. The NBD binds and hydrolyzes ATP. In the presence of ATP, the NBD allosterically regulates the PBD such that Hsp70 has a more open conformation and has a low affinity for binding substrate peptides. The PBD also allosterically regulates the NBD as well. When substrate is bound to the PBD, the NBD is structurally altered to promote the hydrolysis of ATP. In the ADP bound state, Hsp70 is tightly bound to the target substrate and is able to prevent aggregation and provide an environment that is optimized for folding. It is the iterative cycling between these two states, which are controlled by nucleotide binding, hydrolysis, and release, that results in substrate folding [4].

This iterative cycling of Hsp70 is subject to regulation by cofactors. Hsp70s never function alone, but rather require the presence of a J protein, and most often, a nucleotide exchange factor (NEF). The basal ATPase activity of Hsp70 is too low to allow for the efficient capture of substrate peptides in the rapidly changing environment of the cytosol [2]. The J protein solves this problem in part by having its own peptide-binding motif to interact with a substrate and either deliver or help stabilize it in complex with Hsp70.. The J protein then in turn stimulates Hsp70s ATPase activity thus facilitating client capture. Client peptide release is achieved via the action of the NEF,

which encourages release of the bound ADP to be replaced by ATP. ADP dissociation allows the client to attain its active conformation, or undergo another round of capture and folding, all the while being prevented from aggregating or interfering with other proteins in the environment. The activities of Hsp70 with the J protein and NEF comprise what is called the core ‘Hsp70 machine’ [5].

The Hsp70 machine does work with other chaperone networks to maintain protein homeostasis, to include the chaperonins (Hsp60/TRIC), small heat shock proteins (small Hsp), and the Hsp90 system. Hsp70 can work simultaneously or sequentially with these other networks to (re)fold, dissaggregate, assemble, or degrade proteins.

The degradation of proteins that are unable to properly fold has previously been shown to involve the chaperone networks [6-9]. Which chaperones are involved and there precise role is now only beginning to be understood, but it is clear that this role is dependent on subcellular localization and the specific properties of the misfolded protein in question. Recently in Heck et al., a number of misfolded substrates in the cytoplasm were demonstrated to be degraded and ubiquitinated in a molecular chaperone dependent manner [6]. To expand upon this work, we have more precisely characterized the function of chaperones in the degradation of a subset of those misfolded proteins. We have found that while Hsp70 function is required for degradation of all CQC substrates studied to date and the cochaperones involved in modulating Hsp70 activity may be substrate

dependent. Some cochaperones that modulate Hsp70 activity are shown to be involved in CQC, while others are not, suggesting that the identity of the cochaperones working with these chaperone machines is dependent on the structural properties of the misfolded protein. Additionally, we show that certain isoforms of Hsp70 are responsible for promoting the bulk of degradation and ubiquitination. We also demonstrate that the Hsp90 chaperone network is also functioning in CQC and furthermore, its promotion of degradation precedes CQC substrate ubiquitination. Lastly, our work strongly implies that the role of molecular chaperones in CQC goes beyond merely maintaining substrate solubility, but rather their presence is required prior to and perhaps even during ubiquitination. Additional work on how these chaperones are recruiting Ubr1 will be enlightening in order to understand in greater detail the interplay between chaperones and ligases.

Results

Truncated phosphogluconate dehydrogenase (tGnd1) is a newly discovered model CQC substrate. It has been shown to be ubiquitinated and degraded by the Ubr1 CQC system in a Hsp70 chaperone dependent manner. We set out to further characterize the role of chaperones in ubiquitin proteasome system degradation of tGnd1-GFP, as well as the related short-tGnd1 (stGnd1), and compare and contrast them with other well-characterized CQC substrates. tGnd1-GFP comprises 70% of the protein sequence of the wild-type Gnd1 protein, and this results in its recognition by the Ubr1 degradation pathway [6]. stGnd1, is only 30% of the wild-type Gnd1 sequence and shares the same Ubr1-chapeone dependencies on degradation and ubiquitination [6].

In the yeast cytosol there are four homologues of Hsp70, termed Ssa1-4, involved in post-translation proteostasis. Since each isoform has redundant function we used a strain that was null for *Ssa2-4* and either had a functional *Ssa1* or a temperature sensitive (TS) allele *ssa1-45*. In the absence of Hsp70 activity, tGnd1-GFP degradation is strongly stabilized (Fig. 3-1A). Similarly, stGnd1 is also stabilized in this same Ssa compromised background. However, the *ssa1-45* allele is unable to promote the degradation of stGnd1 even at the permissive temperature, despite this allele being competent

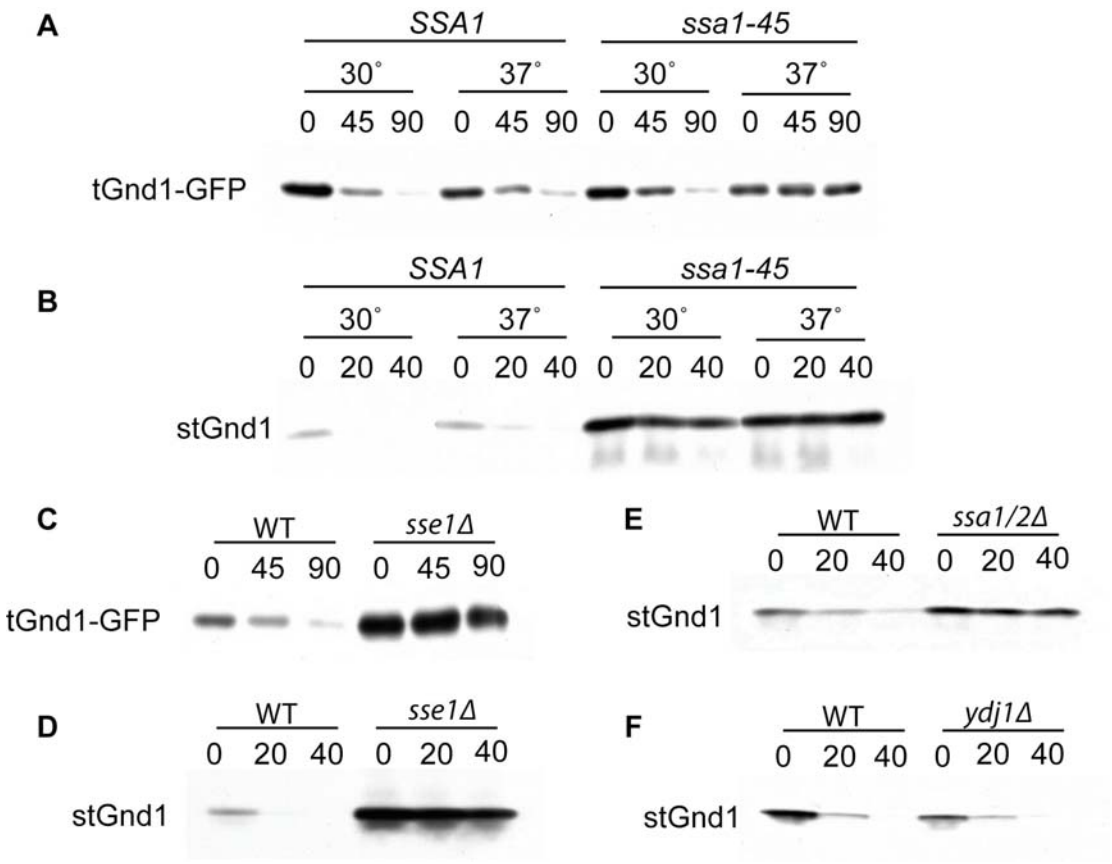

Figure 3-1: The Hsp70 chaperone machine is required for Cytoplasmic Quality Control Degradation. (A) tGnd1-GFP degradation in *ssa2*Δ*ssa3*Δ*ssa4*Δ nulls with either WT *SSA1* or temperature sensitive *ssa1-45* present, evaluated by cycloheximide chase at 30°C or 37°C, followed by anti-GFP immunoblotting. (B) stGnd1 degradation in same strains as in (A). (C) Cycloheximide chase of tGnd1-GFP degradation in *sse1*Δ strain. (D) Cycloheximide chase of stGnd1 degradation in *sse1*Δ strain. (E) Cycloheximide chase of stGnd1 degradation in *ssa1*Δ*ssa2*Δ. (F) Cycloheximide chase of stGnd1 degradation in *ydj1*Δ strain.

for cell survival and being able to perform its role in CQC on a variety of substrates at 30˚C (Fig 3-1B, 2-2A). The only reported difference between tGnd1-GFP and stGnd1 as CQC substrates is the lack of San1 recognition of stGnd1 [6]. We have hypothesized that this difference might be attributable to the manner in which San1 recognizes its substrates and is discussed later, but the inability of the ssa1-45 allele also indicates that stGnd1 is being identified by the CQC machinery in slightly alternative ways than tGnd1-GFP. Previously, tGnd1-GFP had been shown to require Ssa1 and Ssa2 for the bulk of degradation, so we tested stGnd1 in the double null for those proteins as well. In the *ssa1/2*Δ, stGnd1 is strongly stabilized (Figure 3-1E).

In yeast there are a number of NEFs that modulate Ssa activity. The Ssa-NEF Sse1 has been shown to involved in CQC previously. In an sse1Δ, both tGnd1 and stGnd1 are stabilized (Fig 3-1C, D). It is likely that this is the result of its function on Ssa proteins, but Sse1 also has a holdase activity that has been shown to bind directly to Ssa client proteins and could serve as means of recognition by CQC machinery [10].

Efficient Ssa activity is also dependent on J domain containing proteins to promote ATP hydrolysis and at times direct binding and delivery to the Ssa machine. Ydj1 is a yeast J domain containing protein and has been implicated in CQC previously [6, 7]. In a *ydj1*Δ null, stGnd1 degradation is unaffected (Fig. 3-1F). This was unexpected since CPY‡-GFP degradation and ubiquitination are dependent in part on Ydj1 presence (Fig. 2-2). Furthermore,

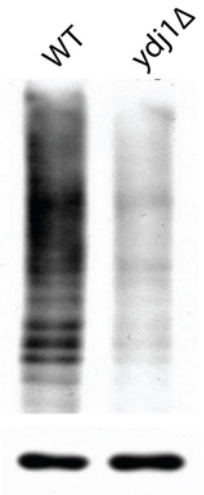

Figure 3-2: Ubiquitination of tGnd1-GFP in vitro requires J-domain protein Ydj1.

Immunopurified FLAG-tGnd1-GFP was incubated with cytosol from Wild-type or *ydj1*Δ strains for 1 h at 30˚C and evaluated for tGnd1-GFP ubiquitination.

tGnd1-GFP ubiquitination *in vitro* is reduced in the *ydj1*Δ (Fig. 3-2). This difference suggests stGnd1 requires slightly alternative machinery for efficient degradation. The lack of effect with *ydj1*Δ likely means one of the other 22 J domain-containing proteins in yeast is responsible for binding and stimulating Ssa activity in this instance [5].

Another major chaperone network involved in quality control is the Hsp90 complex. In yeast, there are two cytoplasmic homologues that provide Hsp90 activity, Hsp82 and Hsc82. The functions of both are redundant and the chaperone activity provided by them together is essential, so loss of function is studied with temperature sensitive mutants in a *hsp82*Δ*hsc82*Δ double null background. At the non-permissive temperature, all CQC substrates under investigation are stable in the absence of Hsp90 activity (Fig 3-3). Hsp90 activity has previously been shown to be involved in the degradation of VHL (L158P), the Von-Hippel-lindau tumor suppressor protein [7]. When expressed in yeast, it cannot fold, and it is dependent on both Hsp70 and Hsp90 activity for degradation. Also, VHL degradation requires Sse1, similar to CPY‡-GFP, tGnd1-GFP, and stGnd1. Given these similarities we hypothesized that Sti1, an Hsp70/90 cochaperone, that is required for VHL degradation, would also be required for degradation of our substrates. Sti1 is a tetratricopeptide repeat (TPR) domain containing protein. It use its TPR domains to interact with Hsp70 and Hsp90 via their own TPR domains, while stimulating Hsp70 client release and facilitating transfer to Hsp90. Surprisingly, in a *sti1*Δ*,* degradation of both CPY‡-GFP and tGnd1-GFP were

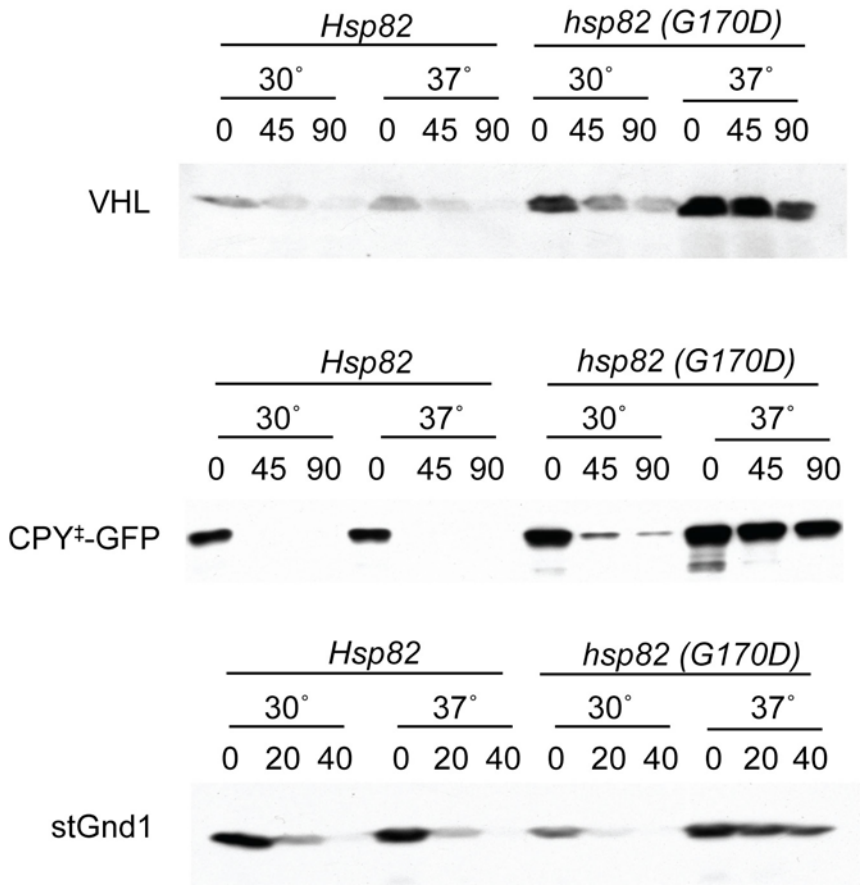

Figure 3-3: Hsp90 activity is required for degradation of CQC substrates.

Cycloheximide chase of strains that were *hsp82*Δ*hsc82*Δ with either a wild-type *Hsp82* or temperature sensitive *hsp82 (G170D)* mutant added back. Degradation of VHL (L158P), CPY‡-GFP, and stGnd1 was assessed at permissive (30˚C) and non-permissive (37˚) temperatures.

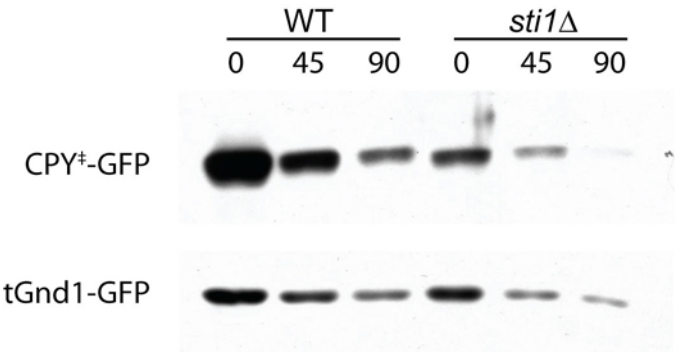

Figure 3-4: Sti1 is not involved in quality control degradation CPY‡-GFP or tGnd1-GFP. Cycloheximide chase of CPY‡-GFP and tGnd1-GFP in wild-type or *sti1*Δ strains.

unaffected (Fig 3-4). It is possible that other cochaperones can fulfill a similar Sti1 function, or perhaps Sse1 activity is able to suffice alone.

Chaperones triage QC substrates to be ubiquitinated and degraded by the 26S proteasome. We wanted to address at which point in chaperone action ubiquitination occurred. In genetic nulls affecting Hsp70 activity, sse1Δ, *ydj1*Δ, and *ssa1/2*Δ, ubiquitination is inhibited (Fig. 2-1, 9) indicating that ubiquitination is occurring after chaperone action. The canonical pathway for Hsp70/90 activity states that Hsp70 transfers client substrates to Hsp90, so we presumed that inactivation of Hsp90 activity would have a similar effect on ubiquitination. Since a straight null is not possible for Hsp90 activity, we used the TS alleles together with a cycloheximide chase to assay for the presence of ubiquitinated species. When cells expressing the Hsp82 (G170D) mutant are grown at the non-permissive temperature there is a strong decrement to degradation (Fig 3-5). This stabilization is not concomitant with a build-up in ubiquitinated substrate. Over the 1.5-hour time course, ubiquitinated tGnd1-GFP disappears in-line with that in wild-type cells. If Hsp90 activity was restricted to post-ubiquitination processes then we would have expected a build-up of ubiquitinated species, much like when the proteasome is inhibited (Fig 2-1). This result is consistent with the other chaperone null results and suggests that ubiquitination requires Hsp90 chaperone activity and occurs after chaperone binding to the target substrate.

The link between chaperones and ubiquitination machinery in quality control is an area of intense study. The best studied link is that of Hsp70 and

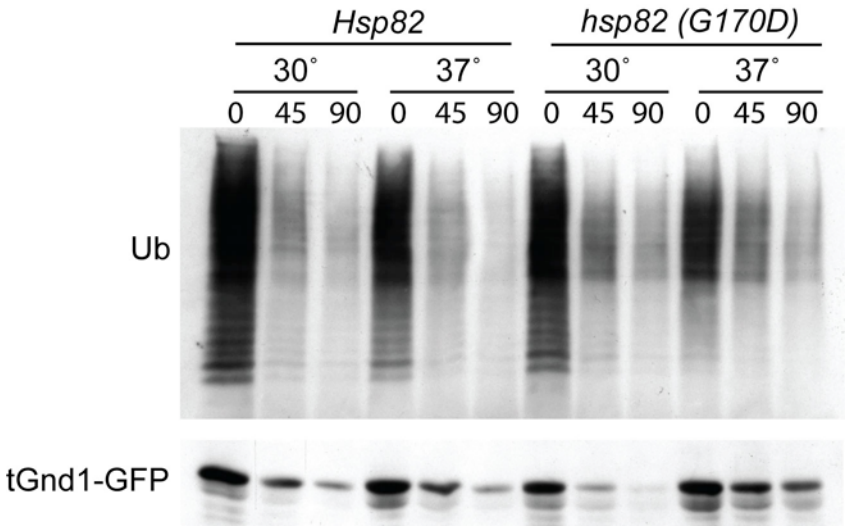

Figure 3-5: Hsp90 inhibition prevents substrate ubiquitination. Cycloheximide strains of strains that were *hsp82*Δ*hsc82*Δ with either a wild-type *Hsp82* or temperature sensitive *hsp82 (G170D)* mutant added back. Cultures were incubated at 30˚ or 37˚C for 1 hour prior to cycloheximide addition. tGnd1-GFP was immunoprecipitated with anti-GFP antibodies at each time point and analyzed for ubiquitination.

CHIP in humans. CHIP is an ubiquitin ligase that interacts with Hsp70 via TPR repeats and antagonizes Hsp70's J-domain stimulated ATPase, thus preventing Hsp70 client binding and shunting those clients down a proteolytic pathway. In Heck et al., it was shown that Ubr1 mediated ubiquitination of CQC substrates was dependent on the activity of chaperones. How Ubr1 is being recruited to these substrates is not known. One model is that chaperones are recruiting Ubr1 and perhaps providing a docking site by which Ubr1 can then ubiquitinate the unfolded substrate. Another model could be that chaperones are merely required to keep the misfolded protein soluble, by in which Ubr1 could then directly interact with the target substrate and ubiquitinate it. We set out to address the latter model by developing a novel *in vitro* ubiquitination assay that uses a misfolded substrate that is unable to be solubilized. We used an immunoglobulin molecule (IgG) that is covalently linked to agarose beads as a substrate for ubiquitination. Applying heat or Guanidine hydrochloride to denature the IgG resulted in significant ubiquitination over untreated IgG beads (Fig 3-6A, B). Furthermore, the ubiquitinated species is covalently attached to the agarose bead indicating that ubiquitination of the denatured IgG molecule is still occurring despite not being soluble. The insolubility of the denatured IgG is confirmed when DTT is absent from the final sample buffer; which prevents ubiquitinated IgG from being released into suspension, thus no ubiquitin signal is detected. However, when DTT is present in the sample buffer a robust ubiquitination signal is observed (Fig 3-6C).

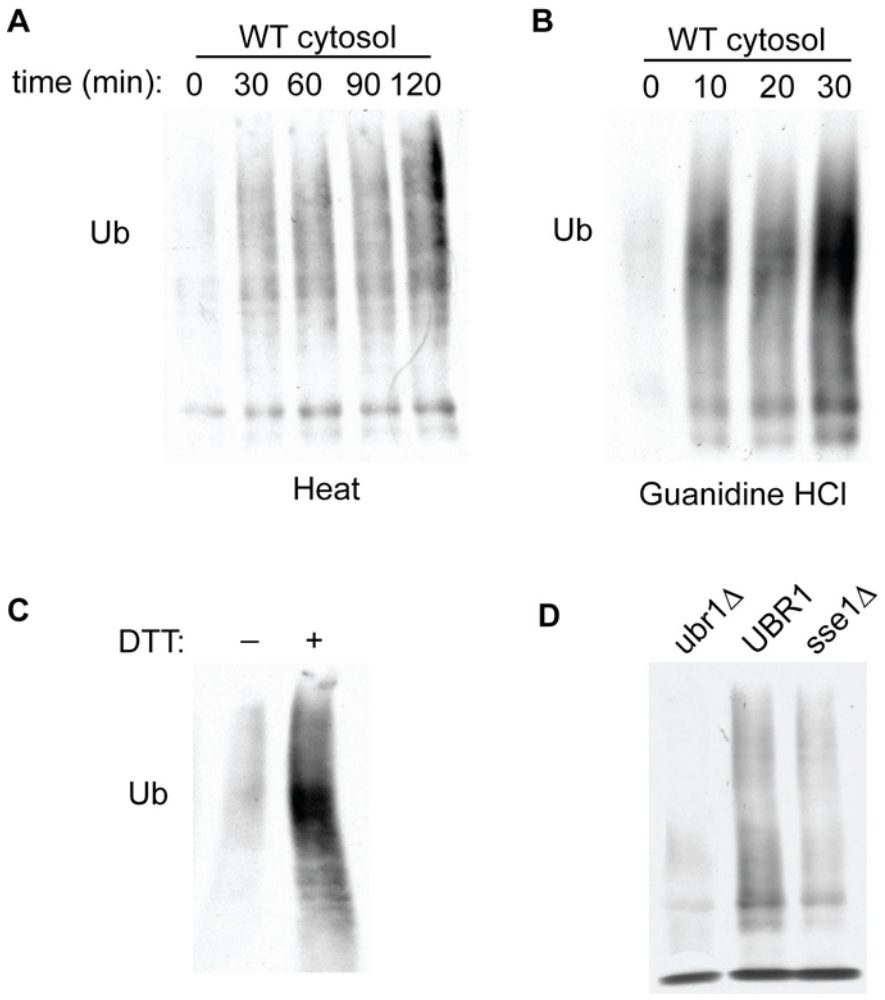

Figure 3-6: Characterization of solid state CQC ubiquitination assay. (A) IgG bound agarose beads were heated to 55˚C for the indicated time course, 0 to 120 minutes. 10uL aliquots of heated beads were removed and used for in vitro ubiquitination reactions consisting of wild-type cytsol and 10mM ATP. Ubiquitination was allowed to proceed for 1 hour. (B) IgG agarose beads were incubated in 6M guanidine HCL for the indicated times. In vitro ubiquitination was carried as in (A). (C) Ubiquitinated IgG is bead bound. In vitro ubiquitination of heated beads (2.5 hrs). Termination of reaction was done with sample buffer w/ or w/o DTT. (D) In vitro ubiquitination of heated beads using cytosol from *ubr1*Δ, *Ubr1*, *sse1*Δ.

Similar to the assay that uses substrate bound by beads in the in vitro ubiquitination assay published in Heck et al, ubiquitination of denatured IgG solid-state substrate is predominately carried out by cytosol containing Ubr1 (Fig3-6D).

This assay allowed us to ask whether the chaperones were required for this ubiquitination reaction to proceed. Cytosol extracted from *ydj11*Δ strains was unable to fully support ubiquitination of this solid-state substrate (Fig3-7A). This result indicates that chaperones need to be present in the cytosol for substrate recognition. A similar result was seen using cytosol from a *sse1*Δ (Fig 3-6D, 3-7B). Therefore, in order for efficient Ubr1-dependent ubiquitination to occur the Hsp70 cochaperones are required. Whether the role of these chaperones is needed for direct substrate binding and presentation or if there modulation of Hsp70 activity is the cause of this ubiquitination deficit is an open question.

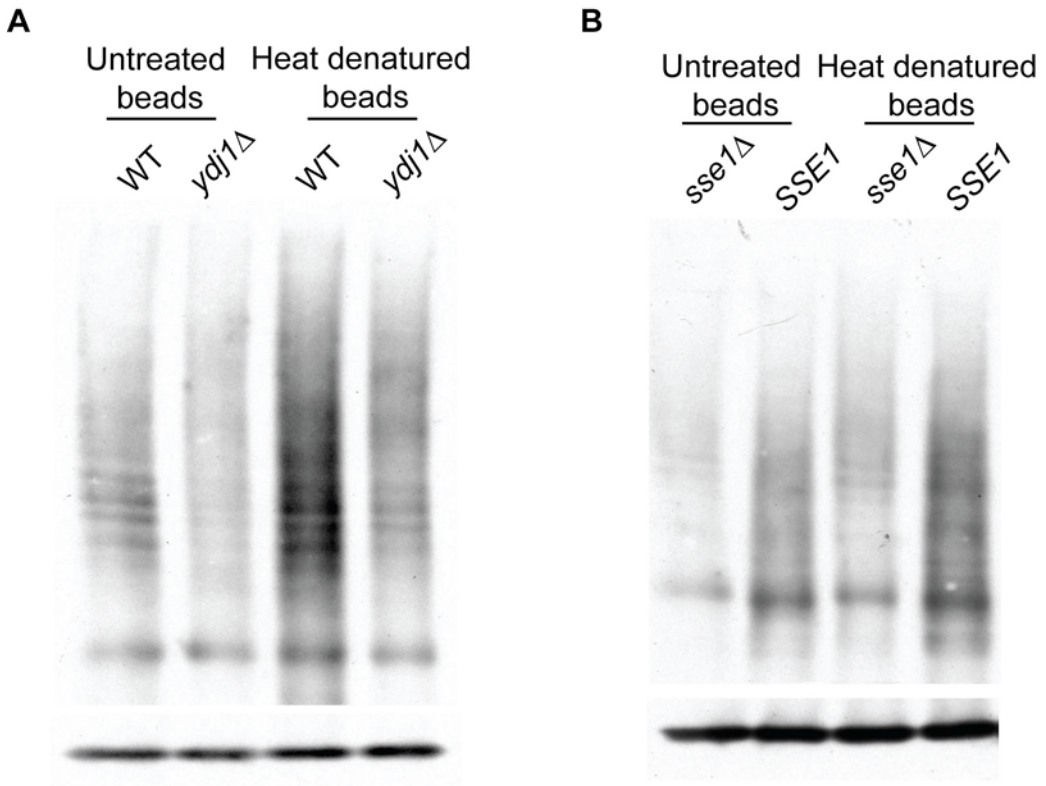

Figure 3-7: Chaperone dependent ubiquitination of insoluble CQC substrate. (A) In vitro ubiquitination of untreated and denatured immobilized IgG with wild-type or *ydj1*Δ cytosol. (B) In vitro ubiquitination of untreated and denatured immobilized IgG with wild-type or *sse1*Δ cytosol.

Discussion

The interplay between molecular chaperone function and that of proteolytic pathways is only beginning to be understood. The prototypical example of UPS components utilizing the chaperone networks for substrate selection is that of CHIP ligase ubiquitination of Hsp70 bound misfolded proteins [11, 12]. Here we have further characterized an additional intersection of UPS components with molecular chaperones. The model substrates CPY‡-GFP, tGnd1-GFP, and stGnd1 require Hsp70 activity for degradation by the UPS. This Hsp70 activity is aided by cochaperones known to modulate Hsp70s ATPase activity. In the absence of these cochaperones, significant decrements to ubiquitination of model substrates is observed. The precise role of these chaperones is an open question though. Both Ydj1 and Sse1 are known to directly bind Hsp70 clients. In some cases Ydj1 binds to the misfolded peptide prior to Hsp70, keeps it soluble and delivers them to Hsp70 [13, 14]. In other cases Ydj1 appears to not directly bind to the substrate, but only be required for stimulating ATP hydrolysis[14]. Sse1 is also postulated to bind misfolded substrates while bound to Hsp70 to help stabilize this interaction[10]. Interestingly, the peptide binding domain of Sse1 not only interacts with target substrates but also seems to have a role in stabilizing itself in order to promote nucleotide exchange on Hsp70. Our data is in agreement with a hypothesis that a majority of CQC degradation proceeds through the Hsp70 chaperone machine, and it is the cochaperones

of Hsp70 that are drivers of specificity that help regulate Hsp70s promiscuous nature.

Hsp90 activity is responsible for folding a subset of Hsp70 client proteins. Hsp90 activity also is required triaging peptides for degradation [7]. Yeast lacking Hsp82/Hsc82 activity are not able to degrade any of the CQC substrates analyzed. The route by which the Hsp90 network is coming into contact with these CQC substrates has yet to be determined. It is likely that the Hsp70 machine transfers the substrates to Hsp90 via the assistance of Sse1 to help stabilize the Hsp70/90 complex. The lack of a degradation phenotype in the *sti1*Δ was surprising given it known role to in promoting Hsp90 dependent degradation of the VHL CQC substrate [7], but there are additional cochaperones that may fulfill this role.

This work also established that the Hsp90 network was required for ubiquitination of target substrates, and was not merely performing a proteasome shuttle function. The ubiquitinated tGnd1-GFP was still degraded in the absence of Hsp90 and no further ubiquitination occurred despite an increase in substrate over the time course. This suggests that Ubr1 will be found in complex with Hsp90 when ubiquitination is occurring.

A pervading question concerning chaperone activity in quality control is to what extent are effects due to chaperone binding the result of keeping a substrate soluble versus presenting the substrate in a manner recognizable to the ligase. The solid-state assay we developed with immobilized denatured IgG attempted to address the presentation/solubilization debate by

determining the level of ubiquitination with or without chaperones present. Cytosols that lacked specific chaperones were unable to ubiquitinate immobilized substrate. This result demonstrates that chaperone activity is required beyond solubilization and is needed in some way to efficiently present the substrate to the ubiquitination machinery.

Materials and Methods

Yeast Strains and Plasmids. The *S. cerevisiae* strains used in this work are listed in Table S1. All strains and plasmids were constructed with standard molecular biology techniques, as described in [15, 16]. Yeast strains were cultured as described in minimal media with 2% dextrose and appropriate amino acid supplements, at 30°C unless otherwise indicated. The majority of strains used were in the BY4741 background, unless otherwise indicated in Table 3-1. Null alleles with coding regions replaced were constructed in the BY4741 background by transforming yeast using the LiOAc method with a PCR product encoding the indicated selection marker and 50bp flanks homologous to the gene to be disrupted (Baudin et al., 1993) or using knockout cassettes in the lab collection. Oligo sequences are available on request.

Degradation assays. Cycloheximide chase degradation assays were performed as previously described (Gardner et al., 1998). Briefly, yeast cells were grown to log phase (approx. OD600< 0.5) and cycloheximide was added to a final concentration of 50ug/ml. At the indicated time points cells were collected by centrifugation and lysed with 0.1ml SUME (1% SDS, 8 M urea, 10 mM MOPS, pH 6.8, 10mM EDTA) with protease inhibitors (260uM ABESF, 142uM TPCK, 100uM Leupeptin, 76uM Pepstatin) and 0.5 mm glass beads followed by vortexing for 2 minutes, followed by addition of 100ul 2x USB (75mM 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 3 min, and then clarified by centrifugation before separation by SDS-PAGE and subsequent immunoblotting with appropriate antibodies.

Degradation experiments involving temperature-sensitive mutants were carried out at 30˚C and 37˚C simultaneously. Pre-incubation of Ssa wildtype and mutant strains at 37˚C was carried out for 30 minutes prior to the addition of cycloheximide. Pre-incubation of Hsp82 wildtype and mutant strains at 37˚C was carried out for 1 hour prior to cycloheximide addition.

Cytoplasm preparation. Cytoplasm for in vitro assays was prepared from the respective genetic backgrounds using an approach modified from our in vitro ERAD assay (Garza et al., 2009): Cells were grown in YPD to an OD600 of 0.8-1.0, and 100 ODs of cells were pelleted. The pellet was washed 2x H2O, once with cold B88 buffer (20 mM HEPES–KOH, pH 7.4, 150 mM KOAc, 250 mM sorbitol, and 5 mM Mg(OAc)2) with protease inhibitors (260uM ABESF, 142uM TPCK, 100uM leupeptin, 76uM pepstatin) and DTT, and resuspension in 100 ul B88 buffer with PI and DTT for lysis by grinding in a mortar and pestle. The mortar and pestle were pre-cooled with liquid nitrogen before addition of the cells. The cells were added to the mortar with a 5 ml of liquid nitrogen. The frozen cells were ground by hand with the pestle. The cells were kept frozen during the process by addition of liquid nitrogen as needed. The ground cells were then placed in a 2 ml tube on ice, and allowed to thaw back to liquid. The resulting cytoplasm was clarified by centrifugation

at 5000 g at 4°C for 5 min. The supernatant was then transferred to a new tube and centrifuged again at 20000 g at 4°C for 15min. A final ultracentrifugation was carried out at 100000 g at 4°C for 60 min. Protein concentration of individual cytoplasms was determined using Bradford Reagent (Sigma-Aldrich). Cytoplasms were kept on ice until use.

In vitro ubiquitination assay. In vitro ubiquitination assays were adapted from [17]. Bead-bound immunoprecipitated substrate was mixed with the isolated cytoplasm from the indicated genetic background in the following way: All cytoplasmic reactions took place in a final volume of 50 ul and were prepared on ice. 150 mg of total protein from the respective cytoplasmic preparation was mixed with 15mM ATP, and 10 ul of FLAG beads bound to pre-IP substrate. The reactions were incubated in a 30°C water bath for 1 hour with periodic agitation. The reaction was terminated by adding 800 ul of IP buffer (15 mM sodium phosphate, 150 mM NaCL, 10 mM EDTA, 2% Trition X-100, 0.1% SDS, 0.5% deoxycholate) with protease inhibitors and 5mM N-ethylmaleimide. The FLAG beads were washed 3x with 1 ml IP wash buffer (50 mM NaCl, 10 mM Tris, pH 7.5), aspirated to dryness, and heated in the presence of sample buffer to 100°C for 3 min before SDS-PAGE and immunoblotting. Immunoprecipitation of substrate prior to in vitro ubiquitination experiments was conducted in the following manner: Strains lacking San1 and Ubr1 and contained FLAG-tGND1-GFP, were grown as described for cytoplasmic preparation above. 10ul of anti-FLAG M2 beads (Sigma Aldrich) were added per 150mg of cytosol, and allowed to nutate

overnight at 4°C. The beads were then pelleted at 1000 rpm for 1min. 3 washes with IP buffer were conducted before final resuspension in B88 reaction buffer.

Preparation of solid state substrate was done using IgG bound agarose beads (Sigma, A0919). Unless otherwise state: Beads were resuspended in B88 buffer, as used in the in vitro ubiquitination procedure above. Beads were then heated in a 55˚C water bath for 2.5 hours and then used immediately or stored at 4˚C for up to three weeks. Untreated controls were only resuspended in B88 buffer. 20uL of resuspended beads was usually used per reaction.

In vivo ubiquitination assay. In vivo ubiquitination of substrates was evaluated by immunoprecipitation (IP) followed by ubiquitin immunoblotting as described in [18]. To assess in vivo ubiquitination: After vortexing in the presence of beads and SUME, 1 ml of IP buffer with protease inhibitors and N-ethylmaleimide was added. The lysate was clarified by centrifugation at 14000 rpm for 5 min. The supernatant was transferred to a new tube and either polyclonal anti-GFP, anti-HA (Covance), or monoclonal anti-FLAG M2 beads (Sigma-Aldrich) were added depending on the substrate. The lysates were nutated overnight at 4°C. In the case of anti-GFP and anti-HA pulldowns, 100ul of protein A sepharose beads were then added and allowed to nutate for an additional 2 hours at 4°C. The beads were then spun down at 1000 rpm, and washed three times with IP wash buffer (50 mM NaCl, 10 mM

Tris, pH 7.5), and aspirated to dryness before addition of electrophoretic sample buffer.

Table 3-1: List of Yeast Strains used in Chapter 3

Name	Genotype	Source
BY4741	*MATa ura3Δ0 leu2Δ0 his3Δ1 met15Δ0*	Resgen Deletion Collection
RHY4622	JN516; *MATα ura3-52 leu2-3 his3-11, 15 trp1-D1 lys2 SSA1 ssa2::LEU2 ssa3::TRP1 ssa4::LYS2 (*P_{TDH3}*-CPY*‡*-GFP, ADE2 URA3)*	[6]
RHY4623	JB67; *MATα ura3-52 leu2-3 his3-11, 15 trp1-D1 lys2 ssa1::ssa1-45 ssa2::LEU2 ssa3::TRP1 ssa4::LYS2 (*P_{TDH3}-CPY‡-GFP, ADE2 URA3)	[6]
RHY6336	BY4741 pRH2081 *(*P_{TDH3}*-CPY*‡*-GFP, URA3)*	[6]
RHY6337	BY4741 *sti1Δ::KanMX* pRH2081 *(*P_{TDH3}*-CPY*‡*-GFP, ADE2 URA3)*	this study
RHY6338	BY4741 *sse1Δ::KanMX* pRH2081 *(*P_{TDH3}*-CPY*‡*-GFP, ADE2 URA3)*	[6]
RHY7447	BY4741	[6]
RHY7448	BY4741 *san1Δ::NatMX*	[6]
RHY7449	BY4741 *ubr1Δ::KanMX*	[6]
RHY7450	BY4741 *san1Δ::NatMX ubr1Δ::KanMX*	[6]
RHY7782	RHY7447 pRH2486 (P_{TDH3}-FLAG-tGnd1-GFP, ADE2 URA3)	[6]
RHY7783	RHY7448 pRH2486 (P_{TDH3}-FLAG-tGnd1-GFP, ADE2 URA3)	[6]
RHY7784	RHY7449 pRH2486 (P_{TDH3}-FLAG-tGnd1-GFP, ADE2 URA3)	[6]
RHY7785	RHY7450 pRH2486 (P_{TDH3}-FLAG-tGnd1-GFP, ADE2 URA3)	[6]
RHY7878	BY4741 sse1Δ::KanMX san1Δ::NatMx	[6]
RHY8075	BY4741 sse1Δ::KanMX	Resgen Deletion Collection
RHY8734	JN516; *MATα ura3-52 leu2-3 his3-11, 15 trp1-D1 lys2 SSA1 ssa2::LEU2 ssa3::TRP1 ssa4::LYS2* pRH2525 (P_{TDH3}-3xHA-stGnd1, URA3)	this study
RHY8735	JB67; *MATα ura3-52 leu2-3 his3-11, 15 trp1-D1 lys2 ssa1::ssa1-45 ssa2::LEU2 ssa3::TRP1 ssa4::LYS2* pRH2525 (P_{TDH3}-3xHA-stGnd1, URA3)	this study

Table 3-1: continued

RHY8736	RHY2804 pRH2486 (P_{TDH3}-FLAG-tGnd1-GFP, ADE2 URA3)	this study
RHY8737	RHY2805 pRH2486 (P_{TDH3}-FLAG-tGnd1-GFP, ADE2 URA3)	this study
RHY8738	BY4741 *ydj1Δ::LEU2* pRH2525 (P_{TDH3}-3xHA-stGnd1, *URA3*)	this study
RHY8739	RHY7447 pRH2525 (P_{TDH3}-3xHA-stGnd1, URA3)	this study
RHY8742	W303 Wild-type (P_{TDH3}-3xHA-stGnd1, URA3)	this study
RHY8743	W303 pRH2525 (P_{TDH3}-3xHA-stGnd1, URA3)	this study
RHY8744	*ade2-1 MET2 LYS2 ura3-1 trp1-1 leu2-3,112 his3Δ hsc82Δ::LEU2 hsp82::LEU2 HSP82::HIS3* (P_{TDH3}-3xHA-stGnd1, URA3)	this study
RHY8745	*ade2-1 MET2 LYS2 ura3-1 trp1-1 leu2-3,112 his3Δ hsc82Δ::LEU2 hsp82::LEU2 HSP82(G170D)::HIS3* (P_{TDH3}-3xHA-stGnd1, URA3)	this study
RHY8732	*ade2-1 MET2 LYS2 ura3-1 trp1-1 leu2-3,112 his3Δ hsc82Δ::LEU2 hsp82::LEU2 HSP82::HIS3* (P_{TDH3}-FLAG-tGnd1-GFP, ADE2 URA3)	this study
RHY8733	*ade2-1 MET2 LYS2 ura3-1 trp1-1 leu2-3,112 his3Δ hsc82Δ::LEU2 hsp82::LEU2 HSP82(G170D)::HIS3* (P_{TDH3}-FLAG-tGnd1-GFP, ADE2 URA3)	this study
RHY8756	*ade2-1 MET2 LYS2 ura3-1 trp1-1 leu2-3,112 his3Δ hsc82Δ::LEU2 hsp82::LEU2 HSP82::HIS3 (*P_{TDH3}*-CPY‡-GFP, ADE2 URA3)*	this study
RHY8757	*ade2-1 MET2 LYS2 ura3-1 trp1-1 leu2-3,112 his3Δ hsc82Δ::LEU2 hsp82::LEU2 HSP82(G170D)::HIS3 (*P_{TDH3}*-CPY‡-GFP, ADE2 URA3)*	this study
RHY8674	*ade2-1 MET2 LYS2 ura3-1 trp1-1 leu2-3,112 his3Δ hsc82Δ::LEU2 hsp82::LEU2 HSP82::HIS3*	this study
RHY8675	*ade2-1 MET2 LYS2 ura3-1 trp1-1 leu2-3,112 his3Δ hsc82Δ::LEU2 hsp82::LEU2 HSP82(G170D)::HIS3*	this study

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