Characterizing Cytosolic Protein Quality Control Pathways in *Saccharomyces cerevisiae* Using Temperature-Sensitive Model Substrates

by

Farzin Khosrow-Khavar

B.Sc. University of Victoria (2006)

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in

The Faculty of Graduate Studies

(Biochemistry and Molecular Biology)

THE UNIVERSITY OF BRITISH COLUMBIA

(Vancouver)

March 2011

Abstract

A large pool of proteins localizes in the cytosol of eukaryotic cells. Proteins in the cell can be misfolded due to cellular stress, mutations, and transcriptional and translational errors. Several E3 ubiquitin ligases have been shown to target misfolded cytosolic proteins for degradation by the proteasome. In this study, we characterized a panel of thermosensitive mutant proteins in *Saccharomyces cerevisiae* from six essential genes. The wild-type alleles of these thermosensitive proteins are stable at restrictive temperature. In contrast, we found that roughly half the tested alleles are significantly degraded at non-permissive temperature in a proteasome-dependent manner. These unstable alleles thus display hallmarks of protein quality control substrates. We found that degradation of mutant protein Pro3-1p, one of the unstable alleles, is dependent on the dual action of Ubr1 and San1 ubiquitin ligases. The single deletions of the ligases do not affect the stability of Pro3-1p. In contrast, double deletion of *UBR1* and *SAN1* leads to significant stabilization of the mutant protein. The increase in stability of Pro3-1 is associated with suppression of thermosensitive phenotype at restrictive temperature, suggesting that the depletion of the essential protein due to its degradation leads to the loss of viability at restrictive temperature.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

AMP	Adenosine monophosphate
ASF	Anti-silencing function
ATP	Adenosine triphosphate
BiP	Immunoglobulin heavy chain binding protein
CAN1	Canavanine resistance
CDC	Cell cycle division
CHIP	Carboxy terminal of hsp70 interacting protein
CLpP	Caseionolytic peptidase
CPY	Vacuolar carboxypeptidase Y
CUE1	Coupling of ubiquitin conjugation to endoplasmic reticulum degradation 1
DER1	Degradation of endoplasmic reticulum 1
DMSO	Dimethyl sulfoxide
DOA10	Degradation of matα2-10
E1	Enzyme 1 (ubiquitin activating enzyme)
E2	Enzyme 2 (ubiquitin conjugating enzyme)
E3	Enzyme 3 (ubiquitin Ligases)
EDTA	Ethylenediaminetetraacetic acid
ELA1	Elongin A
ER	Endoplasmic reticuluum
ERAD	Endoplasmic reticuluum associated degradation

ERDJ1	Endoplasmic reticulum dna J-1
GFP	Green fluorescent protein
GLN1	Glutamine synthetase 1
GRS1	Glycyl-tRNA synthetase 1
GUK1	Guanylate kinase 1
GUS1	Glutamyl-tRNA synthetase 1
HECT	Homologous to E6AP c-terminus
HEPES	(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRD1	HMG-CoA reductase degradation 1
HSP	Heat shock protein
HUL4	Hect ubiquitin ligase 4
HUL5	Hect ubiquitin ligase 5
KAR2	Karyogamy 2
LYP1	Lysine permease 1
NaCl	Sodium chloride
NES	Nuclear export signal
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain Reaction
PIM1	Proteolysis in mitochondria 1
PrA	Vacuolar proteinase A

PRO3	Delta 1-pyrroline 5-carboxyate reductase
QC	Quality control
RING	Really interesting new gene
RKR1	RING domain mutant killed by rtf1 deletion
RTT101	Regulator of ty1 transposition 1
SAN1	Sir antagonist 1
SDS	Sodium dodecyl sulphate
SIR3	Silent information regulator 3
SIR4	Silent information regulator 4
SS	Signal sequence
SSE1	ATPase that is a component of the heat shock protein hsp90
STI1	Stress inducible 1
Tri-CCT	TCP1-ring complex of chaperone containing TCP1
TS (ts)	Thermosensitive
TCL	Total cell lysate
UBC	Ubiquitin conjugating
UBL	Ubiquitin-like
UBR1	Ubiquitin n-recognin 1
UBR2	Ubiquitin n-recognin 2
UBX2	Ubiquitin regulatory X 2
UDP	Uridine 5-diphosphate

UTP	Uridine 5-triphosphate
UFD4	Ubiquitin fusion degradation protein 4
UFO1	UV f-box HO
UGP1	UDP-glucose pyrophosphorylase
UPS	Ubiquitin proteasome system
URA3	Uracil requiring 3
VHL	Von hippel lindau

Acknowledgements

Firstly, I would like to thank my supervisor, Dr. Thibault Mayor, for his guidance, support, and scientific insight throughout my studies. In addition to scientific mentorship, I would like to thank my supervisor for instilling the value of hard-work, persistence, and integrity. I would like to thank the past and present member of the Mayor Laboratory for assistance with techniques but also for support and encouragement. My time in the laboratory has been memorable and I would like to thank you all for your friendship. I would also like to thank Dr. Leonard Foster, Dr. Vivien Measday, and Dr. Leanne Howe for partaking in my committee and providing valuable insight during my studies.

I would like to acknowledge Dr. Philip Hieter and member of the Hieter lab, specifically Jan Stoepel and Megan Kofoed for providing us with strains, plasmids, and equipment vital to this project. I would like to thank Jan for enthusiastic dialogue and input on my project. I would like to thank Dr. Chris Loewen and Dr. Barry Young for assistance with the screens and analysis of the data.

I would also like to thank my family for support and encouragement. I would like to thank my siblings for being the best brother and sister I could ask for. I would also to specially thank and acknowledge my parents for being great role models and their unconditional love and support.

Chapter 1: Introduction

The eukaryotic cell has developed critical protein quality control (QC) pathways to reduce the pool of misfolded and/or damaged proteins [1]. When proteins are synthesized, they are folded to their three dimensional conformation. Proteins must maintain their native conformation to remain functional [2]. Proteins can become misfolded due to mutations, transcriptional and translational errors, and cellular stresses such as oxidative and heat-shock stresses. Aberrant proteins can either be refolded back to their native conformation or targeted for proteolysis. Failure to refold or remove these aberrant proteins can lead to the formation of oligomers or aggregates that are associated with human proteopathies such as Parkinson's and Huntington disease [3,4].

1.1 Molecular Chaperones and Protein Quality Control

One prominent protein QC mechanism is the folding of misfolded polypeptides to their native conformation by molecular chaperones to prevent their degradation. Refolding of misfolded proteins is economically advantageous to the cell as protein synthesis is high in energy expenditure. Thus, the cell has adopted mechanisms to maintain the native conformation of proteins through molecular chaperones. Molecular chaperones are localized in every cell compartment where proteins are either synthesized or imported. Molecular chaperones belong to diverse families which includes Hsp40, Hsp60, Hsp70, Hsp90, Hsp100, and small heat shock proteins [1].

The chaperones Hsp70 (Heat-shock protein 70) and Hsp90 are ATP-dependent chaperones and recognize a large number of substrates in the cytoplasm and endoplasmic reticulum. Hsp70 interacts with a diverse array of substrates including misfolded and aggregated proteins through stretches of peptides with hydrophobic or basic residues. Hsp70 binding to substrates is facilitated by Hsp40 and the nucleotide exchange factor Hsp110 [1,5]. Hsp40 triggers ATP hydrolysis by Hsp70 whereas Hsp110 mediates release of ADP and rebinding to ATP. Controlled cycles of ATP hydrolysis and release have been associated with disaggregation of proteins [6]. Hsp60 chaperonin is a member of TRiC-CCT (TCP1-ring complex of chaperone containing TCP1), which is a barrell shape protein complex that provides a folding environment [7]. Hsp60 typically targets subunits of oligomeric complexes with slow folding kinetics [1,8]. In addition, small heat-shock proteins (e.g.,Hsp27) are ATP-independent chaperones that prevent aggregation of misfolded proteins or loosen aggregates, allowing Hsp70 and Hsp100 to refold the proteins [1,9,10].

In addition to assisting refolding, there is also mounting evidence that chaperone proteins can also promote the turnover of misfolded proteins via degradative quality control pathways. For instance, the yeast Sti1 and Sse1 Hsp70 co-factors, are required for the degradation of the misfolded VHL (Von Hippel Lindau) model substrate, but are dispensable for the folding of the protein [11]. It is thought that chaperone proteins can mediate the recognition of misfolded proteins and facilitate their degradation by the ubiquitin proteasome system.

1.2 Ubiquitin Proteasome System

The ubiquitin proteasome system (UPS) plays a major role in degradative QC. In this pathway, misfolded proteins are first conjugated to poly-ubiquitin chains, and then recognized by specific adaptor proteins and receptors that facilitate degradation by the proteasome [12]. Protein ubiquitylation is an ATP driven process catalyzed by a cascade of enzymes: E1 (ubiquitin activating enzyme), E2 (ubiquitin conjugating enzyme), and E3 (ubiquitin ligases). E1 activates the 76 amino acid ubiquitin by hydrolyzing ATP to adenylate ubiquitin at its C-terminus, forming a high-energy anhydride bond (figure 1). The sulfhydryl group of a cysteine residue in the active site of E1 subsequently makes a nucleophilic attack on the anhydride bond, leading to formation of thioester linkage and expelling AMP. The activated ubiquitin is transferred to a cysteine residue in the catalytic active site of E2 ubiquitin conjugating enzymes [13-15]. E3 ligases typically facilitate the conjugation of ubiquitin on substrate lysine residues, resulting in the formation of covalent isopeptide linkage of ubiquitin's C-terminus and the Ɛ-amino group of a lysine in a substrate [16]. E3 ubiquitin ligases are involved in substrate recognition and confer specificity. Ubiquitylation is a reversible process and polyubiquitin chains can be cleaved by proteases known as deubiquitylating enzymes [17].

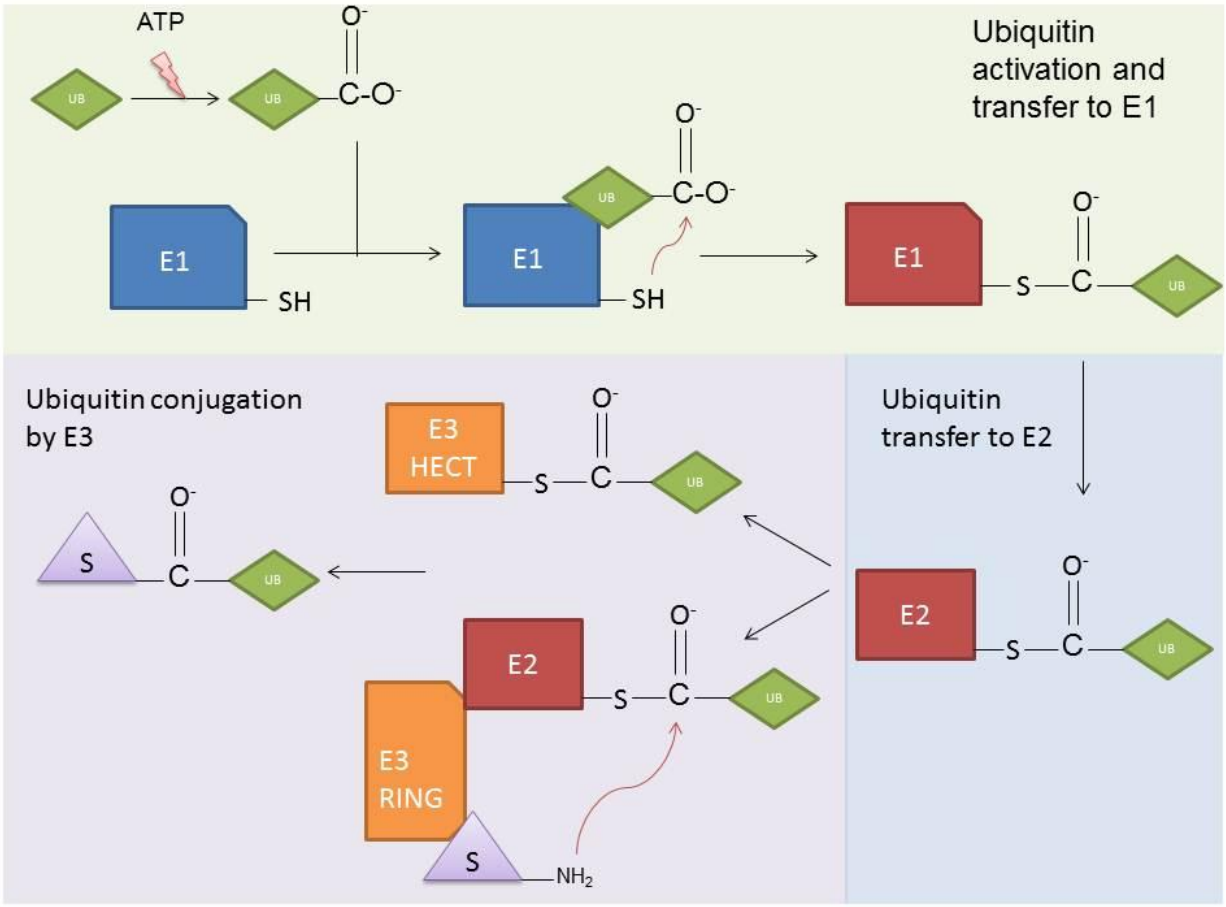


Figure 1: Ubiquitin conjugation pathway. Ubiquitin is activated by E1 (ubiquitin activating enzyme) using ATP as a source of energy. The C-terminus of ubiquitin is adenylated which forms a high energy intermediate. The cysteine residue of the active site of E1 makes a nucleophilic attack and forms a thioester linkage with ubiquitin. Ubiquitin is transferred to an E2 conjugating enzyme by transthiolation. E3 ligases facilitate the binding of ubiquitin to the target lysine residue on the substrate by conferring substrate specificity. RING-E3 ligases simultaneously bind both the substrate and the E2-ubiquitin complex. This brings the substrate lysine residue in close proximity to the E2 active site and facilitates the transfer of ubiquitin to the substrate. HECT ligases first bind ubiquitin and subsequently facilitate the direct transfer of ubiquitin to the substrate [14].

There are more than 90 genes encoding for putative or known E3 ubiquitin ligases in *Saccharomyces cerevisiae.* In mammalian cells, more than 600 genes have been identified that encode for E3 ligases [18]. The E3 ligases target a diverse array of substrates in the cell and play a key role in regulation of diverse cellular processes including DNA repair, cellular trafficking, chromatin remodelling, immune responses and cell cycle progression. E3 ligases are classified into two major classes based on the mechanism by which they facilitate the transfer of ubiquitin from the E2 to the lysine residue on a substrate. RING (Really Interesting New Gene) -type ligases consist of a subunit or domain with a RING motif, which coordinates a pair of zinc ions and recruits the E2 conjugating enzyme [14]. The substrate, recruited by another subunit or domain, is positioned such that a lysine residue on the substrate is in close proximity to the E2 conjugating enzymes. The lysine residue can make a nucleophilic attack on the E2-ubiquitin thioester bond, facilitating the conjugation of ubiquitin on target lysine residues. The second class is made of HECT (Homologous to E6AP C-Terminus) ubiquitin ligases that form a catalytic intermediate with ubiquitin on a conserved cysteine residue (in the HECT domain) and mediate direct covalent attachment of ubiquitin on the substrate [14].

Ubiquitin is synthesized as a precursor and is processed by deubiquitylating protease enzymes to generate a free diglycine motif at the C-terminus, which can be conjugated to target lysine residues of substrates [14,17]. Ubiquitin molecules can also be covalently linked together to form polyubiquitin chains. Chains of ubiquitin are assembled through an isopeptide linkage of the C-terminus of distal ubiquitin and the Ɛ-amino group of ubiquitin lysine. Ubiquitin is an evolutionary conserved 76 amino-acid

long polypeptide and contains seven lysine residues: lysine 6, lysine 11, lysine 27, lysine 29, lysine 33, lysine 48, and lysine 63 [19,20]. All seven residues can be used for isopeptide bond formation (figure 2). The best-characterized residues implicated in polyubiquitin chain formation are lysine 48 and lysine 63. Formation of chains of four or more ubiquitin chains linked through lysine 48 residues is associated with proteasomal degradation of substrates whereas lysine 63 linked polyubiquitin chain is associated with regulation of cellular processes such as DNA repair, endocytosis, and lysosomal degradation [19,21]. Similar to lysine 63 modification, mono-ubiquitylation of proteins is implicated in endocytosis and chromatin remodelling. In addition to ubiquitin, there are a several ubiquitin-like proteins that utilize a similar enzyme cascade as the ubiquitin system. Similar to ubiquitin, attachment of these modifiers has been shown to regulate a diverse array of processes within the cell [22].

The proteasome is a large complex composed of over 30 different proteins [23]. To be recognized and degraded by the proteasome, a substrate-linked ubiquitin chain must contain at least four ubiquitins and typically assembled through lysine 48 [24]. Other linkages (e.g. K11 or K29) have also been shown to target the conjugate for degradation [25]. In contrast, K63-linked ubiquitin chains are not efficiently degraded by the proteasome, because they are rapidly de-ubiquitylated at the proteasome [26]. After binding to the poly-ubiquitylated substrates, the proteasome cleaves off and releases ubiquitin in the cell before unfolding and translocating the substrates into the catabolic chamber for degradation. A large fraction of the proteasome substrates consists of misfolded and nonfunctional polypeptides. Thus, the proteasome is cardinal to cell homeostasis.

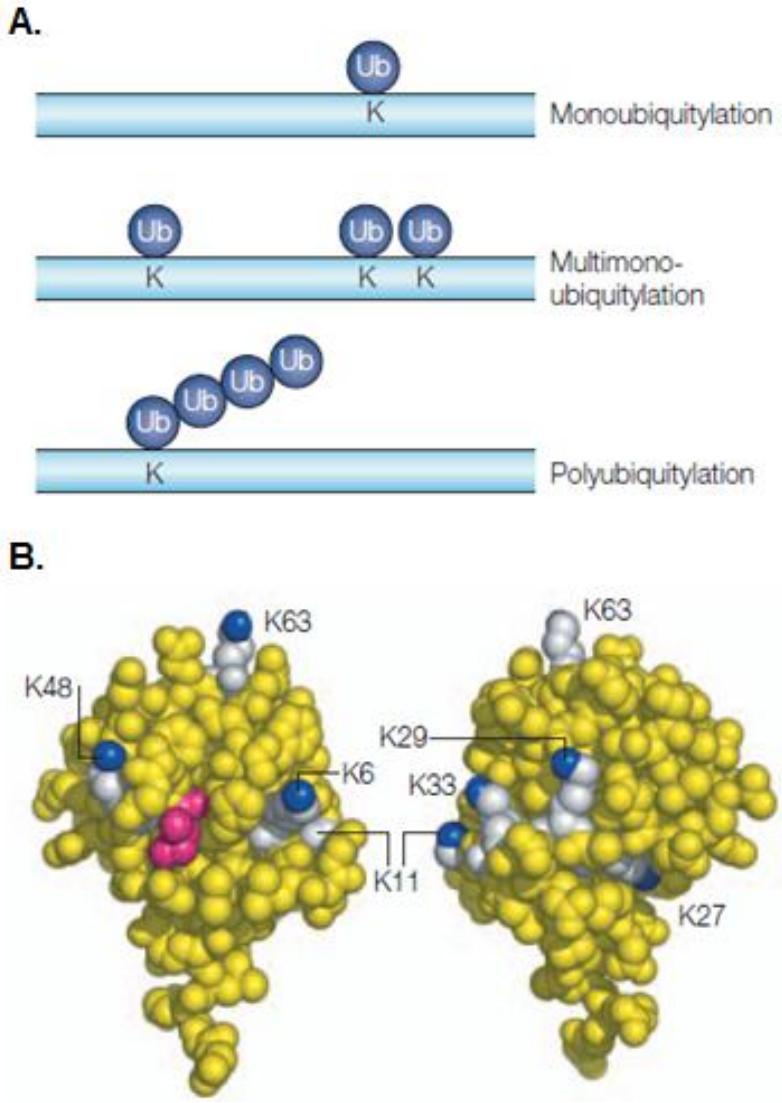


Figure 2. Ubiquitin modification linkages and sites. A. Ubiquitin can be conjugated as monomers, multi-monomer, or polyubiquitin chains on target substrates. Mono-ubiquitylation of substrates has been associated with endocytosis, chromatin remodelling, meiosis, and lysosomal degradation. **B**. Ubiquitin chains can be linked through seven lysine residues (K6, K11, K27, K29, K33, K48, and K63). Lys48 and Lys63 linked chains are the best characterized. Conjugation of chains of four or more ubiquitin molecules linked through Lys48 is associated with proteasome degradation. Lys63-linked polyubiquitin chains have been implicated in regulation of diverse biological processes such as DNA repair, endocytosis, and translation. Figure adapted with permission from Nat Rev Mol Cell Biol. [27].

1.3 Protein QC in Endoplasmic Reticulum

A major role of the ubiquitin proteasome system is to target and degrade misfolded proteins. Degradative protein quality control has been best characterized in the endoplasmic reticulum (ER) [28,29]. When nascent proteins are translocated into the ER, they are modified cotranslationally with a $glucose_3$-$mannose_9$-N-$acetylglucosmaine_2$ glycosylation tag [28,30]. Removal of two terminal glucose residues leads to the recognition and folding of the intermediate polypeptides by lectins, calnexin and calreticulin chaperones. After correct folding, the removal of the last glucose moiety marks the glycoprotein for ER exit [28,30].

When folding by calnexin and calreticulin fails and the polypeptides cannot be folded, two mannose residues of the glycoproteins are trimmed to target them for degradation by a process known as ER associated degradation (ERAD). For instance, it has been previously shown that a mutant form of the enzyme vacuolar carboxypeptidase Y (CPY*), which cannot fold, is retained in the ER and degraded in a proteasome-dependent manner, whereas the wild-type form of the enzyme exits the ER for the secretory pathway after maturation [31,32]. In ERAD, ER-resident proteins are first retrotranslocated in the cytoplasm because there is no ubiquitin and ubiquitin-related enzymes that localize within the ER. In yeast, after retrotranslocation, misfolded ER-proteins are polyubiquitylated by either Doa10 (degradation of Mat-α2-10) or Hrd1 (Hmg-Coa Reductase degradation) [28,30]. Doa10 was identified in a screen for degradation of soluble transcriptional repressor Matα2. Doa10 is composed of 14 transmembrane domains and an N-terminal RING finger domain. Doa10 has been shown to target misfolded proteins with lesions in their cytosolic domains (known as

ERAD-C), and polyubiquitylates the substrates in association with E2 conjugating enzymes Ubc6 and Ubc7 [33,34]. Hrd1 is an E3 ligase with six transmembrane domains and is implicated in polyubiquitylation of substrates with lesions in transmembrane or luminal domains (known as ERAD-M and ERAD-L) [33]. Degradation of these misfolded proteins by Hrd1 is dependent on the Ubc1 and Ubc7 E2s, which are recruited to the ER membrane by interaction with Cue1 (Coupling of ubiquitin conjugation to ER degradation 1) [28,30,33].

The Hrd complex is also associated with Ubx2 (ubiquitin regulatory X), Usa1 (U1-Snp1 associating-1), and Der1. Der1 is a homologue of mammalian Derlin-1 [35]. Der1 is implicated as part of a protein complex that interacts with a diverse array of misfolded proteins and facilitates the translocation of misfolded proteins across the ER membrane [36,37]. It has been proposed that the Sec61 channel mediates retrotranslocation of misfolded ERAD-L substrates and non-glycosylated short-lived transmembrane substrates [38,39]. As misfolded substrates are retrotranslocated across the ER membrane and polyubiquitylated, they are also further extracted from the ER by the AAA+ ATPase Cdc48 complex and subsequently delivered by adaptor proteins to the proteasome for degradation.

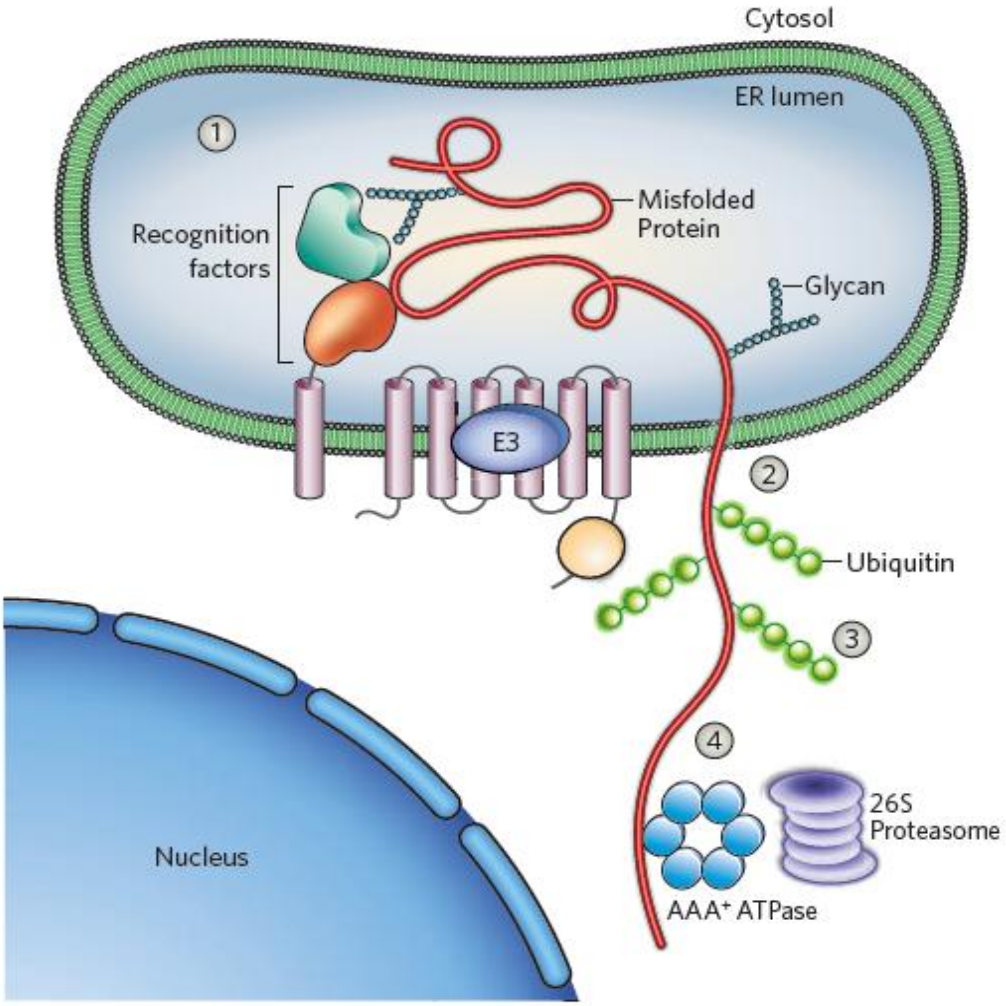


Figure 3. Endoplasmic Reticulum Associated Degradation. Degradation of nascently misfolded proteins within the ER occurs in multiple stages. **1)** Misfolded proteins are recognized by ancillary factors and E3 ligases Hrd1 and Doa10 that are embedded in the ER. **2)** Misfolded proteins are translocated across the ER membrane through channels. It has been proposed that Der1 and ancillary factors provide a channel where misfolded proteins are retranslocated to the cytosol. In addition, the Sec61 translocon has been implicated in displacement of misfolded proteins from the ER. **3)** At the cytosolic surface of the ER, misfolded proteins are ubiquitylated by E3 ligases Hrd1 when substrates have lesions in luminal or transmembrane domains or Doa10 if substrates have lesions in cytosolic domains. **4)** Misfolded proteins are extracted by from the ER membrane by the AAA+ ATPase Cdc48 and guided to the proteasome for degradation. Figure adapted with permission from Nature Insight [28].

The ER protein quality control of misfolded non-glycosylated proteins has been less studied but shown to occur independent of lectins. This process has been shown to be dependent on the chaperones Kar2/ BiP, Hsp70, and ERDj family of proteins that recognize the misfolded protein and target them for retrotranslocation. Previous studies have shown that Kar2 mutants display defects in degradation of CPY* [40]. Similarly Kar2 mutants inhibit *in vitro* degradation of unglycosylated pro-α-factor [41]. In mammalian cells, the fully oxidized form of immunoglobulin light chain (IgκLC) is not transported into the cytosol. BiP is shown to interact with partially oxidized IgκLC and facilitates retrotranslocation, prior ubiquitylation by Hrd1 [42,43]. In mammalian cells, the ERDj family of proteins hydrolyzes ATP and induces binding of Hsp70 to misfolded proteins. When the yeast homologues of ERDj (Jem1 and Scj1) are deleted, the degradation of non-glycosylated CYP* and pro-α-factor is impaired and the proteins aggregate [44].

1.4 Protein QC in Mitochondria

Mitochondria are double membrane organelles that house at least 700 different proteins. The majority of mitochondrial proteins are synthesized by cytosolic ribosomes and preproteins which contain mitochondrial matrix targeting sequences and are transported through the translocon into the mitochondrial matrix. Cellular stress such as oxidative stress and heat-shock stress can affect the folding properties of newly synthesized proteins and proteins imported into the mitochondria [45]. As for the ER, this organelle is lacking ubiquitin and ubiquitin related enzymes. However, in contrast to the ERAD pathway, misfolded proteins in the eukaryotic mitochondria are degraded in the organelle by the AAA+ proteases Pim1 and mtClpP.

Pim1 contains dual proteolytic and ATPase activity. Previous studies have shown that Pim1 in association with Ssc1 mediates the degradation of imported artificial substrates [46]. In yeast, Pim1-mediated substrate recognition is mediated by Hsp78 [47]. Pim1 substrates have been shown to contain unstructured protein sequences of at least 50 amino acids and internal initiation sites [48]. In an independent study, a comparison of wild-type and *pim1Δ* proteome has revealed that the substrates of Pim1 are proteins that contain prosthetic groups and Fe/S clusters [49]. Pim1 has also been shown to target unstable subunits of oligomeric complexes [49]. The second protease active in the mitochondrial matrix is mtClpP. This protease forms a complex with mtClpX that confers ATPase activity. In addition, there are also AAA+ proteases present in the inner mitochondrial membrane, facing either the matrix (m-AAA-protease) or the intermembrane space (i-AAA-protease), which degrade membrane integrated polypeptides [45].

1.5 Degradative Protein Quality Control in Nucleus

A degradative protein quality control pathway has also been found in the nucleus. There are a large number of proteins that reside in the nuclei and misfolding of these proteins can lead to diseases; e.g., accumulation of the nuclear misfolded protein huntingtin is associated with Huntington's disease [50]. The San1 E3 ligase has been shown to specifically target nuclear misfolded proteins in *Saccharomyces cerevisiae.* San1 was shown to act in conjunction with Ubc1 and Cdc34 E2 enzymes to polyubiquitylate misfolded mutant nuclear proteins like Cdc68-1p, Sir4-9p, Cdc13-1p and Sir3-8p [51]. These alleles were previously shown to be unstable at high temperature, presumably due to misfolding. In addition, San1 has been shown to target

the Asf-1 mutant nuclear protein in association with E2 conjugating enzyme Ubc4 in *Saccharomyces pombe*, suggesting that this pathway is conserved in several organisms. Interestingly, a recent study demonstrated that San1 can directly recognize misfolded substrates through disordered N-terminal and C-terminal domains present in the E3 ligase [52,53]. The substrate-recognition domains along with the disordered regions of San1 may allow for flexibility to recognize and ubiquitylate a diverse array of substrates. As discussed below, misfolded proteins that have escaped cytosolic QC can also enter the nucleus and be targeted by San1. Whether this pathway is conserved in higher eukaryotes remains to be determined.

1.6 Protein QC in Cytoplasm

The majority of proteins localize in the cytoplasm, and several E3 ligases have been shown to target cytosolic misfolded proteins. In mammalian cells, CHIP (Carboxyl terminal of Hsc70 Interacting Protein) is a U-box domain (similar to the RING domain) ubiquitin ligase implicated in cytosolic QC. CHIP is shown to interact with Hsp70 and Hsp90 and target the chaperone substrates for degradation [54,55,55,56]. The cytosolic QC system has also been investigated in *Saccharomyces cerevisiae,* in which CHIP is not conserved. Two E2 conjugating enzymes Ubc4 and Ubc5 have been implicated in targeting misfolded proteins in the cytosol. Ubc4 and Ubc5 are heat-inducible E2 conjugating enzymes that interact with the proteasome and are a central component of cellular stress response [57-59]. They have overlapping functions and mediate degradation of short-lived and abnormal proteins [58,59]. In a recent study, Ubc4 and 5 have been shown to act in conjunction to target short-live misfolded proteins in the cytosol [60]. Double deletion of Ubc4 and Ubc5 was shown to eliminate heat-shock

induced polyubiquitylation of misfolded proteins whereas the single deletion does not have a significant effect. It is likely that Ubc4 and Ubc5 act in a redundant manner to target misfolded proteins in the cytosol [60].

The N-end rule ligase Ubr1 targets misfolded proteins in the cytoplasm in combination with other ubiquitin ligases [61-64]. Ubr1 is a 225-KDa RING-type E3 ligase that mediates the degradation of substrates with primary destabilizing residues. This includes substrates with N-terminal unmodified basic residues (Arg, Lys, His) or bulky hydrophobic residues (Leu, Phe, Tyr, or Ile). Ubr1 binds to these substrates using two distinct binding sites [65-68]. The ubiquitylation of N-end rule substrates is mediated by Ubr1 in association with the Rad6 E2 conjugating enzyme [69,70]. Further studies have shown that Ubr1 interacts in a complex with Ufd4 HECT ligase. These two ubiquitin ligases have higher processivity in the polyubiquitylation of N-end rule substrates in comparison to either ligase alone [71].

Ubr1 has been shown to recognize internal degradation signals of misfolded proteins, independently from the recognition of N-end rule substrates. Deletions of both *UBR1* and *SAN1* fully stabilized the cytoplasmic substrate ΔssCPY*-GFP (derived from the misfolded vacuolar carboxypeptidase Y mutant) and truncated cytoplasmic proteins that are unstable [61]. Ubr1 recognition of misfolded proteins was dependent on Hsp70 and Sse1 (ATPase component of Hsp90 chaperone) [61]. This phenomenon was also observed in an independent study, where the engineered cytosolic substrates ΔssPrA (vacuolar proteinase A with deletion of ER signal sequence) and Δ2GFP (green fluorescent protein with truncations in β-barrel structure) was targeted for degradation by San1 [64]. San1 mediated degradation of the truncated substrates was shown to be

accelerated by Ubr1 and was dependent on delivery of the substrates by Hsp70 to the nucleus. Thus, the degradative QC of cytosolic proteins by Ubr1 and San1 seems to occur in a compartment specific manner. Addition of a nuclear export signal (NES) to the substrate ΔssCPY* leads to retention of the substrate in the cytoplasm. This substrate is solely targeted by Ubr1 and not San1. In addition, the stress phenotype by this cytoplasmic substrate is only sensitive to *ubr1*Δ and not *san1*Δ cells. In an independent study, Ubr1 was shown to target newly synthesized misfolded protein kinases located in the cytoplasm. The action of Ubr1 was augmented by Ubr2 (a second RING-E3 that is implicated in N-end rule ubiquitylation) in targeting misfolded cytosolic protein kinases [63]. Further pathways have also been implicated in cytosolic protein QC. The Doa10 ubiquitin ligase involved in ERAD was shown to target temperature-sensitive (*ts*) alleles of the cytosolic Ura3p (Ura3-2p, Ura3-3p) [72]. In addition, Ura3p fused to the CL1 degron (short destabilizing peptide) was similarly targeted by Doa10 in conjunction with the chaperone proteins Ydj1p (Yeast dnaJ 1, chaperone involved in regulation of *HSP90* and *HSP70* functions) and Ssa1-Hsp70p and the E2 conjugating enzymes Ubc6 and Ubc7 [73,74]. In a recent study, the HECT ubiquitin ligase Hul5 was identified as a major player in the ubiquitylation of low solubility cytosolic proteins after heat-shock stress and in physiological conditions. Deletion of *HUL5* reduced growth after heat-shock stress and decreased the degradation of short-lived misfolded proteins in the cell [60]. Another RING-domain E3 ligase, Rkr1/Ltn1, was found associated with the ribosomes and targets nascent non-stop poly-peptides that are stalled during translation for degradation [75]. Altogether, this suggests that there is potentially a large network of different ubiquitin ligases that are involved in degradative QC of misfolded proteins in the cytoplasm.

1.7 Thesis Investigation

Protein QC mechanisms have been classically elucidated using model substrates. In chapter two, we examined whether cytosolic thermosensitive proteins display hallmarks of QC model substrates. The stability of wild-type and mutant proteins was first assessed at restrictive temperature. We next determined whether the ubiquitin-proteasome pathway mediates the degradation of the unstable thermosensitive proteins at restrictive temperature.

In Chapter three, we first performed a screen to identify E3 ligases that may target the unstable mutant proteins for degradation. We hypothesized that these unstable mutants are targeted for degradation by a specific subset of E3 ligases and reasoned that significant stabilization of the thermosensitive substrates could be obtained by deleting the E3 ligases that would lead to the suppression of ts phenotype. We performed a screen in which twelve thermosensitive mutants were crossed to a library of sixty-two single E3 deletion strains in a single array. We describe the results from the screen and the characterization of several false positive hits that we obtained. In the second part of this chapter, we assessed stability of Pro3-1p, a mutant protein that displays classical features of a protein QC substrate, in single or double deletions of E3 ligases implicated in protein QC.

Chapter four summarizes the key findings and offers future possible directions of the project.

Chapter 2. Characterization of Cytosolic Thermosensitive Mutant Proteins

2.1 Introduction

Protein QC pathways have classically been elucidated using mutant model substrates in yeast [11,32,76]. It was previously reported that mutations in open-reading frames affect stability of full-length proteins. For instance, whereas wild-type Ubc9p is a stable protein, several *ubc9 ts* mutant proteins were short-lived and targeted for degradation by the proteasome at the restrictive temperature [77]. Similarly, a *ts* mutant of Ubc4p, with a single point mutation, was also found to be rapidly degraded at the non-permissive temperature [57]. Several other *ts* mutants were subsequently found unstable, like the nuclear proteins Cdc68-1p, Sir4-9p, Cdc13-1p, Sir3-8p and the ER protein Msp2-1p [51,73]. In contrast, the wild-type alleles of these proteins are stable. It is likely that mutations in these proteins affect their folding and three dimensional configuration. Whereas wild-type proteins are not recognized by any degradative QC machinery, the mutant alleles are recognized by E3 ubiquitin ligases and targeted for degradation to the proteasome. In fact, deletions of the E3 ligase *SAN1* and *DOA10* leads to significant stabilization of *cdc68-1 and of msp2-1* mutants respectively [51,73].

In this chapter, we examined whether cytosolic thermosensitive mutant proteins exhibit hallmarks of protein QC substrates. We examined the stability of a panel of *ts* mutants at restrictive temperature and determined whether the mutant proteins are targeted to the UPS pathway. Remarkably, we found that a large portion of the tested *ts* alleles were unstable.

2.2. Methods

2.2.1 Yeast Strains and Plasmids

Media preparation and molecular biology techniques were performed using standard procedures. Yeast strains used in this chapter are listed in Appendix 1. The *ts* alleles employed in this study were generously provided by Dr. Hieter and were previously obtained by the diploid shuffle method to integrate randomly PCR-mutagenized essential genes in their endogenous locus that were then selected for conditional *ts* lethality [78,79]. In this study, the yeast *ts* strains were tagged C-terminally by homologous recombination using the 13myc::KanMX6 module [80]. The deletions were generated by knocking out the specific gene by homologous recombination [81] or crossing the *ts* stains with the gene deletion collection carrying the SGA reporter (c*an1Δ::STE2pr-spHIS5 , lyp1Δ, his3Δ1, leu2Δ0, ura3Δ0, met15Δ0, geneX::NatMX4*) [82,83]. The diploids were selected, sporulated, and spores were tetrad dissected and selected for haploids with the appropriate markers.

2.2.2 Antibodies

Immunoblots were performed with 9E10 monoclonal mouse antibody (UBC Antibody Facility, 1:7,000 dilution) against myc-tagged proteins and anti-PGK monoclonal rabbit antibody as loading control (1:10,000 dilution; gift from J. Thorner, University of California). Anti-Goat anti-mouse and Goat anti-rabbit (Mandel Scientific, 1:10,000) were used as secondary antibodies. Protein bands were quantified by using Li-Cor Odyssey Fluorescent detection system.

2.2.3 Stability Assays

Protein degradation was examined using cycloheximide chase assays. Cells were grown in YPD to exponential phase (OD_{600}=1) at permissive temperature (25°C) before the addition of cycloheximide (100 µg/ml). The cells were then incubated as indicated for 2-3 hours at either the permissive (25°C) or restrictive (37°C) temperatures. For proteasome inhibition experiments, cells were grown to exponential phase in synthetic complete medium (0.17% yeast nitrogen base without ammonium sulphate) supplemented with 0.1% proline and 2% glucose as the carbon source. The overnight grown cultures were diluted to fresh media supplemented with 0.003% SDS at OD_{600} 0.2 [84]. The cells were first pre-treated with 20 µM MG132 or control dimethyl sulfoxide (DMSO) for 30 min and then with 100 µg/ml cycloheximide. Samples were collected at the indicated time points, harvested by centrifugation in a microfuge (5,900 *g*, 30 sec), resuspended in modified laemeli lysis buffer (50 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol) and lysed with glass beads. Protein concentration was determined by Bradford assay (Bio-Rad). Equal amounts of protein were resolved by SDS-PAGE and transferred to nitrocellulose membrane.

2.2.4 Solubility Assessment of Proteins

Yeast cells were grown to exponential phase (OD_{600} =1) and shifted to non-permissive temperature for 30 min. The cells were lysed with glass beads in native lysis buffer consisting of 0.5% NP-40, 20 mM HEPES, 200 mM NaCl, 1 mM EDTA, protease inhibitors (Roche Applied Science), 1 mM phenanthroline and the lysates were then pre-cleared by centrifugation in a microfuge (2,000 *g*, 5 min, 4°C). The lysates were further fractionated to soluble and insoluble fraction by centrifugation (16,000 *g*, 10 min,

4°C). Corresponding volumes of the total cell lysate (TCL), soluble fraction (S1), and pellet (P1) mixed or resuspended in lysis buffer were collected and resolved by SDS-PAGE.

2.3 Results

2.3.1 A large portion of Thermosensitive Mutant Proteins are Degraded at Restrictive Temperature

We reasoned that a large portion of *ts* mutants might be unstable at restrictive temperature. We selected a panel of *ts* alleles of cytosolic proteins (6 genes, 22 alleles) from a mutant library generated by PCR-mutagenesis and monitored their stability [79]. The wild-type and *ts* mutants were C-terminally tagged with 13myc epitopes. We observed that the wild-type alleles tested are completely stable in three-hour chase assays after adding the translation inhibitor cycloheximide to growing the cells at 37°C (figure 4A). We then assessed the viability of the C-terminally tagged mutant alleles on synthetic complete media at permissive (25°C) and restrictive temperatures (37°C) (figure 4B). We found that 20 of the strains maintained the complete loss of viability phenotype at non-permissive temperature, one strain displayed a subdued phenotype (*pro3-2*), and one strain was viable and not further characterized (*guk1-5).* The untagged mutant collection remained *ts* at restrictive temperature on synthetic complete media (data not shown). Thus, the C-terminal tag does not affect the thermosensitive phenotype of the majority of the mutant strains.

We next assessed the stability of the tagged wild-type and *ts* mutant proteins at non-permissive temperature (figure 4C). We observed a different stability pattern for the *ts* mutant alleles. We found that 10 of the 21 assessed proteins displayed 50% or higher reduction in protein levels after 3 h incubation at restrictive temperature. Differential stability was typically observed for different alleles of the same protein, like for the guanylate kinase (*GUK1*). Two alleles (Guk1-3p and Guk1-6p) were completely

degraded over three hour incubation at restrictive temperature, while two other alleles displayed substantial reduction (Guk1-1p, Guk1-2p) and another *ts* allele was stable (Guk1-4p). Overall, we observed about half of the *ts* mutant proteins displayed loss of stability at the restrictive temperature. These results suggest that a large portion of *ts* alleles may be unstable.

2.3.2 The Thermosensitive Mutant Protein Pro3-1p is Preferentially Degraded at Non-Permissive Temperature.

We next sought to examine in more detail the turnover rate of the Pro3-1p *ts* allele because of its pronounced degradation in the first assay (figure 4C). We confirmed that Pro3-1p is completely degraded over three-hour cycloheximide incubation at the non-permissive temperature, while it is fully stable at the permissive temperature (figure 5A). In contrast, the wild-type allele Pro3p is completely stable at both the permissive and restrictive temperatures over the same three-hour chase period (figure 5A). Similarly to the wild-type allele, we found that the second mutant allele Pro3-2p was also fully stable at both the permissive and non-permissive temperatures (figure 5A). Interestingly, this mutant displayed a subdued *ts* phenotype, which may be explained by the higher stability of the protein at 37°C compare to Pro3-1p.

Upon sequencing the mutant alleles, we found that both *pro3-1* and *pro3-2* mutant alleles have four missense mutations and one silent mutation in their open-reading frames (table 1). It is possible that mutations in Pro3-1p affect the conformation of the protein at the restrictive temperature, leading to its misfolding and then degradation. Mutations in Pro3-2p may not perturb the folding to a great extent at the restrictive temperature. In this case the mutant protein, similar to the wild-type, may

maintain its native state. To test this idea, we examined the solubility after centrifugation of the three alleles of Pro3p at the non-permissive temperature. We found that after a 30 min incubation period at 37°C, a significant portion of Pro3-1p is deposited in the insoluble fraction (figure 5B). In contrast, only a small portion of Pro3-2p is insoluble and the wild-type Pro3p is completely soluble in these conditions. These results indicate that, upon shifting the cells to the restrictive temperature, a large portion of Pro3-1p likely misfolds and aggregates.

2.3.3 The Loss of Stability of the Thermosensitive Mutants at Restrictive Temperature is Dependent on the Proteasome.

Based on the previous results, we postulated that the degradation of the poorly soluble Pro3-1p is mediated by a proteasome dependent QC pathway. We therefore tested whether inhibition of the proteasome would impair the degradation of the mutant protein at the restrictive temperature. Incubation of *pro3-1* temperature-sensitive cells with the proteasome inhibitor MG132 prevented the rapid turnover of the mutant protein after shifting the cells at 37°C (figure 5C). This indicates that the Pro3-1p degradation is dependent on the proteasome.

We next examined whether other seven *ts* mutant proteins are targeted to the proteasome (figure 6). Similar to Pro3-1p, we observed a significant increase in stability of the mutant proteins at 37°C upon the inhibition of the proteasome. A large subset of the unstable mutant proteins is completely stabilized by the inhibition of the proteasome whereas a few others are partially stabilized. This suggests that the degradation of a subset of ts mutants is exclusively dependent on the proteasome whereas other mutants may be degraded by another pathway in the cell (e.g. lysosomes).

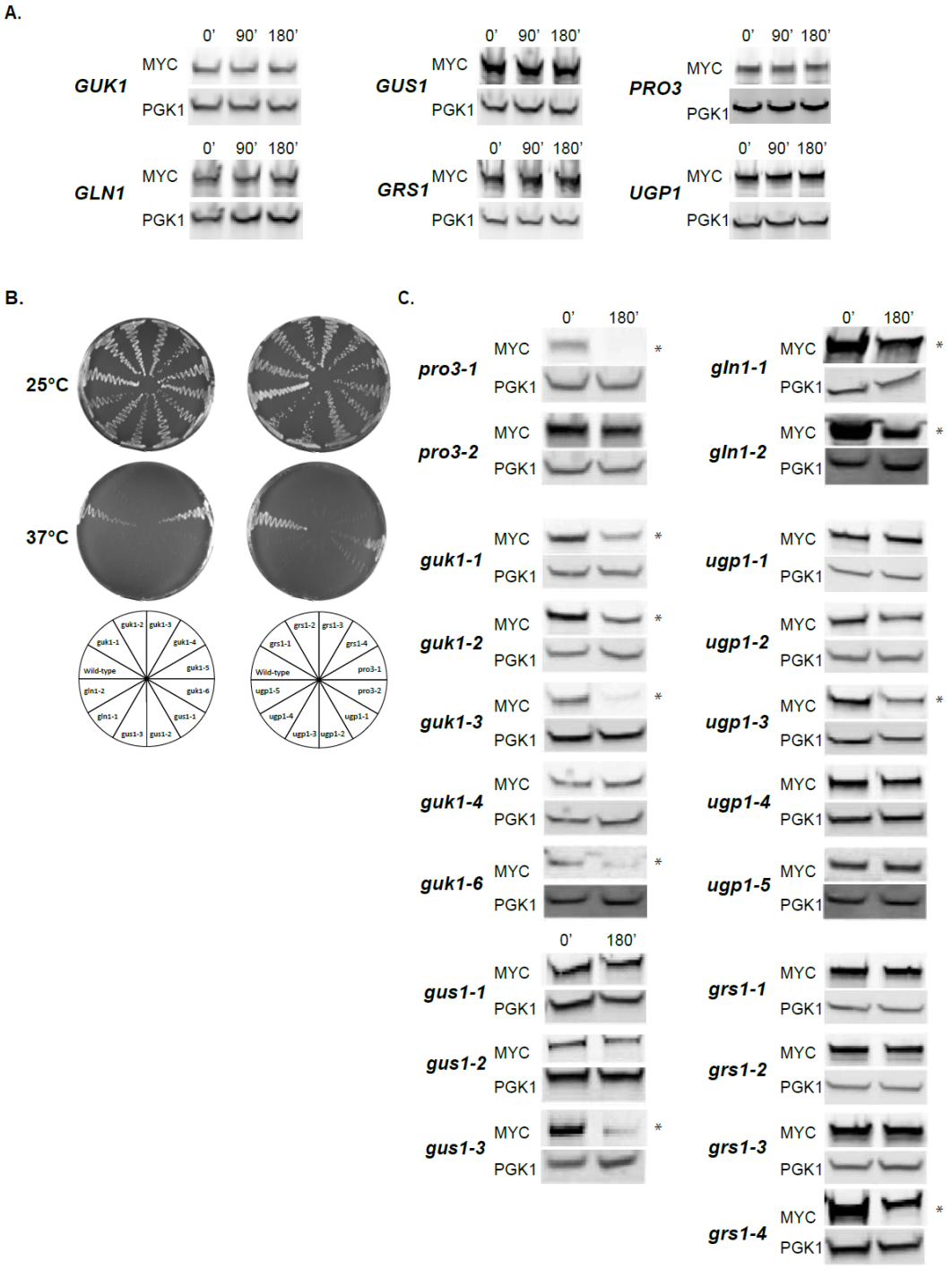


Figure 4. A large fraction of Thermosensitive mutant proteins are degraded at restrictive temperature. (A) The stability of wild-type cytoplasmic proteins was assessed at 37°C using cycloheximide chase assays **(B)** Yeast strains carrying the indicated C-terminally tagged *ts* alleles and wild-type BY4741 background cells were streaked on synthetic complete media and the viability was determined after three days of growth at permissive temperature (25°C) and restrictive temperature (37°C). **(C)** The stability of mutant proteins was determined by a three-hour cycloheximide chase period at 37°C. The lysates were resolved by SDS-PAGE and analyzed by western blotting with 9E10 antibody and PGK1 antibody. * denotes alleles that displayed over a 50% protein level decrease.

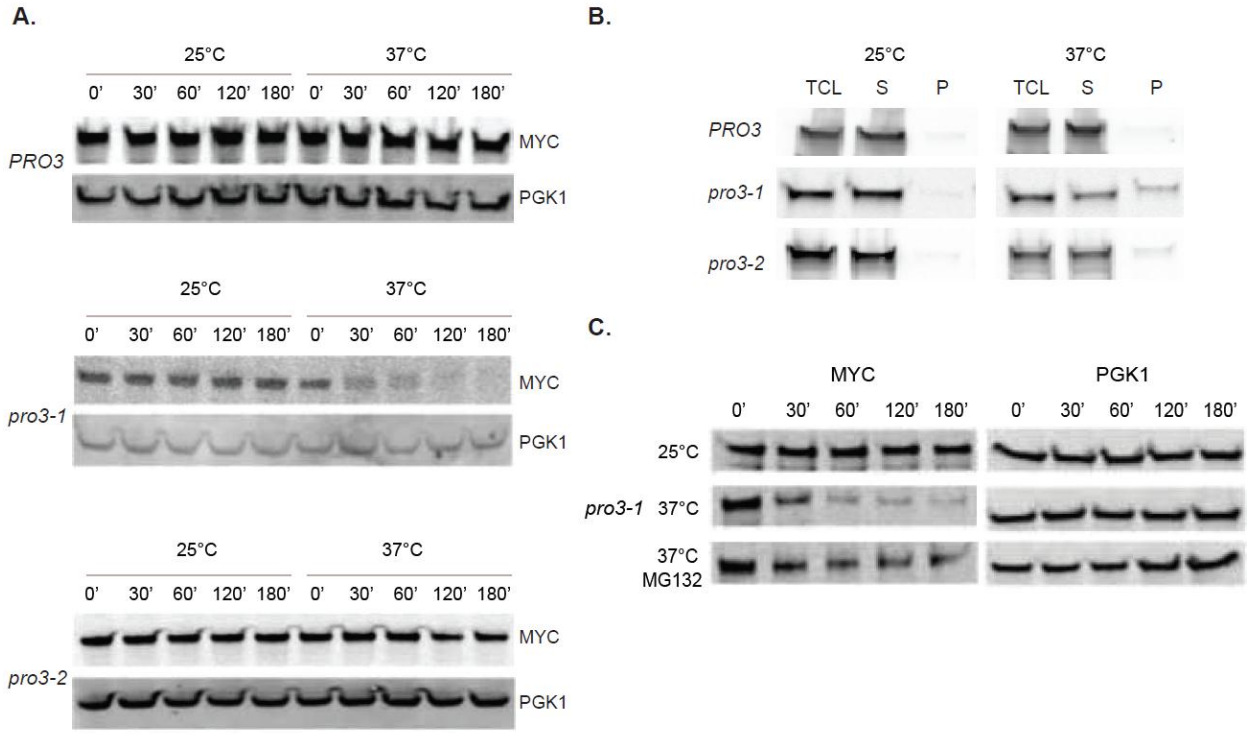


Figure 5. The Delta-1 pyrroline 5-carboxylate reductase (*PRO3*) alleles display variable stability and solubility at restrictive temperature. **A)** Wild-type *PRO3* and Mutant strains *pro3-1, and pro3-2* were grown to exponential phase and the stability of the proteins was assessed over a three-hour time course by cycloheximide-chase analysis at 25°C and 37°C. **(B)** The solubility of wild-type (Pro3p) and mutants (Pro3-1p and Pro3-2p) was assessed prior to and after shifting the cells from 25°C to 37°C for 30 min. An equal portion of each fraction was loaded on SDS-PAGE for western blot analysis with 9E10 monoclonal antibody. **(C)** Mutant strain *pro3-1* was grown to exponential phase and pre-incubated for 30 min with either DMSO or proteasome inhibitor MG132 at 25°C, then treated with cycloheximide and shifted to 25°C and 37°C. The stability of Pro3-1p at the indicated time points was determined by western blot analysis using 9E10 and anti-PGK1 antibodies.

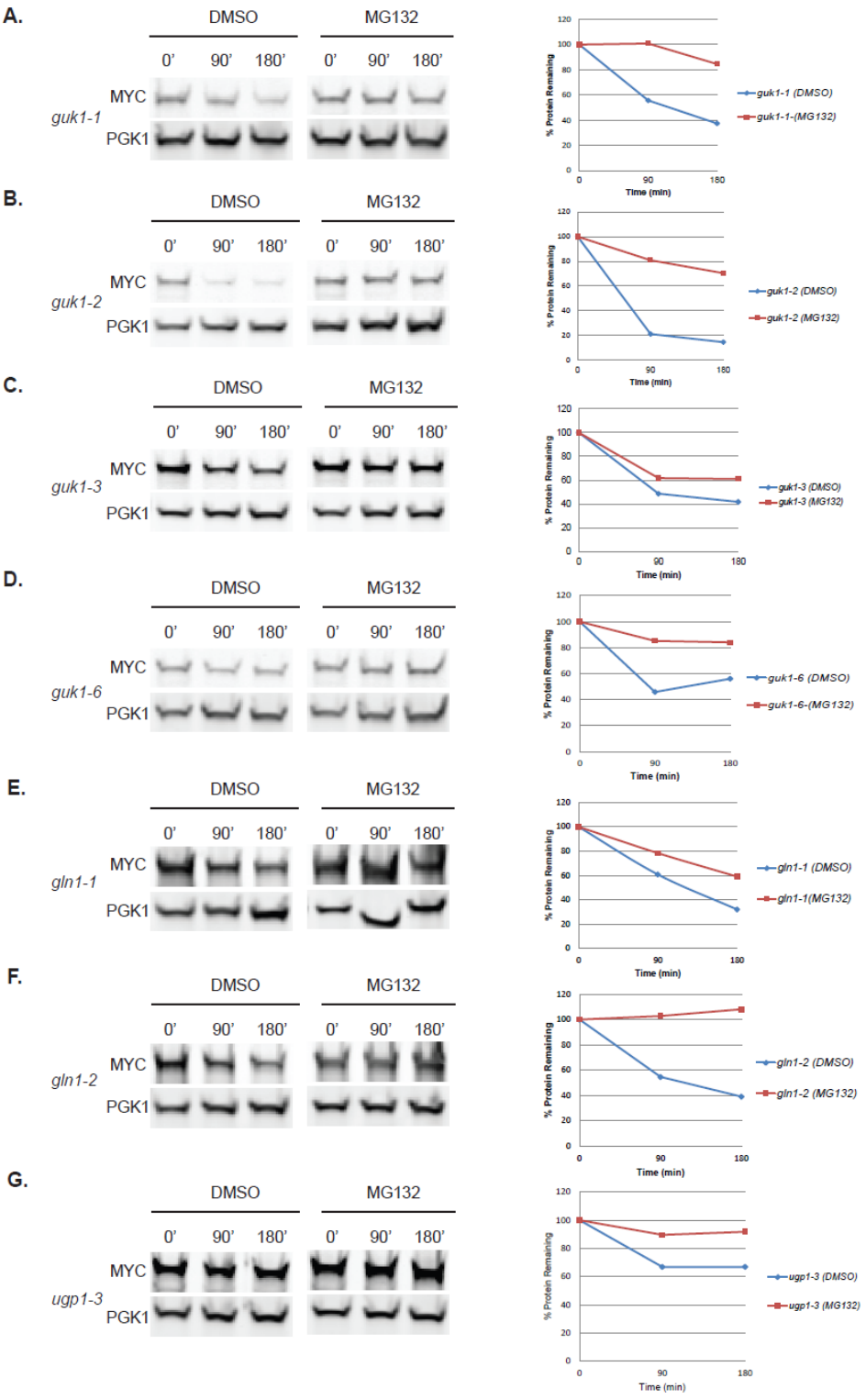


Figure 6. The degradation of *ts* mutant proteins is dependent on the proteasome at the restrictive temperature. Mutant strains with *ts* unstable alleles were pre-incubated for 30 min with either DMSO or proteasome inhibitor MG132 at 25°C. The cells were then treated with cycloheximide and shifted to 37°C. The protein levels at the indicated time points were determined by western blot analysis using 9E10 anti-myc and anti-PGK1 antibodies.

Allele	Total Number of Mutations	Mutation Nucleotide Position	Mutation Amino Acid Position
pro3-1	5	79) G→A 150) A→G(silent) 398) T→C 707) A→T 781) A→G	27) A→T 133) V→A 236) H→L 261) K→E
pro3-2	5	77) A→G 143) C→T 201) T→C(silent) 335) T→C 504) A→T	26) K→R 48) P→L 112) I→T 168) Q→H

Table 1. Number of mutations and mutation position in the primary sequence of *pro3-1* and *pro3-2* alleles. Mutant alleles of *PRO3* (*pro3-1* and *pro3-2*) were PCR amplified from the endogenous loci and the open reading frame was sequenced. The number of missense mutations and the mutation position were determined by alignment of the primary sequence of mutant alleles with wild-type allele.

2.4 Discussion

Thermosensitive mutants are typically generated by introducing point mutations in essential genes, which leads to loss of viability under restrictive conditions [78]. We hypothesized that missense mutations may affect the protein steady-state level of a large portion of *ts* mutants by targeting the essential protein for degradation in the cell. To examine this, we assessed the stability of wild-type and mutant alleles of six essential cytosolic proteins: *PRO3*, *GUS1*, *GUK1*, *GLN1*, *UGP1,*and *GRS1*. Delta 1-pyrroline carboxylate reductase (*PRO3*) is a multimeric enzyme involved in the conversion of delta-1-pyrroline carboxylate to proline in the cytosol of eukaryotic cells [85]. It has been shown that pyrroline 5-carboxylate reductase is a multimer in higher eukaryotes [86]. In yeast, the enzyme is constitutively expressed and is not under transcriptional regulation. Glutamyl tRNA synthetase (*GUS1*) and Glycyl tRNA synthetase (*GRS1*) are cytosolic enzymes that ligate amino acids to cognate tRNA [87,88]. Glutamine synthetase (*GLN1*) is a metabolic enzyme in the cytosol that catalyzes amination of glutamate to form glutamine [89]. Guanylate kinase (*GUK1*) is localized both in the cytosol and nucleus. It converts GMP to GDP and is required for mannose chain elongation in the eukaryotic cell wall [90]. UDP-glucose pyrophosphorylate (*UGP1*) is a cytosolic enzyme involved in the formation of UDP-glucose from glucose-1-phosphate and UTP [91]. All the proteins in this study are in high abundance (>10,000 copies in the cell) and the wild-type alleles were completely stable under restrictive conditions. We hypothesized that the *ts* allele of these proteins may constitute good model substrates to study degradative QC pathways in the cytoplasm.

A *ts* phenotype could be induced by mutations that affect the functionality of the protein by altering catalytic activity, propensity to form functional complexes, or to bind to physiological partners. Alternatively, the mutations could affect the protein steady-state levels by reducing expression levels or the stability (i.e., half-life) of the protein. We tested the stability of a panel of twenty-one corresponding *ts* mutant alleles. We found that mutations in half the mutant alleles lead to a loss of protein stability at restrictive temperature. A protein in an aqueous environment is in equilibrium between the native folded configuration and the unfolded conformation. The native configuration of the protein is determined by covalent interactions, binding energy (including dispersion forces, electrostatic interactions, van der waals forces and hydrogen bonding), and hydrophobic interactions [92-94]. In an aqueous environment, proteins bury hydrophobic residues in the core of the structure while the hydrophilic residues are exposed outwards. This conformation is energetically favourable as the surrounding water molecules have a higher degree of freedom that increases entropy. Protein stability increases as binding energy increases and when entropy increases (i.e. hydrophobic interactions remain intact and remain buried in the core of the protein). It may be possible that missense mutations in the unstable alleles replace key structural residues and perturb the binding energy or hydrophobic interactions, leading to misfolded proteins that are targeted by the degradative pathway. We find that *ts* alleles of *GUK1* are more prone to degradation. Four of the five tested *guk1-ts* mutants are readily degraded at restrictive conditions. This may be accounted for by the high degree of secondary structures in *GUK1* (44% alpha helices and 22% beta strand) [95]. Upon sequencing, we find that the unstable mutants consist of 2-4 missense mutations in the

secondary structure (appendix 3). These mutations may perturb key intermolecular interactions, leading to a malfolded protein that is targeted by degradative pathways.

A major pathway of degradation in eukaryotic cell is mediated by the ubiquitin proteasome system. Misfolded proteins may expose degradation signals or key structural elements that are recognized by E3 ligases. We find that degradation of all the unstable substrates is dependent on the proteasome and thus is a selective process requiring ubiquitylation. Whereas some substrates are exclusively dependent on proteasome-mediated degradation, others were only partially stabilized by the inhibition of the proteasome. A subset of unstable mutants may be targeted by multiple degradative pathways in the cell.

Classically, truncated substrates such as carboxypeptidase Ysc (CPY) have been used to elucidate the protein QC pathways in the ER and cytoplasm [61,76]. While a portion of proteins may become truncated in the cell due to events such as protease processing and transcriptional and translational errors, the use of these substrates does not encapsulate the full spectrum of substrates targeted by the protein QC system *in vivo.* The panel of *ts* mutants that we characterized in this study may be advantageously used as QC model substrates for two reasons: first, the substrates are expressed from the endogenous loci and thus any interactions are likely to be pertinent to physiological conditions; second, these ts mutants are full-length proteins. Many disease-causing mutations are due to small number of missense mutations in the full-length protein. For instance, missense mutations in α-synuclein leads to protein misfolding, aggregation, and formation of amyloid fibrils, which are toxic to the cell [96,97]. Similarly, we find that as few as two missense mutations can lead to decreased

stability of *ts* mutants linked to a loss of viability in restrictive conditions. Missense mutations in Pro3-1p lead to a significant decrease in stability and accumulation of the protein in the insoluble fraction, whereas wild-type Pro3p remains stable and soluble under restrictive conditions. The panel of unstable alleles displays classical feature of protein QC substrates. The wild-type alleles of the mutants are stable whereas the mutant is degraded. In the case of Pro3-1, the mutations induce a conformational change that leads to lower solubility. We speculate that the misfolded form of Pro3-1 is recognized by a degradative pathway in the cell, and that a similar mechanism prevails for the other alleles. In agreement with this idea, we found that the tested unstable alleles are significantly stabilized by proteasome inhibition, indicating that the degradation of these mutant proteins is dependent on the UPS pathway.

Chapter 3. Screening for E3 Ligases and Interactors of Thermosensitive Mutants

3.1 Introduction

We have demonstrated that a large portion of cytosolic thermosensitive mutant proteins are unstable at restrictive temperature and targeted to the proteasome. It has been shown that stabilization of essential thermosensitive mutant proteins can lead to restoration of viability at restrictive temperature. For instance, deletions of the E3 ligase *SAN1* and *DOA10* leads to significant stabilization of *cdc68-1 and of msp2-1* mutants respectively, and restoration of viability under restrictive conditions [51,73]. This suggests that the temperature-sensitive lethal phenotype of many essential gene mutants may be conditional to the degradation of mutated proteins, which are less stable at higher temperatures. We therefore reasoned that the degradation of cytosolic thermosensitive mutants characterized in this study may lead to depletion of the essential protein and loss of viability at restrictive temperature. We further hypothesized that a subset of these misfolded proteins may have distinct structural features and be targeted by a single E3 ligase. Using this rationale, we searched for ubiquitin ligases targeting the thermosensitive substrates by screening E3 deletion strains that rescue the thermosensitive phenotype at restrictive temperature.

3.2 Methods

3.2.1 Media

The media were prepared according to previous reports [83,98]

Complete Amino acid supplement powder mixture 3 g adenine, 2 g uracil 2 g inositol, 0.2 g para-aminobenzoic acid, 2 g alanine, 2 g arginine, 2 g asparagine, 2 g aspartic acid, 2 g cysteine, 2 g glutamic acid, 2 g glutamine, 2 g glycine, 2 g histidine, 2 g isoleucine, 10 g leucine, 2 g glycine, 2 g methionine, 2 g phenylalanine, 2 g proline, 2 g serine, 2 g threonine, 2 g tryptophan, 2 g tyrosine, 2 g valine. Drop-out (DO) powder mixtures correspond to the combination of the above ingredients minus the appropriate amino acid. Per liter of medium, 2 g of the enriched amino acid supplement powder mixture is used.

Minimal amino acid supplement powder mixture: 3 g adenine, 2 g uracil, 10 g leucine, 2 g tryptophan, 2 g methionine. Per liter of medium, 2 g of complete amino acid supplement powder mixture is used.

Amino-acids supplement for sporulation media 2 g histidine, 10 g leucine, 2 g lysine, 2 g uracil. Per liter of sporulation media, 0.1 g of amino-acid supplement is used.

Sporulation media: 1% potassium acetate, 0.1% yeast extract, 0.05% glucose, 0.01% amino-acids supplement powder mixture

Haploid media (SD - His/Arg/Lys, canavinine, thialysine**):** 0.67% yeast nitrogen base without amino acids 0.2% enriched amino-acids supplement powder mixture (DO – His/Arg/Lys), 2% glucose, 50 mg/L canavanine, 50 mg/L thialysine, 2 % bactoagar

Complete haploid selective media (SD - His/Arg/Lys, MSG, canavanine, thialysine, G418, cloNAT)**:** 0.17% yeast nitrogen base without amino acids or ammonium sulphate, 0.1% MSG (L-glutamic acid sodium salt hydrate), 0.2% enriched amino-acids supplement powder mixture (Dropout - His/Arg/Lys), 2% glucose, 50 mg/L canavanine, 50 mg/L thialysine, 200 mg/L geneticin (G418), 100mg/mL neurseothricin (cloNAT), 2% bactoagar

Minimum haploid selective media (SD - His/Arg/Lys, MGS, canavanine, thialysine, G418, cloNAT)**:** 0.17% yeast nitrogen base without amino acids or ammonium sulphate, 0.1% MSG (L-glutamic acid sodium salt hydrate), 0.2% minimum amino-acids supplement powder mixture, 2% glucose, 50 mg/L canavanine , 50 mg/L thialysine, 200 mg/L G418, 100 mg/mL cloNAT, 2% bactoagar

3.2.2 Stability Assays

Stability assays performed were conducted as described in Section 2.2.3

3.2.3 Thermosensitive Suppression Screen

Thirteen yeast strains corresponding to twelve different thermosensitive alleles (*MAT*a *ts-13myc::kanMX6 CAN1 LYP1)* were pinned and arrayed into 1536 density format on Omni-tray plates using Singer Rotor Robot (figure 7). The mutant strain *grs1-4* was arrayed twice as an internal control for the consistency of thermosensitive suppression. The strain *ugp1-4,* a stable thermosensitive allele, was used as a negative control. Suppression of thermosensitive phenotype in haploid strains with *ugp1-4* mutant allele and single E2/E3 deletion could not be due to an increase in stabilization of the protein and likely to occur by an indirect mechanism. In addition, we also included the *cdc68-1* allele that can be rescued by *san1*Δ [51]. However, the *ts* phenotype of *cdc68-1* was not rescued in our conditions, in agreement with previous observations (Dr. R. Gardner, personal communications). A library of 62 single E3 ligase deletions and two E2 conjugating enzyme deletions constituted the query strain (Y7092 background: *MAT*α *E2/E3*Δ*::natMX6 can1*Δ*::STE2pr-HIS5 lyp1*Δ) and was also arrayed into 1536 density. All manipulations performed at room temperature and plates were grown at 25°C. The mutant array was crossed to the query deletion collection by pinning on YPD plate for one day. The resulting zygotes were selected for on YPD plate containing cloNAT and G418 to select for diploids [83,98]. The heterozygous diploids were transferred to a plate with enriched sporulation media and sporulated for five days. This media contains reduced levels of carbon and nitrogen leading to meiosis and formation of haploid meiotic spore progeny. Spores were subsequently transferred to a media containing three selection factors (histidine, thialysine, and canavanine) for the haploid *MATa* progeny and selecting against the diploid as described previously [83,98].

Histidine in the media selects for germination of *MATa* haploids because only cells with *STE2pr-HIS5* reporter survive. Thialysine and canavinine, toxic analogues of lysine and arginine, select for haploid cells that carry the *lyp1Δ* and *can1Δ* makers (i.e., select against *lyp1Δ/LYP1* and *can1Δ/CAN1* diploid cells) [83,98]. The cells were selected on the haploid media plate for two days, allowing germination of the haploid *MATa* progeny. The cells were then re-selected on a new haploid media plate for one day. The *MATa* meiotic progeny were transferred to media containing the haploid selection, G418, and cloNAT. This allowed for selection of *MATa* meiotic progeny that carry the mutant thermosensitive gene (Kan^R) and the deletion of the specified E3 ligase (Nat^R). The haploid cells were then pinned on plates under three different conditions for two days: 1) Minimal haploid selective media (with G418 and cloNAT) at 35°C, 2) Complete haploid selective media (with G418 and cloNAT) at 37°C, and 3) Minimal haploid selective media (with G418 and cloNAT) at 37°C.

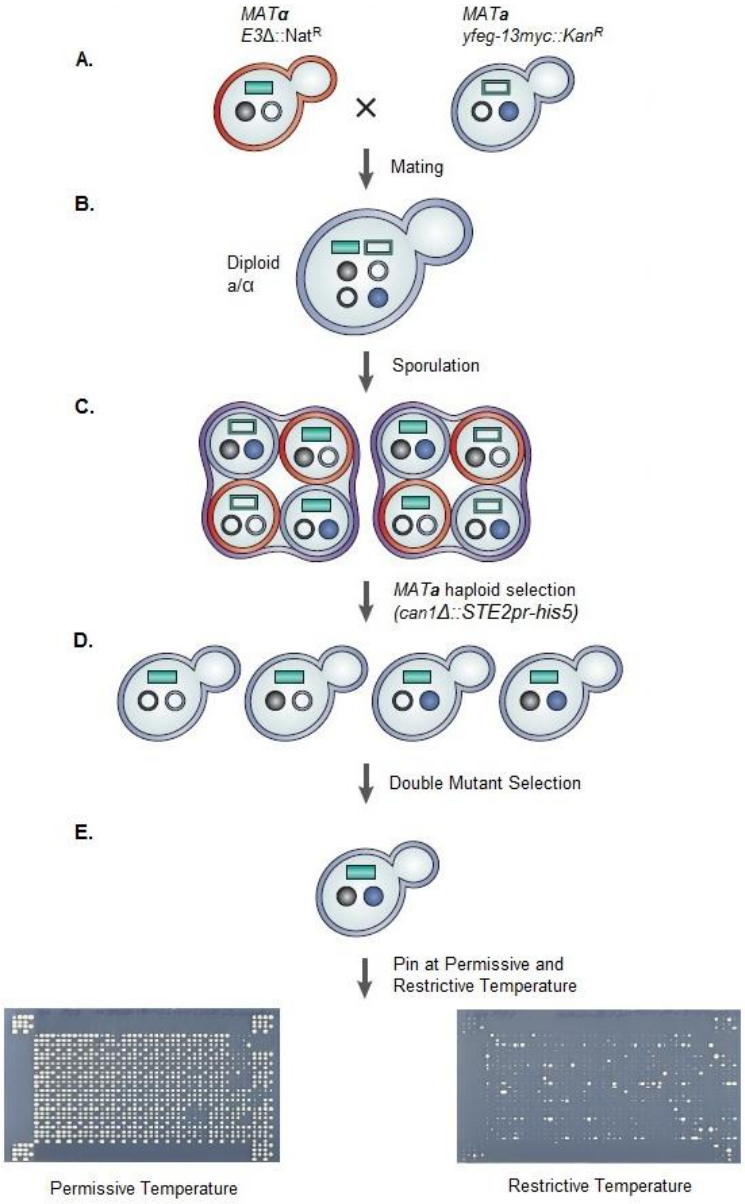


Figure 7. Thermosensitive Suppression Screen. A. Twelve different thermosensitive mutants were arrayed into 1536 density. The mutant array was crossed to a collection of 62 single E3 ligase deletions and two E2 conjugating enzymes deletions deletion query strains arrayed into 1536 density. **B.** The diploids are selected on YPD plate media containing geniticin and neurseothricin for one day. **C.** The heterozygous diploids are transferred to a sporulation plate for 5 days. **D.** *MATa* haploid progeny are germinated in synthetic complete media plate (-His, -Lys, -Arg) with canavanine and thialysine for two days. Histidine dropout selects for germination o*f MATa* haploids because only cells with *STE2pr-his5* reporter will survive. Thialysine and canavanine, toxic analogues of lysine and arginine, select for haploid cells that carry the *lyp1Δ* and *can1Δ* makers. The haploid cells are pinned on the same media again for one day to select further for the *MATa* progeny (and select against diploids). **E.** The cells are finally pinned on a plate with *MATa* selection, geneticin, and neurseothricin media for one day to select for *MATa* progeny with thermosensitive mutant and the single E2/E3 deletion. The cells are pinned for one more round of selection on the same drug selection media. Finally, cells are pinned on haploid selective media and grown at permissive and restrictive temperatures to assess suppression of thermosensitive phenotype. Figure adapted with permission from Nature Reviews, Genetics, 2007 [98]

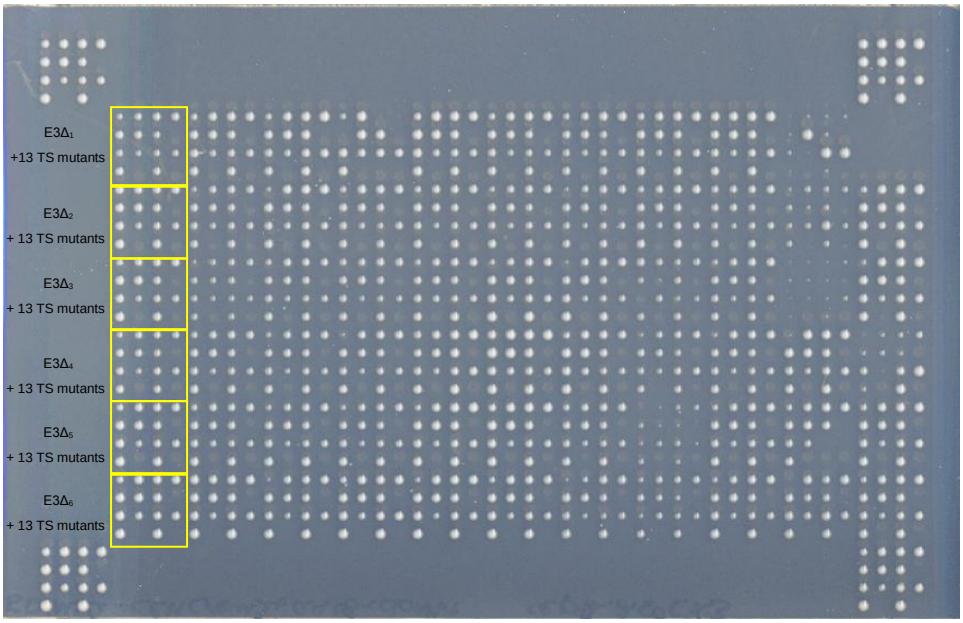


Figure 8. Thermosensitive Suppression Array. A panel of thirteen mutant strains was array into 1536 density format and crossed to a query strain of 62 single deletions of E3 ligases and two E2 conjugating enzymes deletion strains in 1536 format. The progeny haploid cells with the mutant allele and E3 deletion are selected for as described in the methods. In this array, each box corresponds to a single E3 and the corresponding thermosensitive alleles. Each colony in a highlighted box represents a colony with a thermosensitive allele and single E3 deletion combination. In addition, five small arrays (one in each corner of the plate and one within the E2/E3 array) only containing the thirteen mutant strains and no E3 deletion were analyzed to monitor the false positive rates.

3.3 Results

3.3.1 Hits Obtained From Thermosensitive Suppression Screen

A panel of thirteen thermosensitive mutant strains (*pro3-1, guk1-1, guk1-2, guk1-3, guk1-6, gus1-3, gln1-1, gln1-2, ugp1-3, ugp1-4, grs1-4 (2x), cdc68-1*) were crossed to a collection of sixty-two strains carrying a single deletion of an E3 ligase (appendix 2) and two strains with single *UBC4* and *UBC5* deletions in a single array. The strain *grs1-4* was used twice in the same array as an internal control for the consistency of thermosensitive suppression. We reasoned that the thermosensitive phenotype of a large subset of mutant strains is due to the degradation and depletion of the essential protein under restrictive conditions. We further hypothesized that a subset of thermosensitive proteins are targeted by an E3 ubiquitin ligase under restrictive conditions and that deletion of the E3 should stabilize the protein and rescue the *ts* phenotype. The mutant strain *ugp1-4*, a stable mutant allele of *UGP1*, was used as a control to determine whether suppression of the *ts* phenotype could occur by an indirect mechanism independent of an increase in protein stabilization. Using a pin-robot, we crossed each tested *ts* allele with each strain carrying a single E3 deletion on a 845-dense array (13 x 65; figure 8). In addition, we also included four small arrays of the thirteen mutant strains crossed to wild-type cells carrying out the NAT^R cassette at the *HO* loci to measure the rate of false positives (appendix 2). After sporulation, haploid cells containing both a *ts* allele and a single E3 deletion were selected. Haploid cells carrying both a *ts* allele and E3 deletion were tested under three different restrictive conditions to obtain a list of putative ligases targeting the cytosolic mutant proteins (figure 9; appendixes 4-6).

In the first condition, haploid cells were pinned on minimal haploid selective media (with G418 and cloNAT) and grown for two days at 35°C on three separate replicate plates (Figure 9). There were a total of 444 positive hits corresponding to growing colonies in the three replicates (160, 139 and 145 hits, respectively). We excluded, two of the twelve substrates (*pro3-1*, *grs1-4* (1), and *grs1-4* (2) from further analysis because their false positive rate was above 10% (25.5%, 14.9% and 23.4%, respectively; appendix 3). The same trend was observed when we analyzed the number of hits for these *ts* alleles in the E3 deletion suppression screen. After removing these two substrates from the analysis, the total number of hits was reduced to 128 (table 1). The deletion of the *UFO1* E3 ligase, gave the most hits (10 hits, 7.8% of total hits; appendix 4). Two *ts* alleles (*guk1-6* and *ugp1-3*) were suppressed across the three replicates by the deletion of *RTT101, UFO1* and *ELA1* (appendix 4). Nine *ts* alleles were also suppressed by an E3 deletion in two out of three replicates (appendix 4). The strain *grs1-4* was arrayed twice as a control for consistency in the suppression of the *ts* phenotype at restrictive temperature. There was some variability between hits from these two duplicates. However, as this allele was prone to inducing false positive hits, interpretation of the variability is more complicated.

In the second condition, the pinned cells were grown on the same haploid complete haploid selective media, but at a more stringent restrictive temperature (37°C). Under these conditions, there were 495 hits obtained across three separate replicates (154, 175, and 166, respectively). Similarly to the previous condition, results from the same three *ts* alleles (*pro3-1, grs1-4 (1), and grs1-4 (2)*) were excluded due to their high false positive rates (appendix 5). After exclusion of these alleles, the number

of hits was reduced to 183 (table 1). The deletion of the *UFO1* E3 ligase generated the most hits (18 hits, 9.8% of total hits; appendix 5). The *ts* allele *guk1-6* was suppressed across three replicates by deletion of *RTT01*. The *ts* alleles *gus1-3*, *ugp1-4*, *guk1-6*, and *gln1-1* were suppressed by an E3 deletion in two out of three replicates (appendix 5).

In the third condition, the cells were pinned on minimum haploid selection media containing only 5 essential amino acids at 37°C. Under these conditions, 125 hits were positive across three replicates (41, 48. and 36 hits). Again data from *pro3-1* and *grs1-4* were omitted because their false positive rates were above 10%, and a list of 50 hits was obtained (table 1). Overall, this condition was the most stringent and produced the least number of hits. The deletion of *UFO1* E3 ligase generated the most hits (7 hits, 14% of the total hits). The *ts* allele *guk1-6* was suppressed across three replicates by deletion of *RTT101.* The *ts* alleles *ugp1-4*, *guk1-6*, and *gln1-1* were suppressed on two out of three replicates by deletion of *UFO1* (appendix 6).

The stable mutant *ugp1-4* was used as a negative control where suppression of thermosensitive mutant is likely to occur by an indirect mechanism independent of an increase of the substrate stabilization. We did not found any particular E3 deletion that induced a higher number of hits with *ugp1-4*. Conversely, *ugp1-4* in the negative controls (crossed with HOΔ*::NatMX6)* produced a non-negligible number of false positive hits (~4%) indicative that this allele was itself prone to reversion.

We established a final list of potential candidate suppression hits for further analysis and validation (table 2). For this list, we only consider hits that were identified in at least two of the three conditions tested and present in at least two of the three replicated plates in each condition.

3.3.2 Characterization of the Top Hits of the Screen

There were five strong hits (table 2) that we decided to further characterize for validation. These hits correspond to the suppression of the phenotype of four different *ts* alleles (*guk1-6*, *gln1-1*, *grs1-3*, and *gus1-3*) by the single deletion of four ubiquitin ligases (*RTT101*, *UFO1*, *ELA1*, and *HUL4*). Overall, the ligases Rtt101, Ufo1, and Ela1 comprised the largest percentage of positive hits in comparison to other putative E3 ligases examined in the screen (appendix 4-6). This suggests that deletion of these ligases may significantly increase the stability of a large number of thermosensitive proteins. Alternatively, these deletions may also indirectly suppress the thermosensitive phenotype of mutant strains.

To further validate the hits from the screen, we independently generated the haploid strains with the thermosensitive allele and single E3 deletion by either random spore analysis or direct knockout of the E3 ligase. We both assessed the stability of the mutant proteins and re-tested whether the E3 deletion could rescue the *ts* phenotype (table 2). The stability of the top hits was assessed by cycloheximide chase analysis at 37°C (figure 10). The deletion of these ligases did not significantly alter the stability of the corresponding thermosensitive mutant proteins. In addition, deletion of the ligases did not lead to suppression of thermosensitive phenotype under restrictive conditions for three days (37°C and 35°C; data not shown). This suggests that the deletion of the ligases does not affect the stability of the protein and may not provide a general mechanism to suppress the thermosensitive phenotype as observed in the screen. Rtt101 is an E3 ubiquitin ligase implicated in DNA repair and anaphase progression [99,100]. Ufo1 is an E3 ubiquitin ligase that is a subunit of the Skp1-Cdc53-F-box

receptor. Ufo1 binds to the HO endonuclease, leading to its ubiquitylation and degradation [101]. It is possible that deletion of these ligases leads to the perturbation of recombination events, which could, for instance, allow the reversion of the mutation. This may account for the false positive hits obtained with these ligases. Accumulation of intragenic or extragenic mutations during the screen may also account for suppression of thermosensitive phenotype during the screen. All together, these data indicate that the suppression screen that we established is most likely not appropriate for the identification of genes rescuing unstable *ts* mutants, as we obtained a large number of false positives.

3.3.3 Double Deletion of *UBR1* and *SAN1* Significantly Stabilizes Pro3-1p and Suppresses Thermosensitive Phenotype of *pro3-1* Mutant Strain at Restrictive Temperature

We next sought to determine which ubiquitin ligase involved in a cytoplasmic QC pathway may be required for targeting Pro3-1p for degradation by the proteasome. Pro3-1p displays hallmark features of protein QC substrates. It is a mutant protein with short-half and its degradation requires ubiquitylation. However, the *ts* phenotype was often reverted when we performed the screen. To circumvolve this issue, we directly assessed the stability of Pro3-1p in cells containing single deletion of *SAN1, UBR1*, *UBR2*, and *HRD1*, which are the major ubiquitin ligases involved in the degradation of cytoplasmic misfolded proteins. We found that none of the tested single deletion had a meaningful effect on the stability of Pro3-1p when the cells were shifted at the restrictive temperature with cycloheximide (figure 11A). Ubr1 and San1 have been previously shown to act in conjunction to target for degradation the artificial cytosolic substrates

ΔssCPY*-GFP, ΔssPrA and Δ2GFP [61,64]. Therefore, we next decided to examine whether double deletion of *UBR1* and *SAN1* could affect the stability of Pro3-1p at the non-permissive temperature. We found that there was a significant reduction of the turnover of Pro3-1p in the absence of E3 ligases Ubr1 and San1 in comparison to control cells (figure 11B). This indicates that together, Ubr1 and San1 play a major role in the targeting of the Pro3-1p QC substrate for degradation.

We then assessed whether the loss of viability of the *pro3-1* strain at the restrictive temperature is caused by the degradation and depletion of the essential protein. We hypothesized that the reduced turnover of the mutant Pro3-1p could affect the conditional phenotype. We assessed the viability of different *pro3-1* strains carrying out single and double deletions of *UBR1* and *SAN1* on synthetic complete media. We found that the single deletions of *UBR1* and *SAN1* did not suppress the *ts* phenotype, while the double deletion of the ligases restored the growth at the non-permissive temperature (figure 11C). This suggests that the loss of viability of *pro3-1* strain is most likely due to the QC degradation of the misfolded polypeptide, leading to a depletion of the essential protein at restrictive temperature.

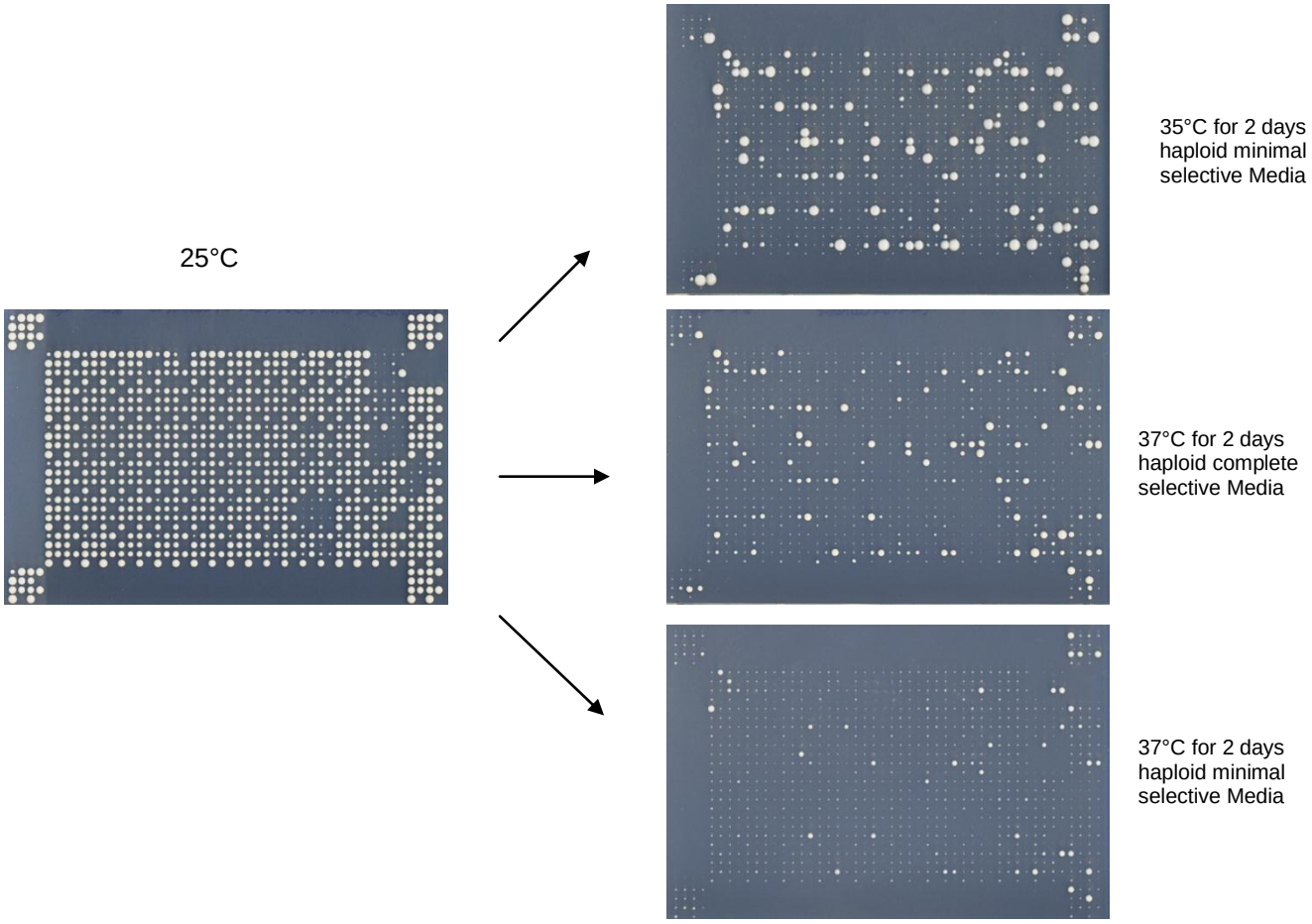


Figure 9. Thermosensitive suppression screen conditions. Haploid cells selected for thermosensitive allele and E3 deletion were pinned under three different restrictive conditions and grown for two days. The cells were grown at 35°C or 37°C in haploid selective media with 17 amino acids (synthetic complete) or haploid selective media with 5 amino acids (synthetic minimal). The colonies were scored under each conditions. The number of positive hits per thermosensitive strain was used to determine an exclusion criteria (appendixes 3-5) for each condition.

Condition	Restrictive Conditions	Total Hit	Total Hits After Exclusion
1	35°C minimal selective media	444	128
2	37°C, Complete selective media	495	183
3	37°C, minimal selective media	125	50

Table 2. Thermosensitive suppression screen summary. The total number hits were determined across three replicates for each condition. The total number of hits was also determined after exclusion of thermosensitive substrates *grs1-4* (in duplicate) and *pro3-1* in all three conditions.

E3 ligase	Substrate	Number of Positive Conditions (Hits)	Validation by Viability	Validation by Western Blot
RTT101	*guk1-6*	3 (9)	No Rescue	No Stabilization
UFO1	*guk1-6*	3 (7)	No Rescue	Not stabilization
HUL4	*gus1-3*	3 (6)	No Rescue	No Stabilization
UFO1	*gn1-1*	2 (5)	No Rescue	No Stabilization
ELA1	*gr1-3*	2 (4)	No Rescue	Not determined

Table 3. Main hits from the thermosensitive suppression screen and their characterization. The main hits were selected if the *ts* phenotype was rescued in at least two out of the three restrictive conditions and in least two out of three replicates in each condition. Haploid strains with the thermosensitive allele and E3 ligase deletion were generated independently for further characterization: 1) phenotype on synthetic complete media was assessed at 35°C and 37°C for three days, 2) stability of the mutant allele was assessed at 37°C as shown in figure 10.

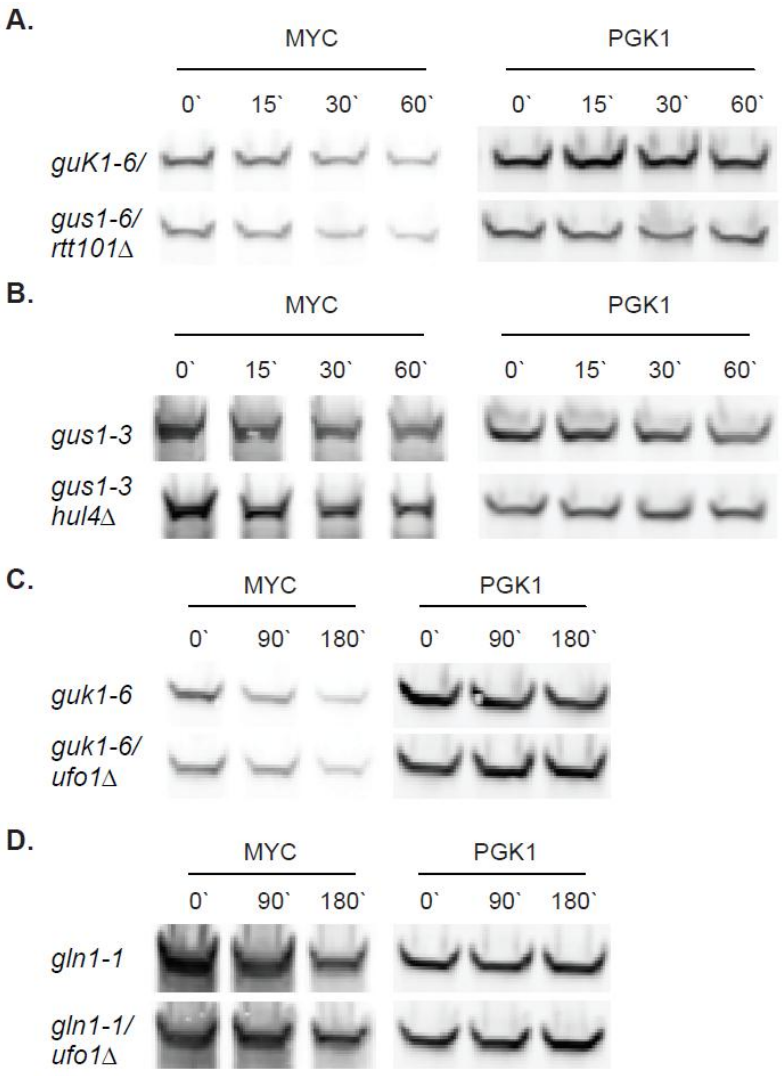


Figure 10. Thermosensitive suppression screen validation. Haploid cells with corresponding thermosensitive mutant and E3 deletion were obtained. The stability of **A.** *guk1-6/ rtt101*Δ **B.** *gus1-3/hul4*Δ **C.** *guk1-6/ ufo1*Δ*,* and **D.** *gln1-1/ufo1*Δ in the presence or absence of the indicated E3 was assessed at 37°C by cycloheximide chase analysis for the indicated time period.

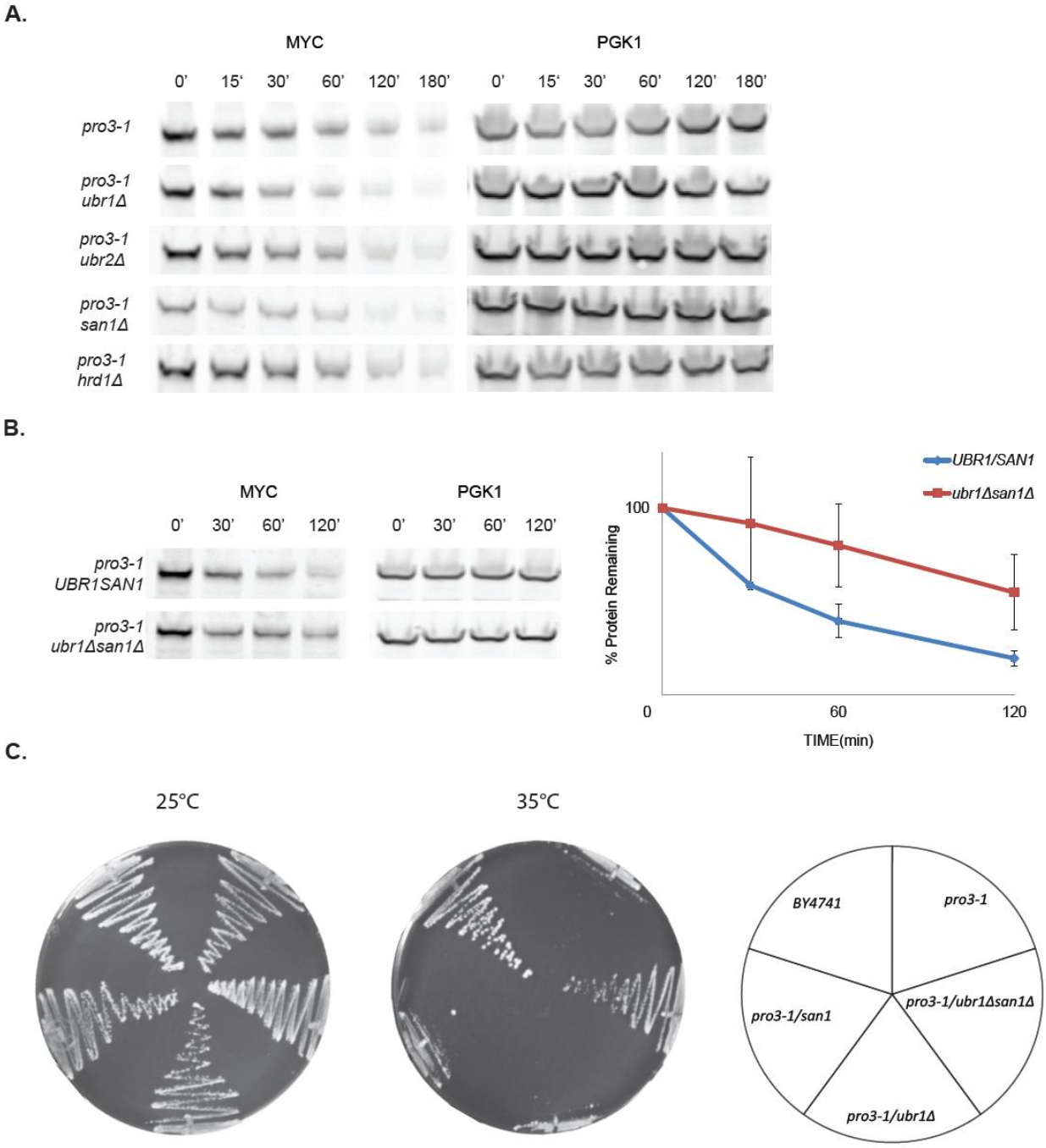


Figure 11. Deletion of *UBR1* and *SAN1* leads to a significant stabilization of Pro3-1p and the suppression of *ts* phenotype of *pro3-1* mutant at restrictive temperature Haploid *pro3-1* strains with the indicated single deletions **(A)** or double deletions **(B)** were grown to exponential phase and the stability of Pro3-1p was assessed by cycloheximide chase analysis at the indicated time at 37°C. The stability of Pro3-1p was determined by western blot analysis using 9E10 and anti-PGK1 antibodies (left panel). Levels of Pro3-1p in the presence or absence of both *UBR1 and SAN1* was measure in three replicate and indicated with standard deviations (right panel). **C)** Viability of haploids *pro3-1* strain with the indicated deletions was assessed on synthetic complete media at 25°C and 35°C for 3 days.

3.4 Discussion

It has been demonstrated previously that stabilization of protein levels of several nuclear and ER mutant proteins leads to suppression of thermosensitive phenotype under restrictive conditions [51,73]. Thus, the depletion of essential proteins leads to loss of viability for these thermosensitive mutant strains. Based on this principle, a panel of eleven unstable cytosolic thermosensitive alleles and the corresponding sixty-two E3 deletion strains (and two E2 deletion strains) were generated in a single array. We hypothesized that a subset of the unstable mutants may be targeted by a single E3 ligase and that deletion of this E3 ligase could suppress the thermosensitive phenotype. In principle, the assay should have been advantageous for two reasons. First, the screen was high throughput as twelve unstable mutants of *ts* alleles could be crossed to a collection of sixty-two putative E3 ligase deletions in a single array. Classically, E3 ligases implicated in degradative QC have been unravelled by assessing only a few E3 ligases at the time. Second, our assay did not rely on identifying protein-protein interactions. Polyubiquitylation by E3 ligases is a transient process and occurs on a millisecond timescale [102] and direct interactions between the substrate and the ligase are often difficult to detect.

The suppression of thermosensitive phenotype varied greatly based on the stringency of the restrictive conditions used (table 2). The amino acid composition of the media (17 amino acids in enriched complete media, 5 amino acids in synthetic complete media) and the restrictive temperature significantly alter the number of hits obtained in the screen. The amino acid composition of the media may affect various metabolic pathways in the cell. Insufficient complement of amino acids may stress the cell further

under restrictive conditions. Supplementation with a full complement of amino acids may compensate for lack of the essential protein. Many metabolic pathways are redundant in the cell and thus the essential metabolite can be synthesized when a full complement of amino acids is present. Suppression of thermosensitive phenotype is also dependent on the restrictive temperature. It may be possible that at high restrictive temperature (37°C), the protein misfolds to a greater extent, leading to denaturation and complete loss of function. In contrast, under semi-restrictive temperature (35°C), the stabilized misfolded protein retains function. Protein denaturation is a reversible process. The cell requires less energy to refold intermediate structures that are misfolded than proteins that are fully denatured [103].

In our study, we obtained a list of five hits that were positive in at least two out of three conditions tested and two out of three replicates under each condition. Among these identified E3s, *RTT101, UFO1,* and *ELA1* obtained the highest percentage of the positive hits among the sixty-two putative E3 ligases in our collection (appendixes 4-6). There are two possible explanations for our analysis. First, these ligases may have a broad role in protein QC and may target a large number of unstable mutants tested in our study. Conversely, deletion of these ligases may act as general suppressors where the thermosensitive phenotype is suppressed through an indirect mechanism.

To validate the hits, the strains were independently regenerated and tested. To date, we were not able to confirm a single hit from the suppression screen. We found that deletion of *RTT101* and *UFO1* did not significantly alter the stability of *guk1-6*. Similarly, deletion of *UFO1* and *HUL4* did not affect the stability of *gln1-1* and *gus1-3* respectively. We found that deletion of these ligases also did not suppress *ts* phenotype

at restrictive temperature. It may be possible that deletion of these ligases affects the random segregation of alleles and recombination events that occur during meiosis. For instance, Rtt101 is an E3 ligase implicated in DNA repair and anaphase progression [99,100,104]. Deletion of *RTT101* may affect the expression of a diverse array of proteins that compensate for the loss of the essential protein. Further, it may be possible that intragenic/extragenic suppressors were obtained in the array of cells during the screening procedure (that occurred over a three week period). The presence of extragenic mutations may lead to suppression of thermosensitive phenotype and account for the false-positive hits obtained from the screen. Further, intragenic mutations acquired during the screening process may affect the folding and stability of the protein. It has been shown that missense mutations can increase the stability of mutant proteins. In this case, the increased stability of the mutant protein may lead to viability at restrictive temperature [103]. Overall, this data indicates that the tested strategy to identify new ubiquitin ligases involved in degradative QC may not be adequate.

Our screen was based on the principle that single deletion of E3 ligases could stabilize the unstable cytosolic mutant proteins. However, it has been recently shown that E3 ligases may work in conjunction to target cytosolic substrates. For instance, the cytosolic protein quality control substrate ΔssCPY is targeted in parallel by Ubr1 and San1 [61]. These ligases have also been shown to target the cytosolic substrates ΔssPrA and Δ2GFP [64]. Further, the E3 ligase Ubr2 has been shown to augment Ubr1 activity in targeting newly synthesized unfolded protein kinases located in the cytoplasm [63]. A second caveat of the screen was that the entire collection of the putative E3

ligases was not included in our deletion collection. To date, over 90 genes have been shown to encode for known or putative E3 ligases in *Saccharomyces cerevisiae*, including thirteen E3 genes that are essential for viability in enriched medium [18]. The array of ligases tested in our collection included sixty-two E3 ligase deletion strains and thus did not encapsulate the full-spectrum of E3 ligases in *Saccharomyces cerevisiae.*

We next examined which E3 is involved in targeting Pro3-1p. To date, six of seven E3 ligases involved in protein QC have been either exclusively or partially implicated in the cytosol of *Saccharomyces cerevisiae* [51,60,61,63,64,72,73,75]. We found that single deletion of four of these E3 ligases implicated in degradative QC did not affect stability of thermosensitive substrate Pro3-1p (other three deletions are currently being test). However, double deletion of *UBR1* and *SAN1* led to a significant stabilization of the substrate under restrictive conditions. We also found that the double deletion of *UBR1* and *SAN1* restored the growth under restrictive conditions, while single deletions of *UBR1* and *SAN1* did not. This suggests that the loss of viability of the *pro3-1* mutant strain is due to the QC mediated degradation and depletion of the misfolded essential protein at restrictive temperature. In the cytoplasm, Ubr1 recognizes and ubiquitylates misfolded substrates in a chaperone dependent manner, while San1 directly targets the same substrates after nuclear transport of the substrates into the nucleus by the chaperone Sse1 and Hsp70 [61]. It was shown that the Ubr1 and San1 mediated degradation is substrate specific. The degradation of a truncated version of the enzyme 6-phosphogluconate dehydrogenase (Gndp1-GFP) is mostly dependent on San1 while degradation of a different truncation of the same substrate (stGnd1-GFP) is dependently solely on Ubr1 [61]. Thus, the degree to which substrates are recognized

by Ubr1 and San1 is substrate specific. Interestingly, San1 was shown to interact with the truncated version of the cytosolic protein Pro3 in a chaperone independent manner [53]. Ubr1 and San1 may utilize a different mechanism to target distinct misfolded substrates or different features among misfolded proteins to assure misfolded proteins in the cells are eliminated and do not accumulate as toxic oligomers in the cell.

Chapter 4. Conclusion and Future Directions

There exists a large pool of protein in the cytoplasm of eukaryotic cells. Subcellular localization studies have assigned the largest number of open reading products to the cytosolic compartment in budding yeast [105]. Further, mass spectrometry analysis of mouse liver fractions shows that about half the proteins identified are localized to the cytoplasm [106]. Physiological stress in the cell such as oxidative stress, osmotic stress, and heat-shock stress can affect the conformation of the large pool of proteins in the cytoplasm and lead to production of aberrant proteins [1]. In addition, errors in cellular processes such as translation, metabolic processes, and protein sorting and processing in the cytosol can further increase the pool of aberrant proteins in this compartment. It is sensible that the cell would evolutionarily adopt selective pathways to dispose of the aberrant proteins produced in the cytoplasm.

In this study, we showed that many cytosolic thermosensitive mutant proteins display hallmarks of protein quality control substrates at restrictive temperature. We found that half the mutant alleles examined are significantly degraded at restrictive temperature. We have also shown that Pro3-1 is stabilized and the *ts* phenotype of *pro3-1* is suppressed in the absence of Ubr1 and San1, suggesting that Pro3-1 is targeted for degradation by these two E3 ligases.

Ubr1 and San1 have been recently implicated in cytosolic degradative QC. In this study, we find that the degradation of Pro3-1p is dependent on both ligases. The degree to which Ubr1 and San1 mediate degradation is dependent on the mutant protein. Ubr1 was shown to augment the degradation of truncated cytosolic substrates but was insufficient by itself [64]. A future objective is to determine the extent to which

Ubr1 and San1 function together in cytosolic QC. We have characterized a panel of ten additional mutant proteins that display classical features of protein QC model substrates. It will be interesting to see what fraction of the unstable thermosensitive alleles are targeted by the dual action of Ubr1 and San1.

A further question to be examined is the mechanism of action of Ubr1 and San1 in cytosolic QC. It has been shown that Ubr1 recognition of misfolded substrates is chaperone-dependent [61], while San1 was recently shown to directly interact with a truncated version of the cytosolic protein Pro3 [53]. Ubr1 and San1 may utilize a different mechanism to recognize the same misfolded substrates. It has been recently shown that the E3 ligase Ufd4 functions to increase the processivity of Ubr1 in the N-end rule pathway [71]. It may be possible that Ubr1 functions in a similar manner with San1. For instance, the misfolded protein could be first ubiquitylated by Ubr1, transported in the nuclei and further ubiquitylated by San1. The panel of cytosolic mutant proteins can be used to investigate the mechanism of action of Ubr1 and San1 and infer the QC mechanism on a large array of targets in the cytosol. For instance, pull down experiments could be done to assess ubiquitylation levels of the substrates in different strains (e.g. *san1*Δ or *ubr1*Δ) or different compartment (e.g. cytoplasm vs. nuclei).

Finally, the panel of model substrates obtained can be used to screen for other E3 ligases involved in cytosolic QC. To date six ligases have been implicated in quality control in the cytoplasm [51,60,61,63,64,72,73,75]. Given the large pool of proteins in the cytosol, it is likely that more ligases will be unravelled in cytosolic QC. In our study, we find that deletion of *UBR1* and *SAN1* significantly stabilizes the mutant protein Pro3-

1. However, the protein is not fully stabilized when this pathway is deleted. It may be possible that other ligases mediate the degradation of Pro3-1. Misfolded substrates may be targeted by multiple degradation pathways in the cell.

References

[1] Buchberger, A, Bukau, B, and Sommer, T. (2010). Protein quality control in the cytosol and the endoplasmic reticulum: brothers in arms. *Mol. Cell* 40, 238-52.

[2] Hartl, FU and Hayer-Hartl, M. (2009). Converging concepts of protein folding in vitro and in vivo. *Nat. Struct. Mol. Biol.* 16, 574-81.

[3] Hol, EM and Scheper, W. (2008). Protein quality control in neurodegeneration: walking the tight rope between health and disease. *J. Mol. Neurosci.* 34, 23-33.

[4] Roth, J, Yam, GH, Fan, J, Hirano, K, Gaplovska-Kysela, K, Le Fourn, V, Guhl, B, Santimaria, R, Torossi, T, Ziak, M, and Zuber, C. (2008). Protein quality control: the who's who, the where's and therapeutic escapes. *Histochem. Cell Biol.* 129, 163-77.

[5] Pratt, WB, Morishima, Y, Peng, HM, and Osawa, Y. (2010). Proposal for a role of the Hsp90/Hsp70-based chaperone machinery in making triage decisions when proteins undergo oxidative and toxic damage. *Exp. Biol. Med. (Maywood)* 235, 278-89.

[6] Liberek, K, Lewandowska, A, and Zietkiewicz, S. (2008). Chaperones in control of protein disaggregation. *EMBO J.* 27, 328-35.

[7] Yam, AY, Xia, Y, Lin, HT, Burlingame, A, Gerstein, M, and Frydman, J. (2008). Defining the TRiC/CCT interactome links chaperonin function to stabilization of newly made proteins with complex topologies. *Nat. Struct. Mol. Biol.* 15, 1255-62.

[8] Saibil, HR. (2008). Chaperone machines in action. *Curr. Opin. Struct. Biol.* 18, 35-42.

[9] Mogk, A, Schlieker, C, Friedrich, KL, Schonfeld, HJ, Vierling, E, and Bukau, B. (2003). Refolding of substrates bound to small Hsps relies on a disaggregation reaction mediated most efficiently by ClpB/DnaK. *J. Biol. Chem.* 278, 31033-42.

[10] Haslbeck, M, Franzmann, T, Weinfurtner, D, and Buchner, J. (2005). Some like it hot: the structure and function of small heat-shock proteins. *Nat. Struct. Mol. Biol.* 12, 842-6.

[11] McClellan, AJ, Scott, MD, and Frydman, J. (2005). Folding and quality control of the VHL tumor suppressor proceed through distinct chaperone pathways. *Cell* 121, 739-48.

[12] Finley, D. (2009). Recognition and processing of ubiquitin-protein conjugates by the proteasome. *Annu. Rev. Biochem.* 78, 477-513.

[13] Schwartz, DC and Hochstrasser, M. (2003). A superfamily of protein tags: ubiquitin, SUMO and related modifiers. *Trends Biochem. Sci.* 28, 321-8.

[14] Kerscher, O, Felberbaum, R, and Hochstrasser, M. (2006). Modification of proteins by ubiquitin and ubiquitin-like proteins. *Annu. Rev. Cell Dev. Biol.* 22, 159-80.

[15] Welchman, RL, Gordon, C, and Mayer, RJ. (2005). Ubiquitin and ubiquitin-like proteins as multifunctional signals. *Nat. Rev. Mol. Cell Biol.* 6, 599-609.

[16] Pickart, CM and Eddins, MJ. (2004). Ubiquitin: structures, functions, mechanisms. *Biochim. Biophys. Acta* 1695, 55-72.

[17] Soboleva, TA and Baker, RT. (2004). Deubiquitinating enzymes: their functions and substrate specificity. *Curr. Protein Pept. Sci.* 5, 191-200.

[18] Li, W, Bengtson, MH, Ulbrich, A, Matsuda, A, Reddy, VA, Orth, A, Chanda, SK, Batalov, S, and Joazeiro, CA. (2008). Genome-wide and functional annotation of human E3 ubiquitin ligases identifies MULAN, a mitochondrial E3 that regulates the organelle's dynamics and signaling. *PLoS One* 3, e1487.

[19] Ikeda, F and Dikic, I. (2008). Atypical ubiquitin chains: new molecular signals. 'Protein Modifications: Beyond the Usual Suspects' review series. *EMBO Rep.* 9, 536-42.

[20] Peng, J, Schwartz, D, Elias, JE, Thoreen, CC, Cheng, D, Marsischky, G, Roelofs, J, Finley, D, and Gygi, SP. (2003). A proteomics approach to understanding protein ubiquitination. *Nat. Biotechnol.* 21, 921-6.

[21] Herrmann, J, Lerman, LO, and Lerman, A. (2007). Ubiquitin and ubiquitin-like proteins in protein regulation. *Circ. Res.* 100, 1276-91.

[22] Winget, JM and Mayor, T. (2010). The diversity of ubiquitin recognition: hot spots and varied specificity. *Mol. Cell* 38, 627-35.

[23] Voges, D, Zwickl, P, and Baumeister, W. (1999). The 26S proteasome: a molecular machine designed for controlled proteolysis. *Annu. Rev. Biochem.* 68, 1015-68.

[24] Chau, V, Tobias, JW, Bachmair, A, Marriott, D, Ecker, DJ, Gonda, DK, and Varshavsky, A. (1989). A multiubiquitin chain is confined to specific lysine in a targeted short-lived protein. *Science* 243, 1576-83.

[25] Xu, P, Duong, DM, Seyfried, NT, Cheng, D, Xie, Y, Robert, J, Rush, J, Hochstrasser, M, Finley, D, and Peng, J. (2009). Quantitative proteomics reveals the function of unconventional ubiquitin chains in proteasomal degradation. *Cell* 137, 133-45.

[26] Jacobson, AD, Zhang, NY, Xu, P, Han, KJ, Noone, S, Peng, J, and Liu, CW. (2009). The lysine 48 and lysine 63 ubiquitin conjugates are processed differently by the 26 s proteasome. *J. Biol. Chem.* 284, 35485-94.

[27] Hicke, L, Schubert, HL, and Hill, CP. (2005). Ubiquitin-binding domains. *Nat. Rev. Mol. Cell Biol.* 6, 610-21.

[28] Hirsch, C, Gauss, R, Horn, SC, Neuber, O, and Sommer, T. (2009). The ubiquitylation machinery of the endoplasmic reticulum. *Nature* 458, 453-60.

[29] Anelli, T and Sitia, R. (2008). Protein quality control in the early secretory pathway. *EMBO J.* 27, 315-27.

[30] Hoseki, J, Ushioda, R, and Nagata, K. (2010). Mechanism and components of endoplasmic reticulum-associated degradation. *J. Biochem.* 147, 19-25.

[31] Finger, A, Knop, M, and Wolf, DH. (1993). Analysis of two mutated vacuolar proteins reveals a degradation pathway in the endoplasmic reticulum or a related compartment of yeast. *Eur. J. Biochem.* 218, 565-74.

[32] Jung, G, Ueno, H, and Hayashi, R. (1999). Carboxypeptidase Y: structural basis for protein sorting and catalytic triad. *J. Biochem.* 126, 1-6.

[33] Carvalho, P, Goder, V, and Rapoport, TA. (2006). Distinct ubiquitin-ligase complexes define convergent pathways for the degradation of ER proteins. *Cell* 126, 361-73.

[34] Vashist, S and Ng, DT. (2004). Misfolded proteins are sorted by a sequential checkpoint mechanism of ER quality control. *J. Cell Biol.* 165, 41-52.

[35] Knop, M, Finger, A, Braun, T, Hellmuth, K, and Wolf, DH. (1996). Der1, a novel protein specifically required for endoplasmic reticulum degradation in yeast. *EMBO J.* 15, 753-63.

[36] Lilley, BN and Ploegh, HL. (2004). A membrane protein required for dislocation of misfolded proteins from the ER. *Nature* 429, 834-40.

[37] Ye, Y, Shibata, Y, Yun, C, Ron, D, and Rapoport, TA. (2004). A membrane protein complex mediates retro-translocation from the ER lumen into the cytosol. *Nature* 429, 841-7.

[38] Willer, M, Forte, GM, and Stirling, CJ. (2008). Sec61p is required for ERAD-L: genetic dissection of the translocation and ERAD-L functions of Sec61P using novel derivatives of CPY. *J. Biol. Chem.* 283, 33883-8.

[39] Scott, DC and Schekman, R. (2008). Role of Sec61p in the ER-associated degradation of short-lived transmembrane proteins. *J. Cell Biol.* 181, 1095-105.

[40] Plemper, RK, Bohmler, S, Bordallo, J, Sommer, T, and Wolf, DH. (1997). Mutant analysis links the translocon and BiP to retrograde protein transport for ER degradation. *Nature* 388, 891-5.

[41] Brodsky, JL, Werner, ED, Dubas, ME, Goeckeler, JL, Kruse, KB, and McCracken, AA. (1999). The requirement for molecular chaperones during endoplasmic reticulum-associated protein degradation demonstrates that protein export and import are mechanistically distinct. *J. Biol. Chem.* 274, 3453-60.

[42] Okuda-Shimizu, Y and Hendershot, LM. (2007). Characterization of an ERAD pathway for nonglycosylated BiP substrates, which require Herp. *Mol. Cell* 28, 544-54.

[43] Skowronek, MH, Hendershot, LM, and Haas, IG. (1998). The variable domain of nonassembled Ig light chains determines both their half-life and binding to the chaperone BiP. *Proc. Natl. Acad. Sci. U. S. A.* 95, 1574-8.

[44] Nishikawa, SI, Fewell, SW, Kato, Y, Brodsky, JL, and Endo, T. (2001). Molecular chaperones in the yeast endoplasmic reticulum maintain the solubility of proteins for retrotranslocation and degradation. *J. Cell Biol.* 153, 1061-70.

[45] Voos, W. (2009). Mitochondrial protein homeostasis: the cooperative roles of chaperones and proteases. *Res. Microbiol.* 160, 718-25.

[46] Wagner, I, Arlt, H, van Dyck, L, Langer, T, and Neupert, W. (1994). Molecular chaperones cooperate with PIM1 protease in the degradation of misfolded proteins in mitochondria. *EMBO J.* 13, 5135-45.

[47] Rottgers, K, Zufall, N, Guiard, B, and Voos, W. (2002). The ClpB homolog Hsp78 is required for the efficient degradation of proteins in the mitochondrial matrix. *J. Biol. Chem.* 277, 45829-37.

[48] von Janowsky, B, Knapp, K, Major, T, Krayl, M, Guiard, B, and Voos, W. (2005). Structural properties of substrate proteins determine their proteolysis by the mitochondrial AAA+ protease Pim1. *Biol. Chem.* 386, 1307-17.

[49] Major, T, von Janowsky, B, Ruppert, T, Mogk, A, and Voos, W. (2006). Proteomic analysis of mitochondrial protein turnover: identification of novel substrate proteins of the matrix protease pim1. *Mol. Cell. Biol.* 26, 762-76.

[50] Hatters, DM. (2008). Protein misfolding inside cells: the case of huntingtin and Huntington's disease. *IUBMB Life* 60, 724-8.

[51] Gardner, RG, Nelson, ZW, and Gottschling, DE. (2005). Degradation-mediated protein quality control in the nucleus. *Cell* 120, 803-15.

[52] Hampton, RY. (2011). San1-mediated quality control: substrate recognition "sans" chaperones. *Mol. Cell* 41, 2-3.

[53] Rosenbaum, JC, Fredrickson, EK, Oeser, ML, Garrett-Engele, CM, Locke, MN, Richardson, LA, Nelson, ZW, Hetrick, ED, Milac, TI, Gottschling, DE, and Gardner, RG. (2011). Disorder targets misorder in nuclear quality control degradation: a disordered ubiquitin ligase directly recognizes its misfolded substrates. *Mol. Cell* 41, 93-106.

[54] Connell, P, Ballinger, CA, Jiang, J, Wu, Y, Thompson, LJ, Hohfeld, J, and Patterson, C. (2001). The co-chaperone CHIP regulates protein triage decisions mediated by heat-shock proteins. *Nat. Cell Biol.* 3, 93-6.

[55] Qian, SB, McDonough, H, Boellmann, F, Cyr, DM, and Patterson, C. (2006). CHIP-mediated stress recovery by sequential ubiquitination of substrates and Hsp70. *Nature* 440, 551-5.

[56] Meacham, GC, Patterson, C, Zhang, W, Younger, JM, and Cyr, DM. (2001). The Hsc70 co-chaperone CHIP targets immature CFTR for proteasomal degradation. *Nat. Cell Biol.* 3, 100-5.

[57] Tongaonkar, P, Beck, K, Shinde, UP, and Madura, K. (1999). Characterization of a temperature-sensitive mutant of a ubiquitin-conjugating enzyme and its use as a heat-inducible degradation signal. *Anal. Biochem.* 272, 263-9.

[58] Seufert, W and Jentsch, S. (1991). Yeast ubiquitin-conjugating enzymes involved in selective protein degradation are essential for cell viability. *Acta. Biol. Hung.* 42, 27-37.

[59] Seufert, W and Jentsch, S. (1990). Ubiquitin-conjugating enzymes UBC4 and UBC5 mediate selective degradation of short-lived and abnormal proteins. *EMBO J.* 9, 543-50.

[60] Neng, F, Ng, A, Measday, V, and Mayor, T. (submitted). Hul5 HECT Ubiquitin Ligase Plays A Major Role in The Ubiquitylation and Turn Over of Cytosolic Misfolded Proteins. .

[61] Heck, JW, Cheung, SK, and Hampton, RY. (2010). Cytoplasmic protein quality control degradation mediated by parallel actions of the E3 ubiquitin ligases Ubr1 and San1. *Proc. Natl. Acad. Sci. U. S. A.* 107, 1106-11.

[62] Eisele, F and Wolf, DH. (2008). Degradation of misfolded protein in the cytoplasm is mediated by the ubiquitin ligase Ubr1. *FEBS Lett.* 582, 4143-6.

[63] Nillegoda, NB, Theodoraki, MA, Mandal, AK, Mayo, KJ, Ren, HY, Sultana, R, Wu, K, Johnson, J, Cyr, DM, and Caplan, AJ. (2010). Ubr1 and ubr2 function in a quality control pathway for degradation of unfolded cytosolic proteins. *Mol. Biol. Cell* 21, 2102-16.

[64] Prasad, R, Kawaguchi, S, and Ng, DT. (2010). A nucleus-based quality control mechanism for cytosolic proteins. *Mol. Biol. Cell* 21, 2117-27.

[65] Varshavsky, A. (2008). Discovery of cellular regulation by protein degradation. *J. Biol. Chem.* 283, 34469-89.

[66] Hwang, CS and Varshavsky, A. (2008). Regulation of peptide import through phosphorylation of Ubr1, the ubiquitin ligase of the N-end rule pathway. *Proc. Natl. Acad. Sci. U. S. A.* 105, 19188-93.

[67] Du, F, Navarro-Garcia, F, Xia, Z, Tasaki, T, and Varshavsky, A. (2002). Pairs of dipeptides synergistically activate the binding of substrate by ubiquitin ligase through dissociation of its autoinhibitory domain. *Proc. Natl. Acad. Sci. U. S. A.* 99, 14110-5.

[68] Xia, Z, Webster, A, Du, F, Piatkov, K, Ghislain, M, and Varshavsky, A. (2008). Substrate-binding sites of UBR1, the ubiquitin ligase of the N-end rule pathway. *J. Biol. Chem.* 283, 24011-28.

[69] Dohmen, RJ, Madura, K, Bartel, B, and Varshavsky, A. (1991). The N-end rule is mediated by the UBC2(RAD6) ubiquitin-conjugating enzyme. *Proc. Natl. Acad. Sci. U. S. A.* 88, 7351-5.

[70] Watkins, JF, Sung, P, Prakash, S, and Prakash, L. (1993). The extremely conserved amino terminus of RAD6 ubiquitin-conjugating enzyme is essential for amino-end rule-dependent protein degradation. *Genes Dev.* 7, 250-61.

[71] Hwang, CS, Shemorry, A, Auerbach, D, and Varshavsky, A. (2010). The N-end rule pathway is mediated by a complex of the RING-type Ubr1 and HECT-type Ufd4 ubiquitin ligases. *Nat. Cell Biol.* 12, 1177-85.

[72] Lewis, MJ and Pelham, HR. (2009). Inefficient quality control of thermosensitive proteins on the plasma membrane. *PLoS One* 4, e5038.

[73] Ravid, T, Kreft, SG, and Hochstrasser, M. (2006). Membrane and soluble substrates of the Doa10 ubiquitin ligase are degraded by distinct pathways. *EMBO J.* 25, 533-43.

[74] Metzger, MB, Maurer, MJ, Dancy, BM, and Michaelis, S. (2008). Degradation of a cytosolic protein requires endoplasmic reticulum-associated degradation machinery. *J. Biol. Chem.* 283, 32302-16.

[75] Bengtson, MH and Joazeiro, CA. (2010). Role of a ribosome-associated E3 ubiquitin ligase in protein quality control. *Nature* 467, 470-3.

[76] Wolf, DH and Schafer, A. (2005). CPY* and the power of yeast genetics in the elucidation of quality control and associated protein degradation of the endoplasmic reticulum. *Curr. Top. Microbiol. Immunol.* 300, 41-56.

[77] Betting, J and Seufert, W. (1996). A yeast Ubc9 mutant protein with temperature-sensitive in vivo function is subject to conditional proteolysis by a ubiquitin- and proteasome-dependent pathway. *J. Biol. Chem.* 271, 25790-6.

[78] Ben-Aroya, S, Pan, X, Boeke, JD, and Hieter, P. (2010). Making temperature-sensitive mutants. *Methods Enzymol.* 470, 181-204.

[79] Ben-Aroya, S, Coombes, C, Kwok, T, O'Donnell, KA, Boeke, JD, and Hieter, P. (2008). Toward a comprehensive temperature-sensitive mutant repository of the essential genes of Saccharomyces cerevisiae. *Mol. Cell* 30, 248-58.

[80] Longtine, MS, McKenzie, A,3rd, Demarini, DJ, Shah, NG, Wach, A, Brachat, A, Philippsen, P, and Pringle, JR. (1998). Additional modules for versatile and economical PCR-based gene deletion and modification in Saccharomyces cerevisiae. *Yeast* 14, 953-61.

[81] Rothstein, R. (1991). Targeting, disruption, replacement, and allele rescue: integrative DNA transformation in yeast. *Methods Enzymol.* 194, 281-301.

[82] Baryshnikova, A, Costanzo, M, Dixon, S, Vizeacoumar, FJ, Myers, CL, Andrews, B, and Boone, C. (2010). Synthetic genetic array (SGA) analysis in Saccharomyces cerevisiae and Schizosaccharomyces pombe. *Methods Enzymol.* 470, 145-79.

[83] Tong, AH and Boone, C. (2006). Synthetic genetic array analysis in Saccharomyces cerevisiae. *Methods Mol. Biol.* 313, 171-92.

[84] Liu, C, Apodaca, J, Davis, LE, and Rao, H. (2007). Proteasome inhibition in wild-type yeast Saccharomyces cerevisiae cells. *BioTechniques* 42, 158, 160, 162.

[85] Brandriss, MC and Falvey, DA. (1992). Proline biosynthesis in Saccharomyces cerevisiae: analysis of the PRO3 gene, which encodes delta 1-pyrroline-5-carboxylate reductase. *J. Bacteriol.* 174, 5176.

[86] Meng, Z, Lou, Z, Liu, Z, Li, M, Zhao, X, Bartlam, M, and Rao, Z. (2006). Crystal structure of human pyrroline-5-carboxylate reductase. *J. Mol. Biol.* 359, 1364-77.

[87] Galani, K, Grosshans, H, Deinert, K, Hurt, EC, and Simos, G. (2001). The intracellular location of two aminoacyl-tRNA synthetases depends on complex formation with Arc1p. *EMBO J.* 20, 6889-98.

[88] Delarue, M. (1995). Aminoacyl-tRNA synthetases. *Curr. Opin. Struct. Biol.* 5, 48-55.

[89] Benjamin, PM, Wu, JI, Mitchell, AP, and Magasanik, B. (1989). Three regulatory systems control expression of glutamine synthetase in Saccharomyces cerevisiae at the level of transcription. *Mol. Gen. Genet.* 217, 370-7.

[90] Shimma, Y, Nishikawa, A, bin Kassim, B, Eto, A, and Jigami, Y. (1997). A defect in GTP synthesis affects mannose outer chain elongation in Saccharomyces cerevisiae. *Mol. Gen. Genet.* 256, 469-80.

[91] Lai, K and Elsas, LJ. (2000). Overexpression of human UDP-glucose pyrophosphorylase rescues galactose-1-phosphate uridyltransferase-deficient yeast. *Biochem. Biophys. Res. Commun.* 271, 392-400.

[92] Creighton, TE. (1990). Prediction of Protein Structure and the Principles of Protein Conformation. Gerald D. Fasman, Ed. Plenum, New York, 1989. xiv, 798 pp., illus. $95. *Science* 247, 1351-2.

[93] Creighton, TE. (1990). Protein folding. *Biochem. J.* 270, 1-16.

[94] Darby, NJ and Creighton, TE. (1990). Folding proteins. *Nature* 344, 715-6.

[95] Blaszczyk, J, Li, Y, Yan, H, and Ji, X. (2001). Crystal structure of unligated guanylate kinase from yeast reveals GMP-induced conformational changes. *J. Mol. Biol.* 307, 247-57.

[96] Polymeropoulos, MH, Lavedan, C, Leroy, E, Ide, SE, Dehejia, A, Dutra, A, Pike, B, Root, H, Rubenstein, J, Boyer, R, Stenroos, ES, Chandrasekharappa, S, Athanassiadou, A, Papapetropoulos, T, Johnson, WG, Lazzarini, AM, Duvoisin, RC, Di Iorio, G, Golbe, LI, and Nussbaum, RL. (1997). Mutation in the alpha-synuclein gene identified in families with Parkinson's disease. *Science* 276, 2045-7.

[97] Yonetani, M, Nonaka, T, Masuda, M, Inukai, Y, Oikawa, T, Hisanaga, S, and Hasegawa, M. (2009). Conversion of wild-type alpha-synuclein into mutant-type fibrils and its propagation in the presence of A30P mutant. *J. Biol. Chem.* 284, 7940-50.

[98] Boone, C, Bussey, H, and Andrews, BJ. (2007). Exploring genetic interactions and networks with yeast. *Nat. Rev. Genet.* 8, 437-49.

[99] Baldwin, EL, Berger, AC, Corbett, AH, and Osheroff, N. (2005). Mms22p protects Saccharomyces cerevisiae from DNA damage induced by topoisomerase II. *Nucleic Acids Res.* 33, 1021-30.

[100] Michel, JJ, McCarville, JF, and Xiong, Y. (2003). A role for Saccharomyces cerevisiae Cul8 ubiquitin ligase in proper anaphase progression. *J. Biol. Chem.* 278, 22828-37.

[101] Kaplun, L, Ivantsiv, Y, Bakhrat, A, and Raveh, D. (2003). DNA damage response-mediated degradation of Ho endonuclease via the ubiquitin system involves its nuclear export. *J. Biol. Chem.* 278, 48727-34.

[102] Pierce, NW, Kleiger, G, Shan, SO, and Deshaies, RJ. (2009). Detection of sequential polyubiquitylation on a millisecond timescale. *Nature* 462, 615-9.

[103] Shortle, D. (1996). The denatured state (the other half of the folding equation) and its role in protein stability. *FASEB J.* 10, 27-34.

[104] Scholes, DT, Banerjee, M, Bowen, B, and Curcio, MJ. (2001). Multiple regulators of Ty1 transposition in Saccharomyces cerevisiae have conserved roles in genome maintenance. *Genetics* 159, 1449-65.

[105] Huh, WK, Falvo, JV, Gerke, LC, Carroll, AS, Howson, RW, Weissman, JS, and O'Shea, EK. (2003). Global analysis of protein localization in budding yeast. *Nature* 425, 686-91.

[106] Foster, LJ, de Hoog, CL, Zhang, Y, Zhang, Y, Xie, X, Mootha, VK, and Mann, M. (2006). A mammalian organelle map by protein correlation profiling. *Cell* 125, 187-99.

Appendix 1. List of Mutant Yeast Strains Used in this Study

Name	Genotype
YFK86	*MATa ura3Δ0 leu2Δ0 his3Δ1 lys2Δ0* (or *LYS2) met15Δ0* (or *MET15) can1Δ::LEU2-MFA1pr::HIS3, grs1-1-13myc::KanMX6::URA3*
YFK87	*MATa ura3Δ0 leu2Δ0 his3Δ1 lys2Δ0* (or *LYS2) met15Δ0* (or *MET15) can1Δ::LEU2-MFA1pr::His, grs1-2-13myc::KanMX6::URA3*
YFK89	*MATa ura3Δ0 leu2Δ0 his3Δ1 lys2Δ0* (or *LYS2) met15Δ0* (or *MET15) can1Δ::LEU2-MFA1pr::His3 grs1-3-13myc::KanMX6::URA3*
YFK90	*MATa ura3Δ0 leu2Δ0 his3Δ1 lys2Δ0* (or *LYS2) met15Δ0* (or *MET15) can1Δ::LEU2-MFA1pr::His3 grs1-4-13myc::KanMX6::URA3*
YFK92	*MATa ura3Δ0 leu2Δ0 his3Δ1 lys2Δ0* (or *LYS2) met15Δ0* (or *MET15) can1Δ::LEU2-MFA1pr::His3 pro3-1-13myc::KanMX6::URA3*
YFK93	*MATa ura3Δ0 leu2Δ0 his3Δ1 lys2Δ0* (or *LYS2) met15Δ0* (or *MET15) can1Δ::LEU2-MFA1pr::His3 pro3-2-13myc::KanMX6::URA3*
YFK94	*MATa ura3Δ0 leu2Δ0 his3Δ1 lys2Δ0* (or *LYS2) met15Δ0* (or *MET15) can1Δ::LEU2-MFA1pr::His3 ugp1-1-13myc::KanMX6::URA3*
YFK95	*MATa ura3Δ0 leu2Δ0 his3Δ1 lys2Δ0* (or *LYS2) met15Δ0* (or *MET15) can1Δ::LEU2-MFA1pr::His3 ugp1-2-13myc::KanMX6::URA3*
YFK96	*MATa ura3Δ0 leu2Δ0 his3Δ1 lys2Δ0* (or *LYS2) met15Δ0* (or *MET15) can1Δ::LEU2-MFA1pr::His3 ugp1-3-13myc::KanMX6::URA3*
YFK97	*MATa ura3Δ0 leu2Δ0 his3Δ1 lys2Δ0* (or *LYS2) met15Δ0* (or *MET15) can1Δ::LEU2-MFA1pr::His3 ugp1-4-13myc::KanMX6::URA3*
YFK99	*MATa ura3Δ0 leu2Δ0 his3Δ1 lys2Δ0* (or *LYS2) met15Δ0* (or *MET15) can1Δ::LEU2-MFA1pr::His3 ugp1-5-13myc::KanMX6::URA3*
YFK100	*MATa ura3Δ0 leu2Δ0 his3Δ1 lys2Δ0* (or *LYS2) met15Δ0* (or *MET15) can1Δ::LEU2-MFA1pr::His3 guk1-1-13myc::KanMX6::URA3*
YFK101	*MATa ura3Δ0 leu2Δ0 his3Δ1 lys2Δ0* (or *LYS2) met15Δ0* (or *MET15) can1Δ::LEU2-MFA1pr::His3 guk1-2-13myc::KanMX6::URA3*

Name	Genotype
YFK104	*MATa ura3Δ0 leu2Δ0 his3Δ1 lys2Δ0* (or *LYS2) met15Δ0* (or *MET15) can1Δ::LEU2-MFA1pr::His3 guk1-3-13myc::KanMX6*
YFK105	*MATa ura3Δ0 leu2Δ0 his3Δ1 lys2Δ0* (or *LYS2) met15Δ0* (or *MET15) can1Δ::LEU2-MFA1pr::His3 grs1-4-13myc::KanMX6*
YFK106	*MATa ura3Δ0 leu2Δ0 his3Δ1 lys2Δ0* (or *LYS2) met15Δ0* (or *MET15) can1Δ::LEU2-MFA1pr::His3 guk1-5-13myc::KanMX6::URA3*
YFK108	*MATa ura3Δ0 leu2Δ0 his3Δ1 lys2Δ0* (or *LYS2) met15Δ0* (or *MET15) can1Δ::LEU2-MFA1pr::His3 guk1-6-13myc::KanMX6::URA3*
YFK109	*MATa ura3Δ0 leu2Δ0 his3Δ1 lys2Δ0* (or *LYS2) met15Δ0* (or *MET15) can1Δ::LEU2-MFA1pr::His3 gus1-1-13myc::KanMX6::URA3*
YFK110	*MATa ura3Δ0 leu2Δ0 his3Δ1 lys2Δ0* (or *LYS2) met15Δ0* (or *MET15) can1Δ::LEU2-MFA1pr::His3 gus1-2-ts::URA3, gus1-2::13myc-KanMX6*
YFK111	*MATa ura3Δ0 leu2Δ0 his3Δ1 lys2Δ0* (or *LYS2) met15Δ0* (or *MET15) can1Δ::LEU2-MFA1pr::His3 gus1-3-13myc::KanMX6*
YFK112	*MATa ura3Δ0 leu2Δ0 his3Δ1 lys2Δ0* (or *LYS2) met15Δ0* (or *MET15) can1Δ::LEU2-MFA1pr::His3 gln1-1-13myc::KanMX6*
YFK115	*MATa ura3Δ0 leu2Δ0 his3Δ1 lys2Δ0* (or *LYS2) met15Δ0* (or *MET15) can1Δ::LEU2-MFA1pr::His3 gln1-2-13myc::KanMX6::URA3*
YFK141	*MATa, ura3Δ0, leu2Δ0, his3Δ1, lys2Δ, MET15, CAN1, guk1-6-13myc::KanMX6::URA3*
YFK143	*MATa, ura3Δ0, leu2Δ0, his3Δ1, lys2Δ0, met15Δ0, CAN1, gln1-1-13myc::KanMX6*
YFK145	*MATa, ura3Δ0, leu2Δ0, his3Δ1, LYS2, met15Δ0, CAN1, guk1-1-13myc::KanMX6::URA3*
YFK147	*MATa, ura3Δ0, leu2Δ0, his3Δ1, LYS2, met15Δ0, CAN1, gus1-3-13myc::KanMX6*
YFK148	*MATa, ura3Δ0, leu2Δ0, his3Δ1, lys2Δ0, MET15, CAN1, gln1-2-13myc::KanMX6::URA3*

Name	Genotype
YFK150	*MATa, ura3Δ0, leu2Δ0, his3Δ1, LYS2, MET15, CAN1, pro3-1-13myc::KanMX6::URA3*
YFK152	*MATa, ura3Δ0, leu2Δ0, his3Δ1, lys2Δ0, met15Δ0, CAN1, ugp1-4-13myc::KanMX6::URA3*
YFK156	*MATa, ura3Δ0, leu2Δ0, his3Δ1, lys2Δ0, MET15, CAN1, ugp1-3-13myc::KanMX6::URA3*
YFK159	*MATa, ura3Δ0, leu2Δ0, his3Δ1, lys2Δ0, MET15, CAN1, guk1-3-13myc::KanMX6*
YFK161	*MATa, ura3Δ0, leu2Δ0, his3Δ1, lys2Δ0, MET15, CAN1, guk1-2-13myc::KanMX6::URA3*
YFK163	*MATa, ura3Δ0, leu2Δ0, his3Δ1, lys2Δ0, MET15, CAN1, grs1-4-13myc::KanMX6*
YFK167	*MATa, ura3Δ0, leu2Δ0, his3Δ1, LYS2, met15Δ0, CAN1, cdc68-1-13myc::KanMX6*
YFK169	*MATα, ura3Δ0, leu2Δ0, his3Δ1, met15Δ0, lyp1Δ, can1Δ::STE2pr-Sp_his5, HOΔNatMX6*
YFK189	*MATa pro3-1-13myc::KanMX6::URA3, san1Δ::NatMX6, his3Δ1, leu2Δ0, lys2Δ0* (or *LYS2*), *met15Δ0* (or *MET15*), *ura3Δ0, CAN1*
YFK196	*MATa pro3-1-13myc::KanMX6::URA3, ubr1Δ::NatMX6, his3Δ1, leu2Δ0, LYS2, MET15 (or met15Δ)*
YFK200	*MATa pro3-1-13myc::KanMX6, ubr2Δ::NatMX6, his3Δ1, leu2Δ0, LYS2, MET15 (met15Δ)*
YFK204	*MATa pro3-1-13myc::KanMX6, hrd1Δ::NatMX6, his3Δ1, leu2Δ0, LYS2, MET15 (met15Δ)*
YFK216	*MATa pro3-1-13myc::KanMX6, ubr1Δ::NatMX6, his3Δ1, san1Δ::HisMX6, leu2Δ0, LYS2, MET15 (met15Δ)*

Appendix 2. List of E2 and E3 Single Deletion Strains Used in Thermosensitive Suppression Screen and Arrangement of the Thermosensitive Suppression Screen Array

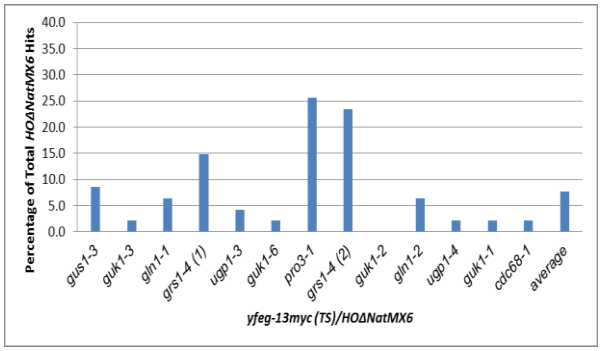
	1	2	3	4	5	6	7	8	9	10	11	12
A	*HO∆ NatMX6*											*HO∆ NatMX6*
B		*HRD1*	*MAG2*	*RAD16*	*TOM1*	*YBR062C*	*YIL001W*	*YDR131C*	*ASI1*	*ELA1*	*MOT2*	
C		*HUL4*	*RAD5*	*UBR1*	*NAM7*	*SAF1*	*YDR049W*	*MFB1*	*ASI3*	*CUL3*	*SLX8*	*TFB3*
D		*HUL5*	*RAD18*	*PSH1*	*SSM4 (DOA10)*	*RKR1*	*YMR258C*	*YDR306C*	*RTT101*	*PEX10*	*GRR1*	*YLR181C*
E		*UBC4*	*DMA2*	*ELC1*	*SAN1*	*UFD2*	*BRP2*	*YLR224*	*VPS8*	*UFD4*	*TUL1*	*YLR148W*
F		*DAS1*	*RMD5 (GID2)*	*HRT3*	*MDM30*	*YKR017C*	*ASR1*	*SKP2*	*DIA2*	*PIB1*	*HO:: NatMX6*	*YDR132C*
G		*YLR352W*	*RAD7*	*CDH1 (HCT1)*	*IRC20*	*YDR266C*	*COS111*	*UBR2*	*BRE1*	*UBC5*	*UFO1*	*YBR158*
H	*HO∆ NatMX6*											*HO∆ NatMX6*

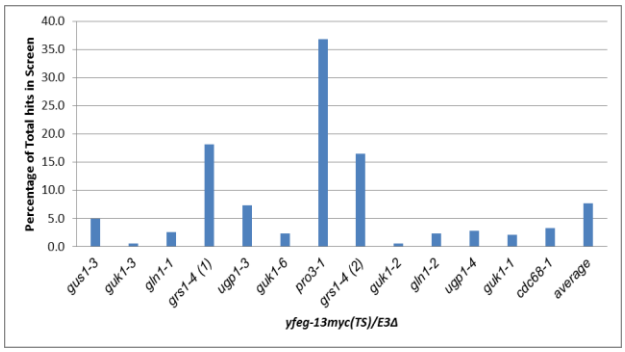
Appendix 3. *GUK1* Thermosensitive Allele Mutations

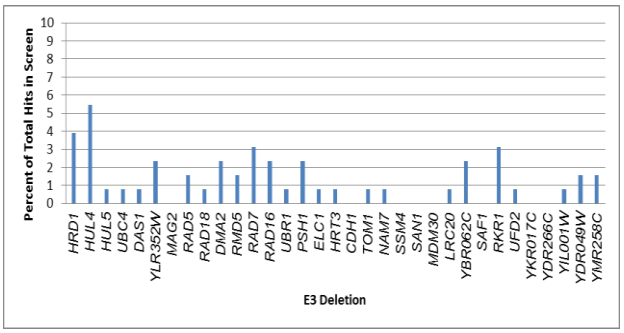
TS Allele	Amino Acid Substitutions	Mutation Position
guk1-1	F→H A→T T→A E→K	59→α-helix 84→α-helix 95→β-sheet 127→α-helix
guk1-2	L→S E→G	22→ α-helix 67→unstructured
guk1-3	M→K I→T S→F	102→ α-helix 121→ β-sheet 143→ α-helix
guk1-4	F→S P→T K→R	59→ α-helix 112→H-bonded turn 177→ α-helix
guk1-5	S→P N→Y	29→ H-bonded turn 77→ β-sheet
guk1-6	I→S E→G	97→ β-sheet 141→bend

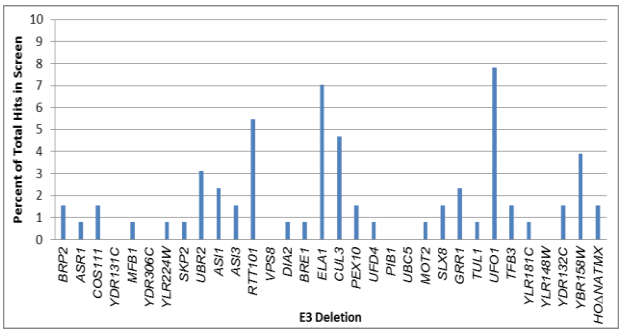
Appendix 3. *GUK1* mutants were PCR amplified from the endogenous loci and the open reading frame was sequenced. The number of missense mutations was determined by alignment of the primary sequence wild-type allele. The mutation position in the primary sequence was determined from *GUK1* crystal structure from RCSB protein data bank [95].

Appendix 4. Thermosensitive Suppression Screen Analysis at 35°C on Haploid Minimal Selective Media







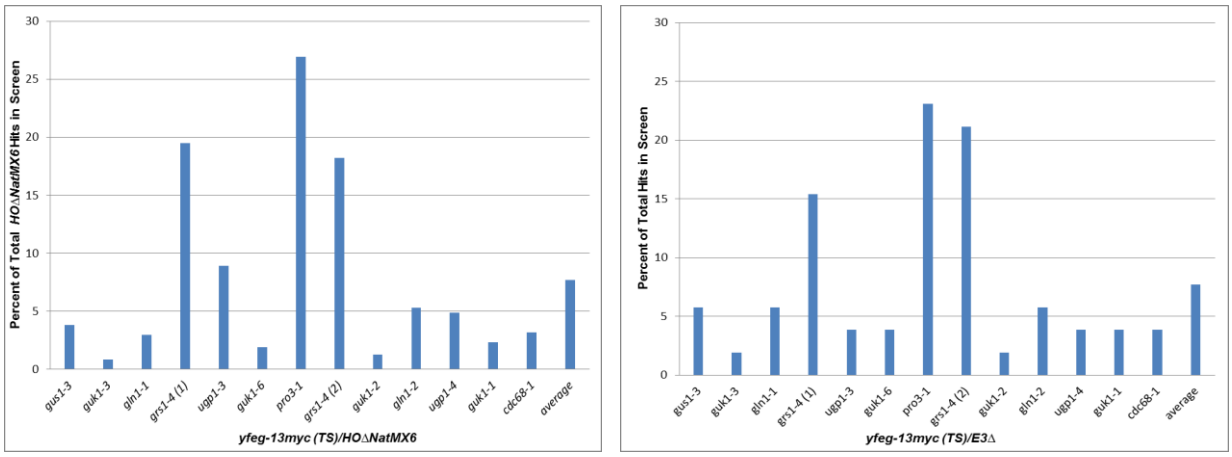


E3 Ligase	Substrate	Total Hits
RTT101	*guk1-6*	3
RAD18	*ugp1-3*	3
UFO1	*guk1-6*	3
HUL4	*gus1-3,gln1-2*	2
HUL5	*gln1-1*	2
YLR352	*ugp1-3*	2
YDR266C	*ugp1-3*	2
RTT101	*ugp1-3,guk1-1*	2
ELA1	*gus1-3,cdc68-1*	2
UFO1	*gln1-2,guk1-1*	2
UFD4	*guk1-3*	2
SLX8	*ugp1-3,ugp1-4*	2
YDR132C	*gln1-2*	2
YDR158W	*ugp1-3,gln1-1,gus1-3*	2
YBR062C	*gln1-1*	2

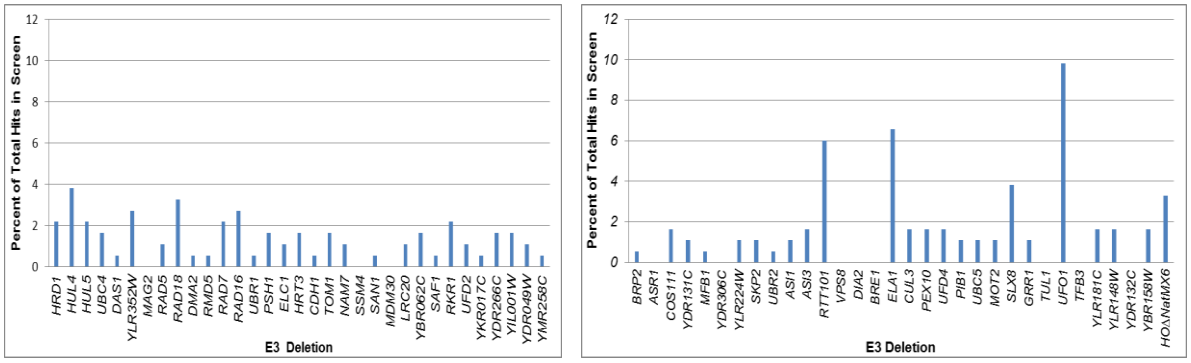
Appendix 4. A. Temperature-sensitive/E3Δ deletion array was pinned on haploid minimal selective media at 25°C and 35°C for two days. The thermosensitive strains were crossed to a strain with deletion of *HO* loci (negative control). The percent positive hits was calculated as a fraction of each thermosensitive strain with *NAT*^{R} integrated at *HO* loci growing at 35°C over the total number of cells with the integrated cassette under the same conditions (left panel). Based on this criteria, mutants *pro3-1* and *grs1-4* were excluded from the analysis. The percent of positive hits per substrate was calculated as the number of positive hits per thermosensitive strain over the total number of positive hits in the screen (right panel). **B.** The positive hits for each ligase Is calcuated as a hit per given ligase deletion strain over the total number of hits in the screen. **C.** Final list of hits was determined for colonies growing on at least two out of three replicates.

Appendix 5. Thermosensitive Suppression Screen Analysis at 37°C on Haploid Complete Selective Media

A.



B.



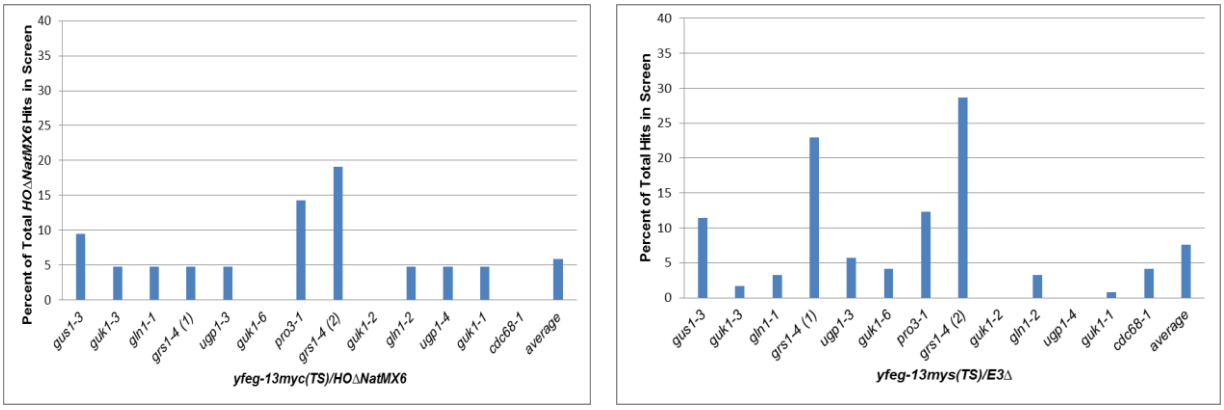
C.

E3 Ligase	Substrate	Hits
RTT101	*guk1-6*	3
HUL4	*gus1-3*	2
RAD7	*gus1-3*	2
YBR062C	*ugp1-4*	2
ELA1	*gus1-3,ugp1-4*	2
CUL3	*ugp1-4*	2
UFO1	*ugp1-4,guk1-6,gln1-1*	2
YBR158W	*ugp1-4*	2

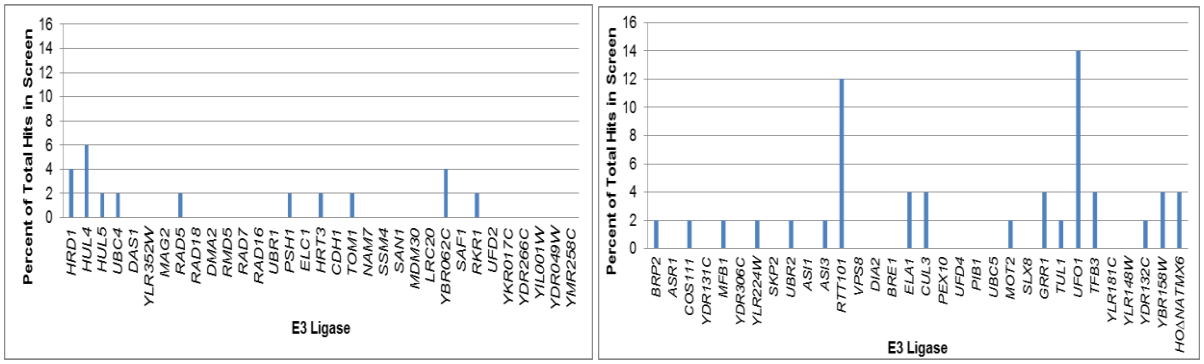
Appendix 5. A. Temperature-sensitive/E3Δ deletion array was pinned on haploid complete selective media at 25°C and 37°C for two days. The thermosensitive strains were crossed to a strain with deletion of *HO* loci (negative control). The percent positive hits was calculated as a fraction of each thermosensitive sensitive strain with *NAT*^{R} integrated at *HO* loci growing at 37°C over the total number of cells with the integrated cassette under the same conditions (left panel). Based on this criteria, mutants *pro3-1* and *grs1-4* were excluded from the analysis. The percent of positive hits per substrate was calculated as the number of positive hits per thermosensitive strain over the total number of positive hits in the screen (right panel). **B.** The positive hits for each ligase is calcuated as a hit per given ligase deletion strain over the total number of hits in the screen. **C.** The final list of hits was determined by growth on at least two out of three replicates.

Appendix 6. Thermosensitive Suppression Screen Analysis at 37°C on Haploid Minimal Selective Media

A.



B.



C.

E3 Ligase	Substrate	Total Hits
RTT101	*guk1-6*	3
HUL4	*gus1-3*	2
UFO1	*ugp1-4,guk1-6, gln1-1*	2

Appendix 6. A. Temperature-sensitive/E3Δ deletion array was pinned on haploid minimal selective media at 25°C and 37°C for two days. The thermosensitive strains were crossed to a strain with deletion of *HO* loci (negative control). The percent positive hits was calculated as a fraction of each thermosensitive sensitive strain with deletion of HO loci growing at 35°7 over the total number of cells with deletion of *HO* loci under the same conditions (left panel). Based on this criteria, mutants *pro3-1*, *grs1-4,* and *gus1-3* were excluded from the analysis. The percent of positive hits per substrate was calculated as the number of positive hits per thermosensitive strain over the total number of positive hits in the screen (right panel). **B.** The positive hits for each ligase Is calcuated as a hit per given ligase deletion strain over the total number of hits in the screen. **C.** The final list of hits was determined by growth on at least two out of three replicates.