

HSP70 Isoforms Ssa1 and Ssa2 are Differentially Required in San1 Mediated
Degradation

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Abstract

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Misfolded proteins have the propensity to aggregate and become toxic to the cell if they accumulate. To counter this detrimental burden, the eukaryotic cell has developed a variety of protein quality control (PQC) mechanisms to eliminate misfolded proteins from the cell. One mechanism is the coordination between chaperones and degradation machinery, which can help the cell rid itself of misfolded proteins. In the cytosol and endoplasmic reticulum, chaperones are often required for the degradation of misfolded proteins. In the nucleus, however, the requirement for chaperones in the degradation of nuclear localized misfolded proteins is not clear. Previously, we found that the yeast ubiquitin-protein ligase San1 ubiquitinates misfolded nuclear proteins for proteasomal degradation. It has been shown that Ssa1 and Ssa2 are the dominantly expressed isoforms of HSP70 and that they are required for the degradation of some substrates that were originally thought to be cytosolic, but were found to be nuclear localized. Here we explored the requirement for Ssa1 and Ssa2 in San1-mediated degradation and found that the involvement of the Hsp70 chaperones Ssa1 and Ssa2 is not universal, and the requirement for Ssa1 and Ssa2 correlates with substrate solubility.

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LIST OF ABBREVIATIONS

PQC Protein quality control
NucPQC Nuclear Protein quality control
HSP Heat Shock Protein
RING Really interesting new gene
NLS Nuclear localization signal
GFP Green fluorescence protein
GBD Gal4 binding domain
GAD Gal4 activation domain
UPR Unfolded protein response
ERAD ER-associated degradation
INQ Intranuclear quality control compartment
IPOD Insoluble protein deposit
UPS Ubiquitin proteasome system

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PREFACE

A portion of the data included in this dissertation has been derived from the following publications as well as unpublished data from our lab obtained by previous lab members:

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DEDICATION

To my parents, Mark and Leigh Jones, for allowing me to pursue a career of my choice. Also, to my fur children Kai (German Shepherd), Sapheria (American Pitbull), and Drogo the Duckling (American Bully). I'd also like to mention my future wife, Kimberly Krupa, for her tremendous support during my time in graduate school. In addition, I also want to dedicate this dissertation to every person and educator who has ever expected, or told me that I'd grow up to be a failure. Without you all I would have never had the drive to prove you all wrong.

"They said he would amount to nothing... they said." –Unknown

CHAPTER I: INTRODUCTION AND SIGNIFICANCE

Protein homeostasis is important to maintain for the survival of the cell. Each protein has a specific, or subset of functions that contribute to the viability of the cell. In order for proteins to function correctly, they must adopt a proper three-dimensional folding state. Due to the fact that the cellular environment is exposed to a variety of intrinsic and extrinsic factors; it is possible for protein synthesis and folding to become impaired. If a protein is not correctly folded it normally loses its function, and can take on aggregation-prone misfolded folded states, which can lead to cellular toxicity. Misfolded proteins can arise from by a variety of different avenues including, but not limited to translational errors, errors in nascent peptide folding, chemical exposure and cellular stress. It is imperative that the cell manages misfolded proteins because they can associate into toxic aggregate forms. Protein aggregation is known to be the underlying cause for 35+ human neurodegenerative disorders including Alzheimer's disease, Parkinson's disease, Huntington's disease, and amyotrophic lateral sclerosis (Skovronsky et al., 2006; Wang et al., 2008).

The cell handles misfolded proteins by employing protein quality control (PQC) systems that can be generally categorized into two classes. The protein folding class mitigates the effects of misfolded proteins through the folding, refolding, segregation and disaggregation activities of protein chaperones (Chen et al., 2011; Verghese et al., 2012). The protein degradation class eliminates misfolded proteins through targeted proteolysis, which in eukaryotes typically involves the ubiquitin proteasome system (Fredrickson and Gardner, 2012). The combined activities of folding and degradation PQC systems provide the cell with a broad range of capabilities essential to manage the

wide variety of misfolded proteins stochastically produced in the cell. This two-class system is well established in the cytosol and the ER, but it is poorly understood in the nucleus. In the following chapters I will discuss how chaperones from the folding class of PQC interact with degradation class machinery to facilitate the removal of misfolded proteins from the nucleus.

The ubiquitination enzymatic cascade

One means by which PQC occurs in the cell is through degradation of misfolded proteins. PQC degradation generally occurs when the misfolded burden becomes so high that it is more energetically favorable for the cell to destroy the protein than repair it (Goldberg, 2003). It is also thought that destruction is invoked when a protein simply can never achieve a correct fold and is constitutively misfolded, like most of the mutants we use. PQC degradation happens most commonly, in *Saccharomyces cerevisiae*, through the action of the ubiquitin-proteasome system (UPS). The UPS post-translationally modifies proteins with the small protein called ubiquitin that acts as a signal for proteasomal recognition. In addition to mediating PQC degradation, ubiquitin is known to have a variety of functions: it can be used as a signal for protein degradation; it can alter protein-protein interactions; and it can also alter protein localization and activity in the cell (Glickman and Ciechanover, 2002; Mukhopadhyay et al., 2007; Schnell et al., 2003). Ubiquitin gets covalently attached to target substrates at lysine residues in an active process called, ubiquitination or ubiquitylation. Ubiquitin is conserved from the budding yeast *Saccharomyces cerevisiae* to mammals, with humans and yeast sharing 96% ubiquitin sequence identity (Ray et al., 2010). Each ubiquitin molecule contains 7

distinct lysine residues that can be used as sites for additional ubiquitin attachment resulting in distinct ubiquitin chains (Glickman and Ciechanover, 2002). Ubiquitination of targets can occur as a monoubiquitination, polyubiquitination or as ubiquitin chains. Ubiquitination can occur in different ways depending on what process ubiquitin is being used for. In addition, each distinct lysine in ubiquitin dictates the type of chain that is formed. For example, ubiquitin chaining at lysines K29 and K48 are usually associated with degradation (Thrower et al., 2000) while lysines K11 and K63 are associated with DNA repair (Matsumoto et al., 2010) and intracellular trafficking respectively (Spence et al., 2005).

Ubiquitination occurs in a cascade that utilizes three enzymatic steps. The first step is the activation of ubiquitin by an E1, or ubiquitin-activating enzyme. Activation of ubiquitin occurs in an ATP dependent manner and results in the formation of a thioester linkage between the E1 and the ubiquitin molecule. Once ubiquitin is activated by the E1, it is transferred to an E2, or ubiquitin-conjugating enzyme. The E2 then interacts with an E3, or ubiquitin ligase, which interacts with the target substrate and facilitates the attachment of ubiquitin from the E2 to a target substrate at a specific lysine residue. There are different types of E3's but the focus of this dissertation will be on an E3 from the really interesting new gene (RING) family called San1, which will be discussed in more detail later in this chapter.

The ubiquitin-proteasome system

The ubiquitin proteasome system (UPS) is one of two major mechanisms by which protein degradation occurs in mammalian cells (Lilienbaum, 2013). The UPS is a two-

part system where a substrate is polyubiquitinated with at least 4 sequential ubiquitin molecules that serve as a flag for the 26S proteasome to recognize and target the substrate for subsequent degradation. Once recognized, the ubiquitin chain gets cleaved, and a variety of peptidases within the proteasome break the ubiquitinated protein into small peptides (Thrower et al., 2000). Further breakdown occurs after release from the proteasome and amino acids are recycled to make new proteins (Finley et al., 2012).

Generally protein synthesis occurs in the cytosol (Schubert et al., 2000). It is estimated that around one-third of proteins synthesized are immediately destroyed by the UPS due to defects or improper synthesis (Schubert et al., 2000). In addition, the UPS generally is thought to act through two arms; the regulated degradation arm and the PQC degradation arm. In regulated degradation, the UPS is responsible for destroying normal proteins (ie cell cycle regulators), as part of regulated degradation during the life of the cell (Asher and Shaul, 2005). In PQC degradation, the UPS targets abnormal or misfolded proteins for the destruction (Gardner et al., 2005; Rosenbaum et al., 2011). Our lab focuses on the destruction of misfolded proteins because they can be toxic to the cell and lead to disease states.

The cell has developed UPS PQC systems in every cellular compartment. The ER, cytosol, mitochondria and golgi are all locations where post production misfolding can occur. Compartmentalized PQC is essential because it gives the cell the ability to get rid of misfolded proteins in the compartments they arise. Each compartmental system can coordinate the refolding or degradation of misfolded proteins through the action of chaperones or compartment localized degradation machinery. These PQC systems are

best understood in the cytosol and ER. Each UPS PQC system is generally characterized and named after the E3 ligase associated with it. The classic examples for the ER and cytosol are the Doa10 and Ubr1 pathways respectively, but there are a variety of UPS PQC systems in the cell. Although UPS PQC systems are best understood in the ER and cytosol, very little is known about how nuclear UPS PQC systems work despite the fact that 80% of proteasomes are located to the nucleus (von Mikecz et al., 2008; Wojcik and DeMartino, 2003; Enenkel, 2014).

The San1-mediated ubiquitin-proteasome system

Our lab discovered the first known, nuclear-localized ubiquitin ligase, San1, that functions in nuclear protein quality control (NucPQC) degradation in the budding yeast *Saccharomyces cerevisiae* (Gardner et al., 2005). This discovery was interesting because San1 was initially implicated as a suppressor for temperature-sensitive phenotypes in *Saccharomyces cerevisiae* (Schnell et al., 1989; Xu et al., 1993). These findings lead to the idea that San1 might function in NucPQC (Gardner et al., 2005). San1 has been shown to target mutant proteins that are folding compromised (Rosenbaum et al., 2011; Prasad et al., 2010; McClellan et al., 2005); but one caveat to this is that many normally folded proteins are marginally stable (Taverna et al., 2002; McClellan et al., 2005). Proteins exist on an energy landscape from high energy unfolded states to low energy folded states (Balchin et al., 2016). Transitioning between these energy states can leave proteins vulnerable to misfolding due to potential for exposure of hydrophobic residues (Balchin et al., 2016). Exposed hydrophobic residues are the feature that renders a partially folded or misfolded protein aggregation prone.

This means that at any give time a normally folded marginally stable protein could potentially become a target of San1 if they are perceived to be a misfolded protein and are accessible by San1.

San1 is an intrinsically disordered RING family E3 ubiquitin ligase. We have proposed that San1's regions of disorder give San1 the broad flexibility to interact with the wide variety of misfolded protein conformations it will likely encounter in the nucleus (Rosenbaum et al., 2011). Based on previous work in our lab, we also know that San1 interacts with regions of exposed hydrophobicity in misfolded substrates to ubiquitinate them (Fredrickson et al., 2011). This is important because we know that regions of exposed hydrophobicity can promote protein aggregation and lead to toxicity depending on the protein (Münch and Bertolotti, 2010; Weids et al., 2016).

After the discovery of San1's function in NucPQC in 2005, other role players in this NucPQC pathway had not yet been discovered. Unlike other ubiquitin ligases shown to function in cellular PQC degradation, our lab found that San1 does not require chaperones for its function in *in vitro* studies (Rosenbaum et al., 2011). However, I will discuss how chaperones, more specifically Ssa1 and Ssa2 of the heat shock protein 70 (HSP70) class, play a role in San1-mediated NucPQC degradation in the following chapters. It is important to note that a previous student in the Gardner lab, Pamela Gallagher, also made contributions in understanding the San1 pathway that mirror my findings. Her focus was on the chaperone Cdc48/p97, which is an AAA-ATPase associated with several cellular functions. She found that Cdc48 was differentially required post-ubiquitination to maintain the solubility of some San1 substrates (Gallagher et al., 2014). Based on her work, one of the over arching questions for my

dissertation was: do chaperones play a role in San1 mediated degradation *in vivo*? This is an important question to answer because the *in vitro* data previously mentioned suggests that chaperones are not involved with San1 function (Rosenbaum et al., 2011).

Until this point, it was not known if chaperone involvement in the San1 UPS was required. My data suggests there is one potential mechanism for chaperones in San1 mediated degradation. In the next chapter I will focus on the history of chaperones and their involvement with PQC degradation machinery. I will also discuss the overall state of the NucPQC field and what we know about how it works.

CHAPTER II: NUCLEAR PROTEIN QUALITY CONTROL (NucPQC)

Overall nuclear protein quality control

Quality control studies in the nucleus have traditionally focused on how the cell maintains the integrity of the nuclear genome and the quality of mRNA prior to export from the nucleus (Eberle and Visa, 2014; Heyer, 2015). By contrast, our understanding on how the cell maintains the quality of nuclear proteins has lagged behind. The protein aspect of overall nuclear quality control is exceptionally important to consider because an escalating failure to remove or repair misfolded nuclear proteins can lead to a deterioration in the integrity of the nuclear genome and the quality of the mRNA produced. This in turn can impact overall cellular protein homeostasis, or proteostasis, due to a reduction in the translational fidelity of the genetic code into its functional complement of proteins. Over the last decade, studies on misfolded nuclear proteins have revealed some of the folding and degradative PQC systems that function in the nucleus. These studies appear to operate on similar overarching principles to PQC systems in the cytosol with chaperones, small heat-shock proteins, and ubiquitin ligases generally managing misfolded nuclear proteins (Guerriero et al., 2013; Fredrickson et al., 2013; Rosenbaum et al., 2011; Heck et al., 2010).

One unique aspect of the nucleus is that it contains a high concentration of DNA. Due to the negative charge of DNA, many structural chromatin proteins have a considerable positive charge, such as histones (Alberts et al., 2002). In addition, the nuclear proteome is enriched for proteins that possess low complexity, intrinsically disordered regions, (Meng et al., 2016), suggesting these proteins have a broad

capacity for conformational flexibility. We think this is important because conformational flexibility gives these proteins the ability to interact with a variety of targets. If these proteins misfold, those flexible interactions could then turn to these proteins into perpetrators for toxic interactions and lead to disease states. Furthermore, chromatin is dynamic, and there is ongoing remodeling that involves continuous assembly and disassembly of DNA-RNA-protein complexes (Langst, 2015). These factors are potentially a considerable source for protein misfolding and aggregation specific to the nucleus because processes involved with assembly and disassembly can be error prone.

Unlike the cytosol, one key PQC challenge the nucleus does not face to any considerable extent is the need to balance the robustness of PQC in the misfolding of damaged proteins with the PQC in the folding of nascent proteins. As I mentioned before, nuclear proteins are synthesized in the cytosol and are imported into the nucleus through the nuclear pore (Grossman et al., 2012). Thus, NucPQC can be primarily focused on proteins that have become misfolded via damage during or after nuclear import, and NucPQC pathways may have evolved to target specific features of damage-induced misfolding that are particularly harmful in the nuclear environment. However, I note there is a possibility that the nucleus engages in limited translation (Pederson, 2013), and may have to manage a low level of translational products. Furthermore, failures in cytosolic PQC can reduce the amount of correctly folded nuclear proteins, which can subsequently burden NucPQC pathways by decreasing the levels of functional nuclear proteins.

PQC degradation in the nucleus

The majority of proteasomes are nuclear localized (Wojcik and DeMartino, 2003), and the ubiquitin-proteasome system is the main route for misfolded protein degradation in the nucleus (Fredrickson et al., 2012). However, a recent study has suggested that nuclear proteins destined for proteasome degradation are first exported from the nucleus and destroyed by cytosolic proteasomes (Chen and Madura, 2014). It is not clear if this is specific to a class of proteins that contain nuclear export signals or general for all nuclear proteins. What is clear is that a number of nuclear-localized ubiquitin ligases have been implicated in the PQC degradation of misfolded nuclear proteins (Jones and Gardner, 2016). For this dissertation, I will limit the discussion to the major yeast ubiquitin ligases involved in the degradation of misfolded nuclear proteins, as yeast has been the most extensively used organism for studies of NucPQC degradation. The best understood yeast ubiquitin ligase involved in NucPQC degradation is San1.

San1's ability to target misfolded nuclear proteins is attributed to large regions of intrinsic disorder within the N- and C-terminal regions of San1 (Rosenbaum et al., 2011). These disordered regions possess multiple substrate-interaction regions in a conformationally plastic scaffold, which we have proposed provides San1 a broad mechanism to target many different misfolded proteins for degradation (Rosenbaum et al., 2011; Fredrickson et al., 2013). San1 has been found to recognize exposed hydrophobicity within its misfolded substrates that would have normally been buried within the protein (Fredrickson et al., 2011). San1 does not appear to favor a particular type of exposed hydrophobic residue; it only requires that the degree of total exposed

hydrophobicity in its substrates is at a threshold that leads to insolubility and aggregation (Fredrickson et al., 2013), which also correlates in many cases with substrate toxicity (Fredrickson et al., 2013; Fredrickson et al., 2011).

San1 is the most well understood nuclear-specific ubiquitin ligase. However, it does have some substrate overlap with the ubiquitin ligase Ubr1 (Heck et al., 2010; Prasad et al., 2010), but Ubr1 most likely acts in the cytosol rather than the nucleus (Heck et al., 2010; Prasad et al., 2010; Eisele and Wolf, 2008; Nillegoda et al., 2010; Summers et al., 2013; Theodoraki et al., 2012). To date it is unknown if there is a San1 homolog in metazoans. No functional equivalents have been found. This is likely due to the extreme intrinsic disorder of San1 (Rosenbaum et al., 2011; Fredrickson et al., 2013), which allows for a high degree of sequence divergence over evolution (Khmelinskii et al., 2014). This information suggests there may be a link between cytosolic and nuclear degradation pathways but more work is needed to understand this possibility.

Residing in the ER/inner nuclear membrane, the ubiquitin ligase Doa10 has also been implicated in NucPQC degradation (Furth et al., 2011; Wang and Prelich, 2009; Ravid et al., 2006; Ravid, 2008). *DOA10* (aka *SSM4*) was first characterized as an mRNA quality control gene (Alfassy et al., 2013). Mutation to *DOA10/SSM4* suppressed the temperature sensitivity of a mutant form of the nuclear Rna14 protein (Alfassy et al., 2013). However, the Doa10 protein is best characterized for its role in ER-associated degradation (ERAD), ubiquitinating both soluble and ER-membrane substrates for degradation by the proteasome (Prasad et al., 2012; Furth et al., 2011; Deng et al., 2006; Theodoraki et al., 2012; Wang and Prelich, 2009; Mandart et al., 1994; Swanson

et al., 2001; Carvalho et al., 2006; Kreft et al., 2006). Similar to San1, Doa10 also recognizes exposed hydrophobicity in its substrates, though the hydrophobicity differs from what San1 recognizes and appears to be presented as an amphipathic helix (Furth et al., 2011; Wang and Prelich, 2009; Ravid et al., 2006, Mandart et al., 1994; Metzger et al., 2008). The exact mechanism by which Doa10 binds its substrates still remains to be elucidated. Doa10 has a mammalian homolog called TEB4/MARCH-VI, but a PQC function has not yet been established for TEB4 (Carvalho et al., 2006).

In addition to Doa10, an inner nuclear membrane ubiquitin-ligase complex called Asi (composed of the yeast ubiquitin ligases Asi1 and Asi3, along with Asi2) has recently been implicated in the NucPQC degradation of misfolded inner nuclear membrane proteins and soluble transcription factors (Gilon et al., 2000; Foresti et al., 2014). The Asi complex seems to function in a parallel pathway with the ER-membrane ubiquitin ligase Hrd1 to destroy misfolded nuclear membrane proteins (Gilon et al., 2000; Foresti et al., 2014; Gauss et al., 2006; Denic et al., 2006; Carvalho et al., 2006; Sasagawa et al., 2007). However, more studies are needed to understand the broader role of the Asi complex in NucPQC, as well as its substrate specificity at the nuclear membrane.

The challenge with NucPQC

The majority of protein synthesis occurs in the cytosol (Wu and Warner, 1971). Conversely, not all proteins stay there (Wu and Warner, 1971). Proteins are shuttled to every cellular compartment to carry out functions required for the viability of the cell. Although efficient, risk associated with post-synthesis damage completely bypasses

translational-level quality control. The ER and cytosol have been well studied in this realm, but in the nucleus much less is known. This is surprising being that the genetic framework for the cell, DNA, is located in the nucleus packaged into chromatin. An escalating failure to remove or repair misfolded nuclear proteins can lead to a deterioration in the integrity of the nuclear genome and the quality of the mRNA produced. This in turn can impact overall cellular proteostasis due to a reduction in the translational fidelity of the genetic code into its functional complement of proteins. This can potentially lead to damage, toxicity and subsequent cell death.

One challenge that the cell faces in NucPQC is the fact that there are a variety of essential sub-nuclear compartments that are required for normal cell function. For example, the nucleolus is localized within the cell nucleus and is the site of ribosome biogenesis (Kressler et al., 2009; Thomson et al, 2013; Woolford and Baserga, 2013). Cajal bodies are also localized to the nuclear space and have been shown to be involved in RNA-related functions, including snRNP biogenesis, mRNA processing, and telomere maintenance (Gall et al., 1999; Zhao et al., 2011). The sub-nuclear compartments are important to consider because they carry out vital functions to cell survival. The key point here is that like other cellular compartments; post-production misfolded proteins can arise in the nucleus. Therefore there must be pathways specific to the nucleus to deal with misfolded protein threats.

To understand how NucPQC system may work, it is important to discuss how misfolded proteins arrive in the nucleus. Nuclear misfolded proteins can arise from a variety of avenues. Normally folded proteins enter the nucleus one of two ways. They can contain nuclear localization signals (NLS) where they are actively transported

across the nuclear membrane by some mechanisms that are known and others that are unknown or they can passively diffuse into the nucleus if they are below the 40kDa threshold for passive entry (Marfori et al., 2010; Rodriguez et al., 2004). One important thing to note here is that the nuclear pore can breakdown as the cell ages. This lowers the selectivity for protein entry which can potentially result in more proteins entering the nucleus (D'Angelo et al., 2009). The interesting part of this transport is that damage or misfolding can occur pre-entry, post-entry or during transit. Naturally misfolded proteins in the nucleus pose a particular threat to the cell due to DNA being in such close proximity. As previous mentioned, there are 35+ diseases where the hallmark of the malady is associated with nuclear specific misfolded protein aggregation (Woulfe, 2007).

This information led us to hypothesize that NucPQC must be robust to ensure proper sequestration or elimination of misfolded protein threats. One idea that has come up in commentary in the field is that the nucleus itself serves as a PQC compartment for misfolded cytosolic proteins (Jones and Gardner, 2016). This thought is centered on the idea that when the burden of misfolded proteins becomes too high in the cytosol, the cell uses other compartments as a means for dynamic and rapid degradation or refolding of the misfolded protein. This may explain why we see some proteins originally thought to be cytoplasmic in the nucleus being degraded by NucPQC (Prasad et al., 2010, Heck et al., 2010). This is an interesting topic because it implies the cell purposefully shuttles misfolded proteins into the nucleus increasing risk of DNA exposure to toxic misfolded proteins. Future studies will be important to determine how

this might be happening and how the cell might balance such a process, but for now I will focus on what we do know.

PQC chaperones in the nucleus

A common characteristic amongst NucPQC ubiquitin ligases is that chaperones have been implicated in the degradation of some substrates (Guerriero et al., 2013; Heck et al., 2010; Prasad et al., 2010; Furth et al., 2011; Wang and Prelich, 2009; Ravid et al., 2006; Ravid et al., 2008; Kikis et al., 2010; Taylor and Dillin, 2011). A few reports demonstrate that chaperones are required for the ubiquitination of misfolded cytosolic proteins destined for NucPQC degradation by San1 (Guerriero et al., 2013; Heck et al., 2010; Prasad et al., 2010). Another study showed that ubiquitination via San1 does not universally require chaperones (Rosenbaum et al., 2011). This is a controversial aspect of NucPQC degradation because little is known about the precise role chaperones play in NucPQC degradation.

The linkage between chaperones and PQC degradation comes from a long history of PQC studies in the ER and cytosol (Buchberger et al., 2010), and has shaped how the PQC field thinks about the compartmental triage of misfolded proteins (Miller et al., 2015). Ubr1 targets substrates in what appears to be a chaperone-dependent manner, with the Hsp70 chaperones Ssa1/Ssa2, the Hsp40 chaperone Ydj1, and the Hsp110 chaperone Sse1 required for Ubr1-dependent degradation (Guerriero et al., 2013; Heck et al., 2010; Prasad et al., 2010; Prasad et al., 2012). Doa10 also targets substrates in what appears to be a chaperone-dependent manner, utilizing Ssa1/Ssa2, Ydj1, and the Hsp40 chaperone Sis1 (Furth et al., 2011; Wang and Prelich, 2009; Ravid et al., 2008;

Kreft et al., 2006; Kikis et al., 2010; Taylor and Dillin, 2011; Johnston et al., 1998; Kaganovich et al., 2008). Hrd1 has also been shown to utilize BiP and Yos9 to carry out its substrate targeting for degradation of luminal and membrane proteins (Sasagawa et al., 2007; Gauss et al., 2006; Denic et al., 2006; Varvalho et al., 2006) However, the role of chaperones in San1-mediated degradation remains a subject of open debate (Rosenbaum et al., 2011; Guerriero et al., 2013; Heck et al., 2010; Prasad et al., 2010; Prasad et al., 2012), the San1 pathway does utilize the AAA-ATPase chaperone Cdc48/p97 for the degradation of select San1 substrates that are highly insoluble (Gallagher et al., 2014).

The linkage between chaperones and PQC degradation comes from a long history of PQC studies in the ER and cytosol (Buchberger et al., 2010), and has shaped how the PQC field thinks about the compartmental triage of misfolded proteins (Miller et al., 2015). It may be that chaperone involvement in NucPQC degradation is important for partitioning misfolded proteins between insoluble aggregates and available monomers that can be recognized by the NucPQC degradation ubiquitin ligases. How chaperones function in NucPQC has remained an open question. My project for this dissertation addresses some of these questions and sheds light on how chaperones in the nucleus could be coordinating with nuclear ubiquitin ligases, more specifically, San1, to facilitate degradation of misfolded proteins.

PQC inclusions in the nucleus

Although each cellular compartment possesses a robust complement of folding and degradative PQC activities, as I briefly discussed before, the burden of misfolded

proteins can exceed the capacity of the compartment's PQC systems during stress or with age (Latouche et al., 2006; Seidel et al., 2012; Bao et al., 2002). When the burden of misfolded proteins overwhelms a compartment's PQC pathways, misfolded proteins can aggregate (Latouche et al., 2006; Seidel et al., 2012; Bao et al., 2002). One way the cell counters the incapacity of PQC pathways is to concentrate misfolded proteins into inclusion bodies that sequester aggregation-prone misfolded proteins. Prominent inclusions include the perinuclear mammalian aggresome (Corbeil-Girard et al., 2005), the perinuclear/intranuclear yeast JUNQ/INQ (Zhang and Qian, 2011; Chartier et al., 2006), and the perivacuolar yeast IPOD (Chartier et al., 2006).

The JUNQ/INQ is a primary nuclear inclusion for misfolded protein sequestration in both yeast and mammalian cells (Zhang and Qian, 2011; Chartier et al., 2006). Originally identified as perinuclear (Chartier et al., 2006), a recent study found that the deposition is intranuclear and bounded by the nuclear membrane (Zhang and Qian, 2011). Furthermore, genotoxic stressors like methyl methanesulfonate (MMS) appear to force important nuclear proteins into this inclusion (Tavanez et al., 2009). Interestingly, *san1* Δ cells were found to be sensitive to MMS in a large-scale genetic screen (Latonen et al., 2011), suggesting that accumulation of misfolded San1 substrates could contribute to increased DNA damage. Alternatively, MMS can methylate Cysteine, Histidine, Lysine, and Arginine residues in proteins (Gidalevitz et al., 2006), which could lead to protein misfolding and the need for San1 to remove MMS-damaged misfolded proteins.

The JUNQ/INQ is dynamic and the misfolded proteins in the JUNQ/INQ are mobile (Chartier et al., 2006). However, the mobility of misfolded proteins in the JUNQ/INQ can

decrease during stress (Woerner et al., 2016; Savas et al., 2012). The aggregase Btn2 is important for localizing misfolded proteins to the JUNQ/INQ (Zhang and Qian, 2011). Furthermore, Hsp70 chaperones and proteasome subunits have also been shown to localize with the JUNQ (Woerner et al., 2016). Thus, the JUNQ/INQ is likely to be the site where misfolded nuclear proteins are stored until they can be destroyed by ubiquitin-proteasome pathways. An important feature of the JUNQ/INQ is that localization of misfolded proteins to this site prevents their passage from the mother cell to the daughter cell during mitosis (Toyama et al., 2013). In this way, the quality of the nuclear environment in the daughter cell can be kept pristine.

In mammalian cells, nuclear inclusions have been studied using proteins with polyglutamine and/or polyalanine-expansions that lead to disorders such as Huntington's, Kennedy's, or oculopharyngeal muscular dystrophy (OPMD) (Nakatsukasa et al., 2008; D'Angelo et al., 2009; Gardner et al., 2013; Kawai et al., 1999; Ouan et al., 2006). In most cases, distinct chaperones have also been found to co-localize with the nuclear inclusions (Nakatsukasa et al., 2008; D'Angelo et al., 2009; Janer et al., 2013; Kawai et al., 1999; Quan et al., 2006), similar to the JUNQ. It currently remains unknown if the nuclear inclusions in these cases are identical to the JUNQ or comprise different structures. Furthermore, different nuclear sub-compartments may have their own unique sites of deposition. For example, an inclusion forms within the nucleolus after proteasome inhibition (Gartner et al., 2014), though it is unknown if the nucleolar inclusion is composed of misfolded proteins. Future work will be needed to understand the breadth and types of inclusions in the nucleus that act as PQC deposition sites.

PQC interplay between the nucleus and cytosol

Finally, it is important to consider that there will likely be significant interplay between nuclear and cytosolic PQC pathways as there is an intimate and dynamic communication between the two compartments through the nuclear pore. One interesting type of PQC interplay that has emerged in the last few years is that misfolded proteins are not always excluded from the nucleus if they are first generated in the cytosol (Guerriero et al., 2013; Park et al., 2010; Heck et al., 2010; Prasad et al., 2012). This seems counterintuitive because cytosolic PQC pathways should prevent misfolded proteins from entering the nucleus where they can do harm. Why are these 'cytosolic' misfolded proteins imported into the nucleus when they should be managed in the cytosol? There are two possibilities. First, it could be that some misfolded cytosolic proteins are below the ~40kDa passive diffusion limit of the nuclear pore (Grossman et al., 2012). This is the case for a number of misfolded cytosolic proteins studied (Guerriero et al., 2013; Park et al., 2010; Heck et al., 2010; Prasad et al., 2012). Second, it could be there are active mechanisms to import misfolded cytosolic proteins to the nucleus if NucPQC degradation is more robust than cytosolic PQC degradation. Both Sis1 and Sse1 are implicated in the nuclear transport of certain misfolded cytosolic proteins (Heck et al., 2010; Prasad et al., 2010; Gallina et al., 2015), suggesting that one function for these chaperones may be to facilitate the trafficking of misfolded cytosolic proteins into the nucleus where San1 can recognize them. In addition, deletion of the small heat shock protein Hsp42 leads to the nuclear localization of misfolded cytosolic proteins (Zhang and Qian 2011), indicating that there are also retention mechanisms in the cytosol to prevent nuclear import. How transport and retention

pathways coordinate to allow or disallow nuclear import of misfolded proteins is a new frontier for NucPQC studies.

Another possibility for interplay between cytosolic and NucPQC systems is that there may be conditions where cytosolic PQC pathways become overloaded and unable to buffer the cytosol against misfolded protein accumulation. For example, it has been shown that expression of toxic polyQ proteins in worms causes loss of function for chaperone-dependent, metastable proteins (Sahin et al., 2014). In addition, protein aggregation in the cytosol can interfere with nucleo-cytosolic transport; transport between the nucleus and cytosol (Guo et al., 2014). It is possible that the nucleus serves as a back-up PQC compartment for cytosolic PQC when the cytosol can't handle a high burden of misfolded proteins. More work is needed to determine if this is the case.

Lastly, some nuclear pore proteins are exceptionally long-lived in non-dividing cells (Iwata et al., 2009; Longshaw et al., 2004), and nuclear pores have been found to deteriorate with age potentially allowing larger proteins to passively enter the nucleus (McClellan et al., 2005). Thus, NucPQC pathways might become increasingly important with age as nuclear pore selectivity breaks down. This has significant ramifications for human health because at least 15 diseases have the common characteristic that they show greater level of misfolded protein aggregation in the nucleus that increases with age, and many of these proteins are not found in the nucleus at youthful ages (Woulfe, 2008). Taken all together, we as a field have learned bits and pieces of how NucPQC works (**Figure 2.1**), but more work is needed to understand how these systems function in their entirety. In the next chapter I will address one of the bigger questions I

speculated on above; how chaperones and the San1 degradation pathway coordinate to facilitate substrate degradation. I will discuss my findings on how HSP70 is differentially involved in San1 mediated degradation.

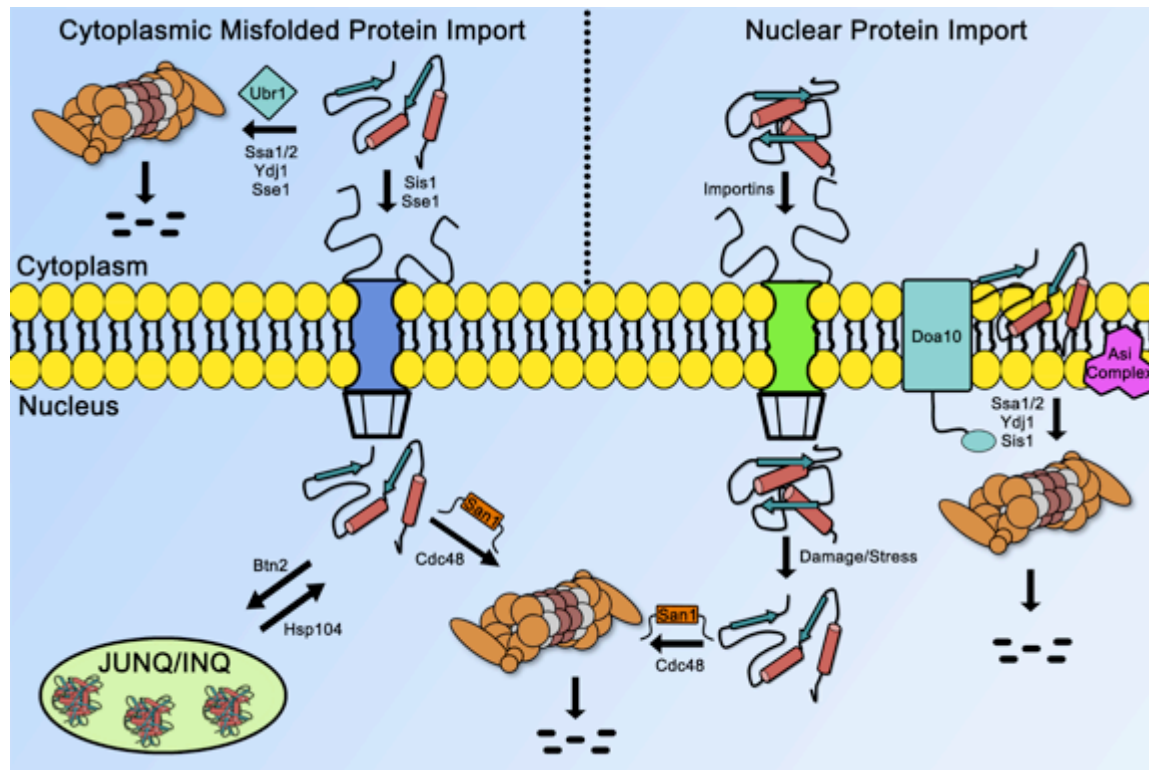


Figure 2.1 NucPQC network as the field currently stands

This figure includes what we know about how NucPQC is functioning as a field. This figure is the culmination of a number of publications but is summarized in (Jones and Gardner, 2016)

CHAPTER III: SSA1 and SSA2 ARE NOT UNIVERSALLY REQUIRED IN SAN1-MEDIATED DEGRADATION

Introduction to the Ssa HSP70 family

Now that I've discussed the background and history of the San1 pathway and chaperone/degradation machinery relationships it is important to discuss my results. My work has been centered on the HSP70 family of chaperones. The HSP70 family of chaperones is an evolutionarily conserved, and ubiquitously expressed cohort of proteins spanning from bacteria to humans (Sharma et al., 2009). In eukaryotes, each member of the HSP70 family is localized to a certain cellular compartment. In the cytosol, HSP70 mainly functions as a molecular chaperone by binding newly synthesized proteins as well as aggregated proteins and assists in their proper folding or refolding (Becker and Craig, 1994; Bukau and Horwich, 1998).

The focus of my research has been on the Ssa family of budding yeast HSP70s. The Ssa family includes 4 isoforms of HSP70: Ssa1, Ssa2, Ssa3 and Ssa4. Ssa1 is basally expressed under normal growth conditions and gets upregulated in heat shock (Werner-Washburne et al., 1989). Ssa2 is 98% identical in sequence identity to Ssa1, but functionally seems to also have a role in nuclear import of tRNA and some enzymes (Takano et al., 2015; Brown et al., 2000). Ssa2 is the most highly expressed at basal conditions while Ssa1 is under heat shock. Ssa2 may function in parallel with Ssa1, however results are not clear (Sharma and Masison, 2011). Ssa3 shares 85% sequence identity to Ssa1. Ssa3 is expressed in times of cellular stress due to heat shock or starvation, but not under normal growth conditions (Werner-Washburne et al., 1989; Werner-Washburne et al., 1987). Ssa4 is also upregulated in heat shock, cold

shock, ethanol exposure and starvation but is also not expressed under normal growth conditions (Boorstein and Craig, 1990; Kandrор et al., 2004; Quan et al., 2004; Werner-Washburne et al., 1989). For my studies I focused on Ssa1 and Ssa2 because they are the dominant forms expressed, and because they have been implicated in the San1 UPS pathway in past studies (Guerriero et al., 2013; McClellan et al., 2005).

Ssa1 and Ssa2 are required for model PQC substrates

As I previously discussed in chapter two, chaperones have been shown to be important for degradation of misfolded proteins in the cytosol and ER, but in the nucleus their role in NucPQC degradation is not well understood. Our previous studies have suggested that San1 does not interact directly with chaperones to facilitate degradation (Rosenbaum et al., 2011). However, Recent studies have revealed that the Hsp70 chaperones Ssa1 and Ssa2 are required *in vivo* for the San1-mediated ubiquitination and degradation of some misfolded proteins (Heck et al., 2010; Prasad et al., 2012; Guerriero et al., 2013). These substrates all lacked canonical nuclear localization sequences so their requirement for San1 was puzzling because San1 is nuclear localized (Gardner et al., 2005). We hypothesized that the requirement for Ssa1 and Ssa2 may have been because Ssa1 and Ssa2 have been shown to be important for nuclear import of some substrates that were previously thought to be cytosolic (McClellan, 2005; Heck et al., 2010; Prasad et al., 2010, 2012).

To test this hypothesis we took 3 non canonical nuclear substrates that were shown to be localized to the nucleus and require San1 for degradation, $\Delta 2$ GFP, GFP with an internal deletion to its well-ordered beta barrel structure (Prasad et al., 2010),

Δ ssPrA, vacuolar Proteinase A with its signal sequence removed which induces misfolding (Prasad et al., 2010) and VHL, the human von Hippel Lindau tumor suppressor that misfolds in the absence of its binding partners elongin b and c (Feldman et al., 1999, Feldman et al., 2003, Hansen et al., 2002, Melville et al., 2003), and attached GFP^{NLS} to them, to form GFP^{NLS}- Δ 2GFP, GFP^{NLS}- Δ ssPrA and GFP^{NLS}-VHL. Attachment of a NLS forced these into the nucleus removed the potential need for Ssa1 and Ssa2 for import. We hypothesized that if these now NLS-plus versions that were formally non canonical (NLS-minus) substrates still required Ssa1 and Ssa2 that it was for a function specific to the nucleus. Cycloheximide chase assays were performed in parent, *ssa1/2* Δ and *san1* Δ cells to determine if these substrates still required Ssa1 and Ssa2 for degradation in the nucleus. As a control we tested whether GFP^{NLS} is a stable protein in our system and we concluded it was (**Figure 3.1**). This was an important control to ensure that GFP^{NLS} itself is stable and isn't influencing the degradation of substrates we attach it to. We queried if these NLS tagged versions still required Ssa1 and Ssa2 for degradation. We determined that all three NLS-tagged substrates required Ssa1 and Ssa2, thus suggesting that the requirement for Ssa1 and Ssa2 was independent of nuclear import (**Figure 3.2**).

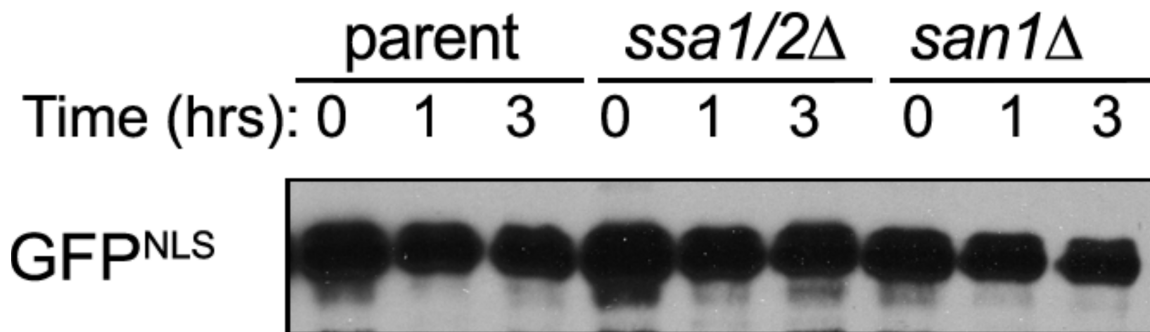


Figure 3.1 GFP^{NLS} is a stable protein. Cycloheximide-chase degradation assays were performed on parent, *ssa1/2*Δ and *san1*Δ cells to assess the stability of GFP^{NLS}. Anti-GFP antibody was used to detect GFP^{NLS}.

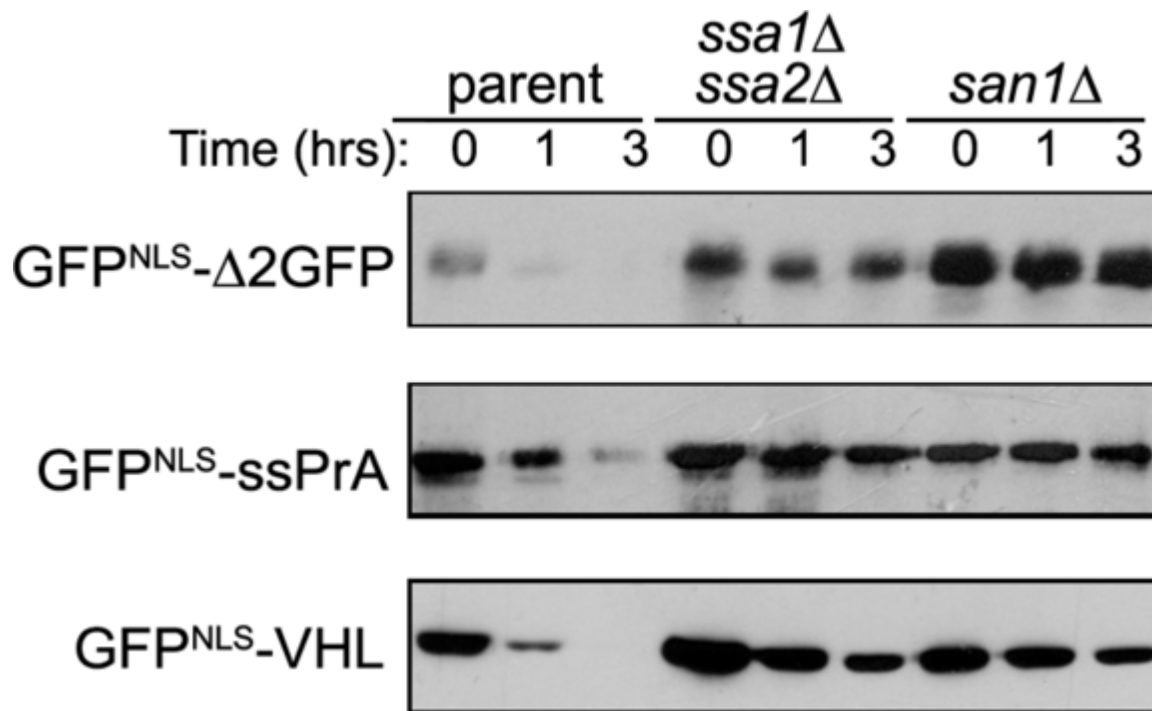


Figure 3.2 Substrates require Ssa1 and Ssa2 for nuclear localized degradation.

Cycloheximide-chase degradation assays were performed on parent, *ssa1/2* Δ and *san1* Δ cells to assess the stability of GFP^{NLS}- Δ 2GFP, GFP^{NLS}- Δ ssPrA and GFP^{NLS}-VHL. For the GFP^{NLS}-substrates expressed from the GAL1 promoter, expression was induced by addition of galactose for 3 hours prior to cycloheximide addition. Time after cycloheximide addition is indication above each line. Anti-GFP antibodies were used to detect each substrate.

Ssa1/Ssa2 are differentially required in San1 mediated degradation

Based on our findings that GFP^{NLS}- Δ 2GFP, GFP^{NLS}- Δ ssPrA and GFP^{NLS}-VHL required Ssa1/Ssa2 for degradation we wanted to determine if all San1 substrates shared this characteristic. To make broader claims for San1 substrates that require Ssa1/Ssa2 we queried five representative substrates from the 40+ known San1 substrates we previously characterized to determine if the Ssa1/Ssa2 requirement was universal (Fredrickson et al., 2011; Gardner et al., 2005; Rosenbaum et al., 2011). The first group of San1 substrates included 5 proteins (Cdc13, Cdc68, Sir3, Sir4, and Ura3) that contain single missense mutations that are thought to cause temperature-dependent misfolding (Fredrickson et al., 2011; Gardner et al., 2005). The second group of San1 substrates consists of 20 proteins with truncations thought to cause misfolding due to the deletion of key amino acid sequences (Rosenbaum et al., 2011; Gallagher et al., 2014). The third group includes 16 small hydrophobic peptides that are thought to display the type of exposed hydrophobicity recognized by San1 in misfolded proteins (Fredrickson et al., 2011; Gallagher et al., 2014).

The five representative substrates that we chose were two proteins that contain single missense mutations, GAD-Cdc68-1, which is the isolated N-terminal domain of Cdc68 (residues 2-468) carrying the structurally destabilizing G132D mutation (Evans et al., 1998) and GAD-Cdc13-1, which is the isolated N-terminal domain of Cdc13 (residues 1-600) carrying the structurally destabilizing P371S mutation (Nugent et al., 1996). For the truncated protein group, we used GFP^{NLS}-Tef2*, which contains residues 190–458 of the translation elongation factor Tef2 fused to GFP^{NLS}, and GFP^{NLS}-Bgl2*, which contains residues 20–313 of the endo- β -1,3-glucanase Bgl2 fused to GFP^{NLS}

(Rosenbaum et al., 2011; Gallagher et al., 2014). For the hydrophobic peptide degrons, we used peptide 6 (RDILVYTYILVYVYI) fused to GFP^{NLS} (Fredrickson et al., 2011; Gallagher et al., 2014).

Our results revealed that Ssa1/2 seem to be intermediately or partially required for San1-mediated degradation of GFP^{NLS}-Tef2*, GFP^{NLS}-Bgl2*, and GFP^{NLS}-Peptide 6 (**Figure 3.3**). In addition, Ssa1/2 did not seem to be required for the degradation of GAD-Cdc68-1 or GAD-Cdc13-1 (**Figure 3.4**). These results created three distinct substrate classification groups. Those that fully require Ssa1/2 for degradation, those that partially/intermediately require Ssa1/2 and those that are degraded independent of Ssa1/2. These different groups lead us to speculate on why we see these differences amongst San1 substrates. We can visualize them all together here (**Figure 3.5**).

As I previously discussed, Pamela Gallagher, a former graduate student in the Gardner Lab, discovered that the AAA-ATPase Cdc48/p97 was required for the degradation of San1 substrates and that it played a role in maintaining substrate solubility before substrates are delivered to the proteasome but after they are ubiquitinated (Gallagher et al., 2014). We know that the proteasome has difficulty degrading aggregated proteins (Holmberg et al., 2004; Snyder et al., 2003; Verhoef et al., 2002). We also know that Cdc48/p97 acts downstream of San1 (Gallagher et al., 2014). It is not known if Ssa1/2 coordinate with Cdc48 to maintain substrate solubility. However, it has been shown previously that the Hsp70 chaperones Ssa1/Ssa2, the Hsp110 chaperone Sse1, and the Hsp40 chaperone Ydj1 cooperate to facilitate the degradation of some San1 substrates by acting upstream of San1 ubiquitination (Guerriero et al., 2013; Heck et al., 2010; Prasad et al., 2010, 2012). Thus we

hypothesized that Ssa1/2 could be important for maintaining San1 substrates in a soluble state upstream of ubiquitination so that San1 can later recognize them to target them for degradation.

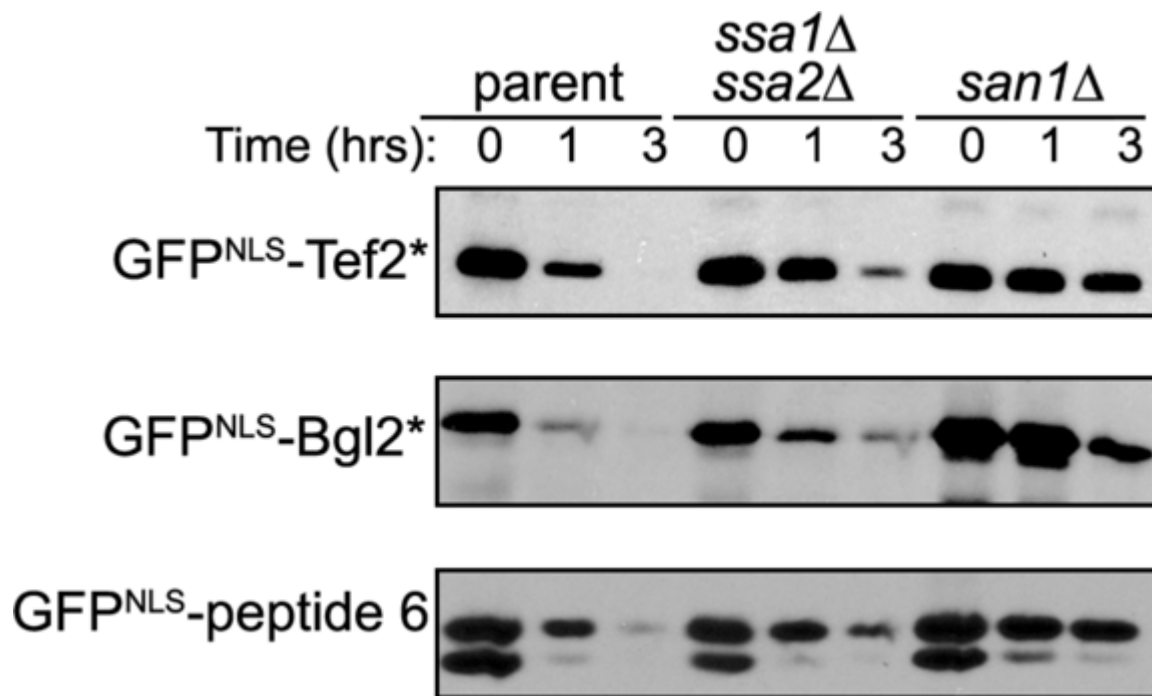


Figure 3.3 Substrates differentially require Ssa1 and Ssa2 for degradation.

Cycloheximide-chase degradation assays were performed on parent, *ssa1/2* Δ and *san1* Δ cells to assess the stability of GFP^{NLS}-Tef2*, GFP^{NLS}-Bgl2* and GFP^{NLS}-Peptide 6. For the GFP^{NLS}-substrates expressed from the GAL1 promoter, expression was induced by addition of galactose for 3 hours prior to cycloheximide addition. Time after cycloheximide addition is indication above each line. Anti-GFP antibodies were used to detect each substrate.

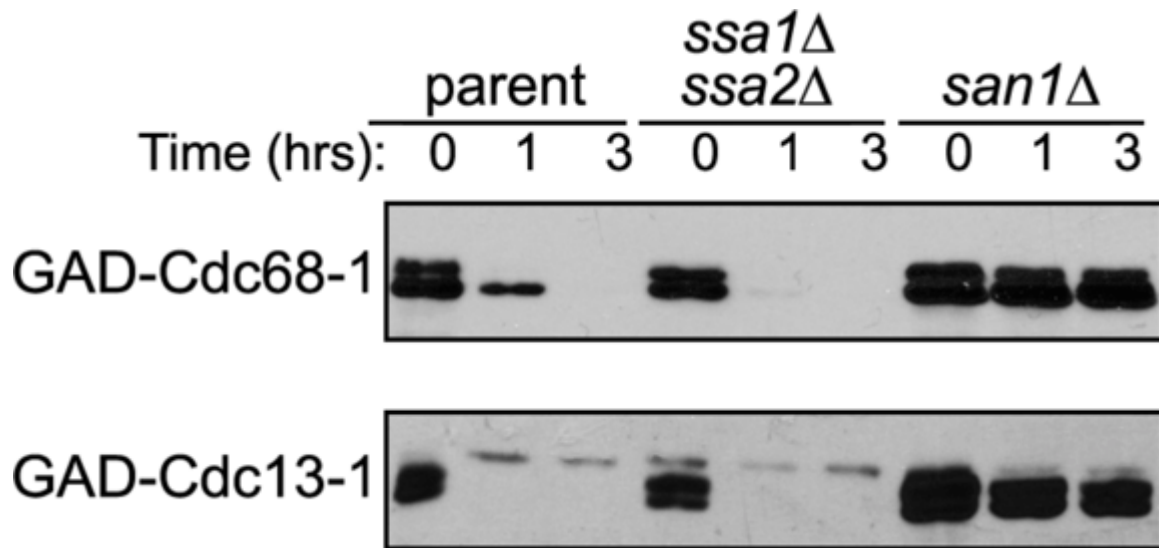


Figure 3.4 Tested substrates do not require Ssa1 and Ssa2 for degradation.

Cycloheximide-chase degradation assays were performed on parent, *ssa1/2* Δ and *san1* Δ cells to assess the stability of GAD-Cdc68-1 and GAD-Cdc13-1. Expression from the ADH1 promoter was constitutive prior to cycloheximide addition. Time after cycloheximide addition is indication above each line. Anti-GAD antibodies were used to detect each substrate.

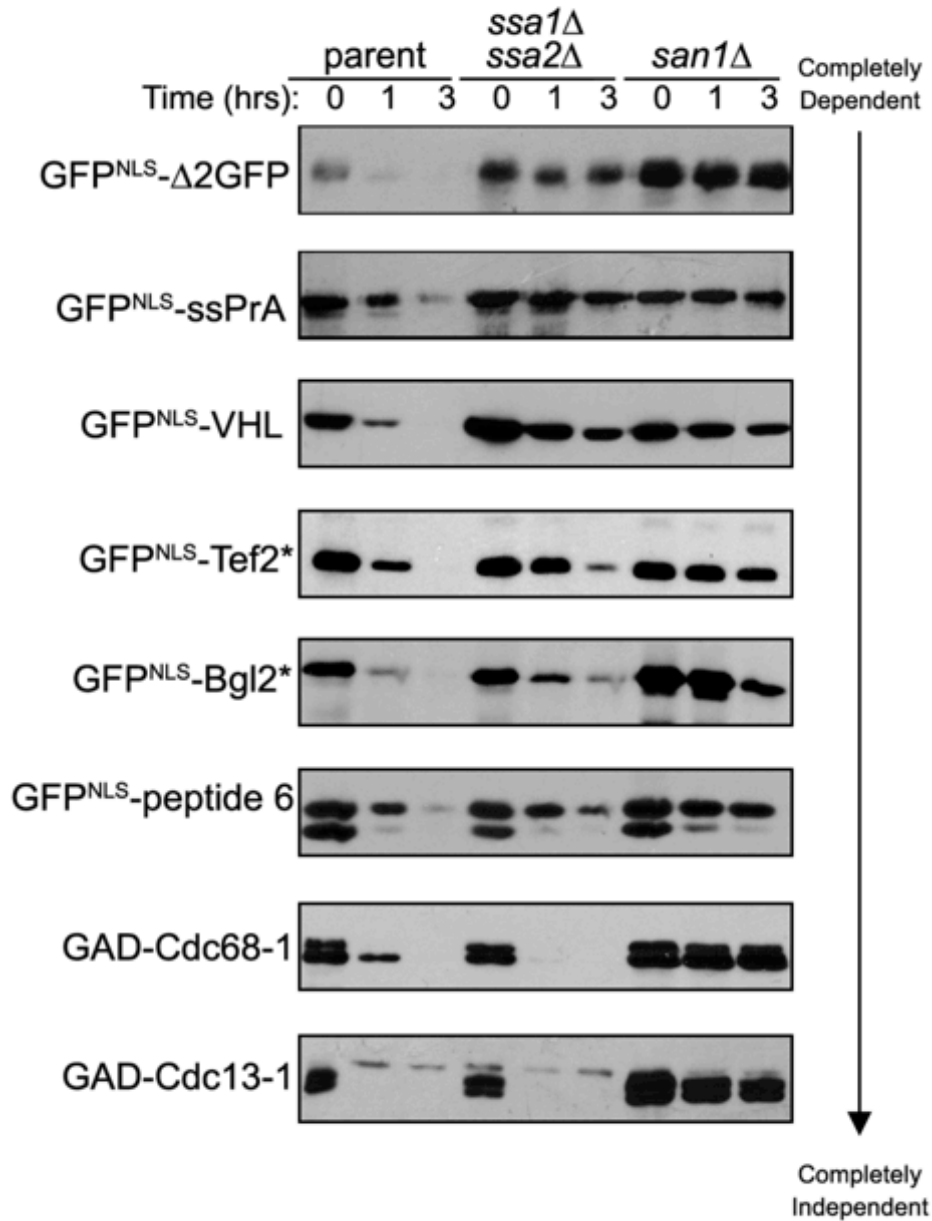


Figure 3.5 Ssa1 and Ssa2 are differentially required for degradation. Summary of the data from figures 3.2, 3.3 and 3.4 showing that.

Substrate solubility correlates with requirement for Ssa1/Ssa2

The variable requirement for Ssa1/Ssa2 sparked our interest in understanding what the underlying cause(s) were for this differential requirement. We aimed to clarify what substrate characteristic might determine Ssa1/2 requirement. Previously, we showed in an *in vitro* luciferase assay that San1 was unable to modify misfolded proteins with ubiquitin for subsequent degradation after luciferase was denatured and aggregated (Rosenbaum et al., 2011). We suspected that this could be because, when luciferase takes on an aggregative state, it is no longer accessible to San1. Our previous findings revealed that San1 recognizes exposed hydrophobicity in its target substrates. We also found that aggregated luciferase in an *in vitro* assay San1 was not able to ubiquitinate aggregated forms of luciferase (Fredrickson et al., 2011; Fredrickson et al., 2013). This led us to think about how San1 maybe become able to target those aggregated substrates.

Based on previous published results and previous work in our lab with Cdc48, we entertained the possibility that the role of chaperones degradation was to maintain the solubility of substrates so they are accessible to degradation machinery. This chaperone function has been documented in previous studies (Chen et al., 2011). Thus, we explored the possibility that substrate solubility plays a role in the requirement for Ssa1/Ssa2. To test this we performed sedimentation assays as described in **Appendix II**. First we determined that GFP^{NLS} was a soluble protein as its known to take on the folding state of proteins it's tagged to (**Figure 3.6**) (Waldo et al., 1999). Then

sedimentation assays were performed with GFP^{NLS}- Δ 2GFP, GFP^{NLS}- Δ ssPrA and GFP^{NLS}-VHL and the five San1 substrates from our catalogue, GAD-Cdc68-1, GAD-Cdc13-1, GFP^{NLS}-Tef2*, GFP^{NLS}-Bgl2* and GFP^{NLS}-Peptide 6 in wild type, *ssa1/2* Δ and *san1* Δ cells to determine relative levels of substrate in the soluble versus insoluble pellet. We found that in the case of GFP^{NLS}- Δ 2GFP, GFP^{NLS}- Δ ssPrA, GFP^{NLS}-VHL, GFP^{NLS}-Tef2*, GFP^{NLS}-Bgl2* and GFP^{NLS}-Peptide 6, when Ssa1/Ssa2 were removed, the level of substrate in the insoluble fraction increased (**Figure 3.7 and Figure 3.8**). However, in the case of GAD-Cdc68-1 and GAD-Cdc13-1 we saw little to no transition of substrate into the insoluble pellet (**Figure 3.9**).

From these results we concluded that the substrates that seem to be differentially dependent on Ssa1/Ssa2 for degradation all displayed a large shift of protein from the soluble to the insoluble pellet in the absence of Ssa1/Ssa2. The data collectively can be seen in (**Figure 3.10**). This data suggests a paradigm shift where chaperones are not universally required for PQC in the nucleus like they have been shown to be in the cytoplasm and ER.

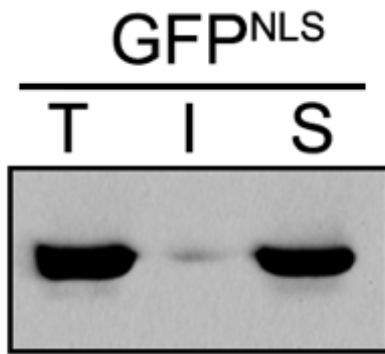


Figure 3.6: GFP^{NLS} is predominantly a soluble protein. Sedimentation assays were performed to determine how GFP^{NLS} partitions between the insoluble pellet fraction (I) and the soluble supernatant (S). Total lysate (T) indicates the total amount of GFP^{NLS} in cell lysates. Anti-GFP antibodies were used to detect GFP^{NLS}. (Previously shown by Pamela Gallagher in unpublished data).

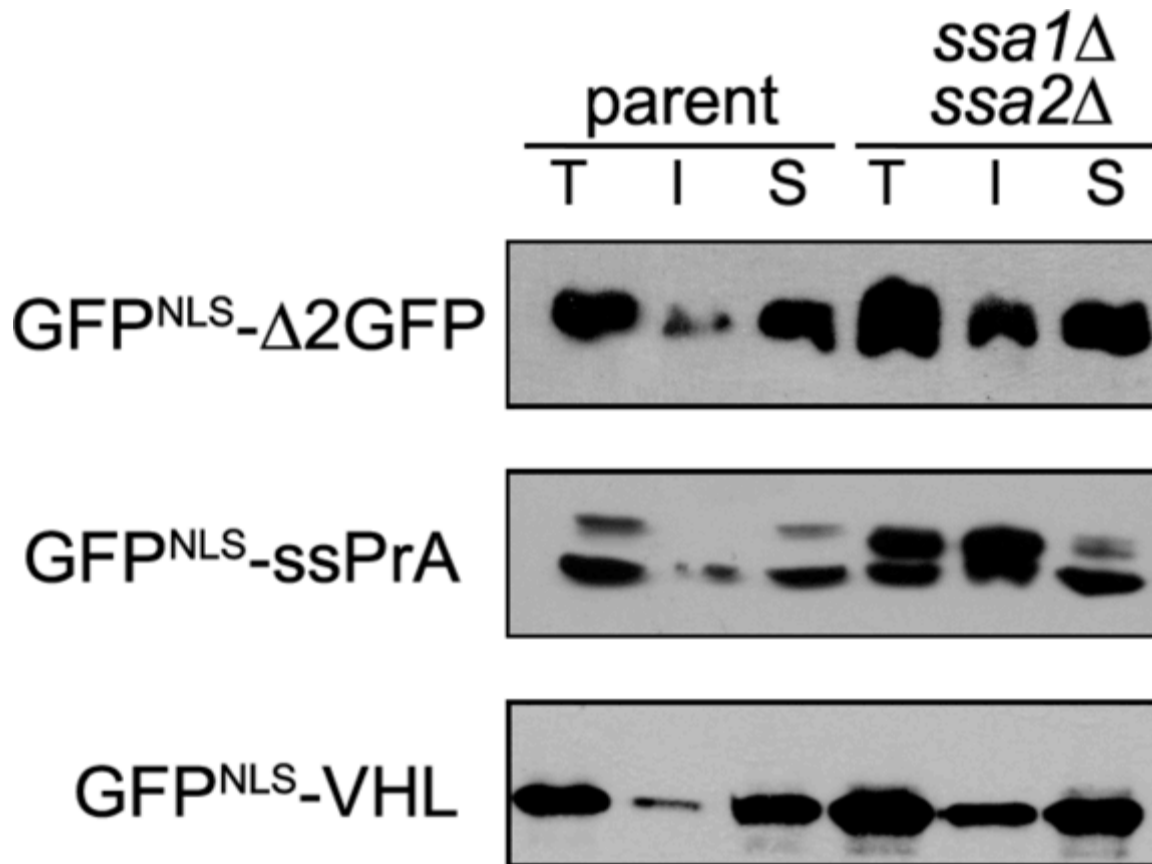


Figure 3.7 Full Ssa1/2 dependency correlates with increased insolubility.

Sedimentation assays were performed to determine how GFP^{NLS}- Δ 2GFP, GFP^{NLS}- Δ ssPrA and GFP^{NLS}-VHL partitions between the insoluble pellet fraction (I) and the soluble supernatant (S). Total lysate (T) indicates the total amount of GFP^{NLS} in cell lysates. Anti-GFP antibodies were used to detect GFP^{NLS}.

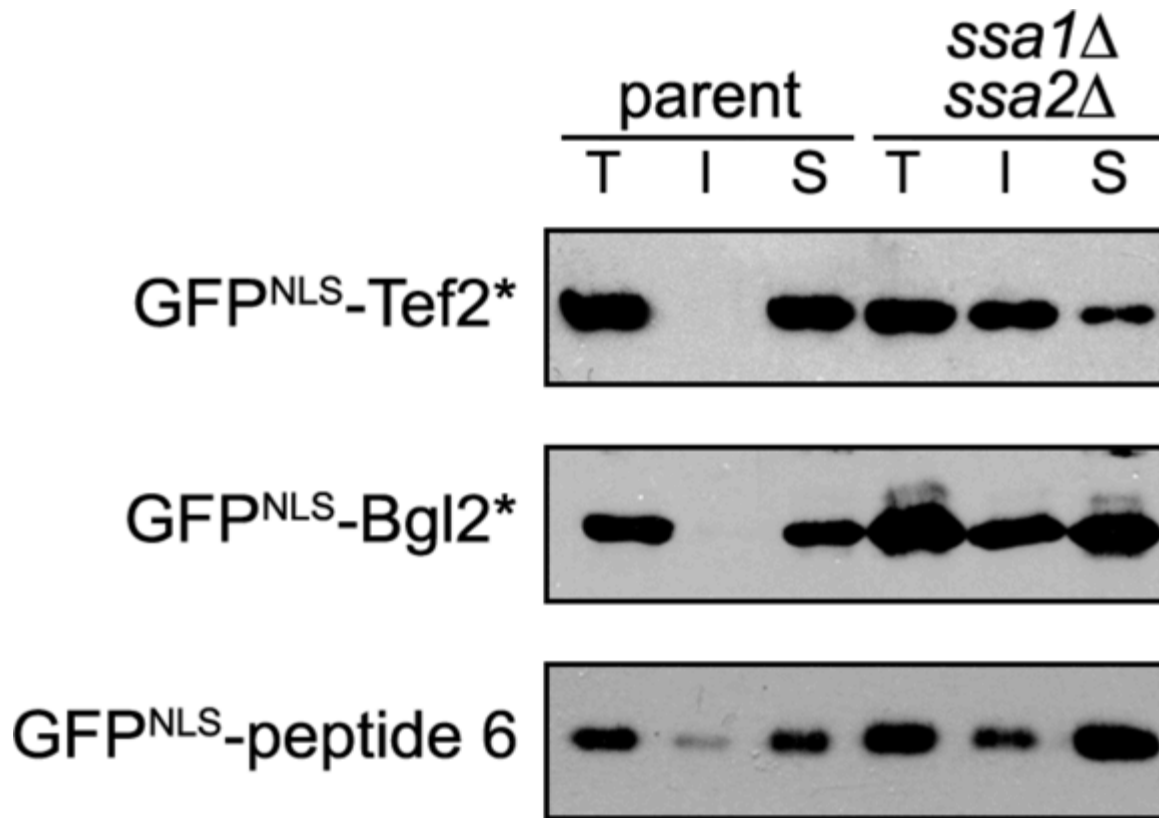


Figure 3.8 Ssa1/2 deletion results in increased insolubility. Sedimentation assays were performed to determine how GFP^{NLS}-Tef2*, GFP^{NLS}-Bgl2* and GFP^{NLS}-Peptide 6 partitions between the insoluble pellet fraction (I) and the soluble supernatant (S). Total lysate (T) indicates the total amount of GFP^{NLS} in cell lysates. Anti-GFP antibodies were used to detect GFP^{NLS}.

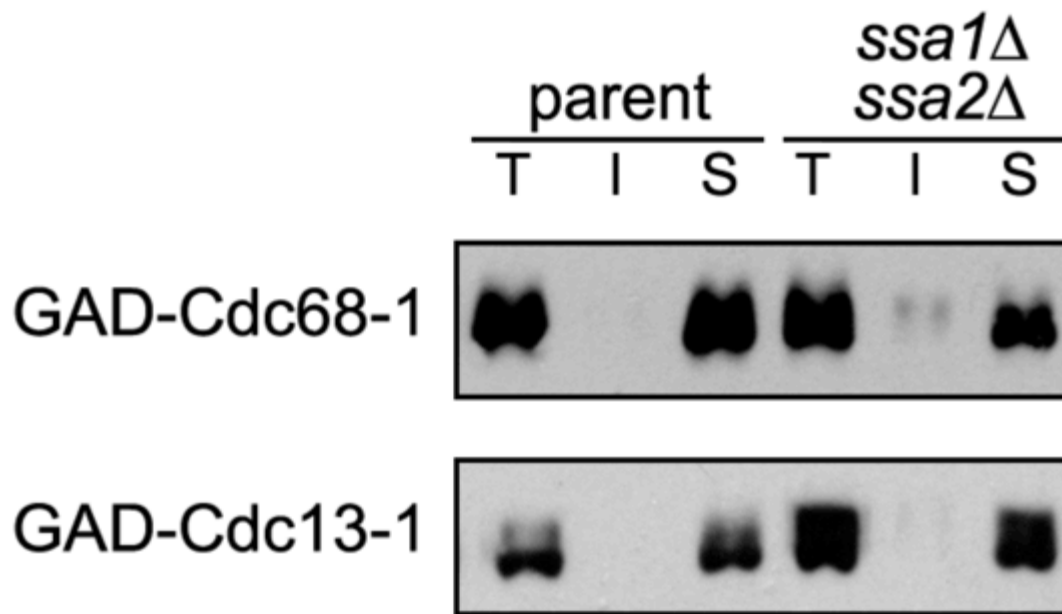


Figure 3.9 Substrates tested show no shift in solubility. Sedimentation assays were performed to determine how GAD-Cdc68-1 and GAD-Cdc13-1 partition between the insoluble pellet fraction (I) and the soluble supernatant (S). Total lysate (T) indicates the total amount of GAD in cell lysates. Anti-GAD antibodies were used to detect GAD.

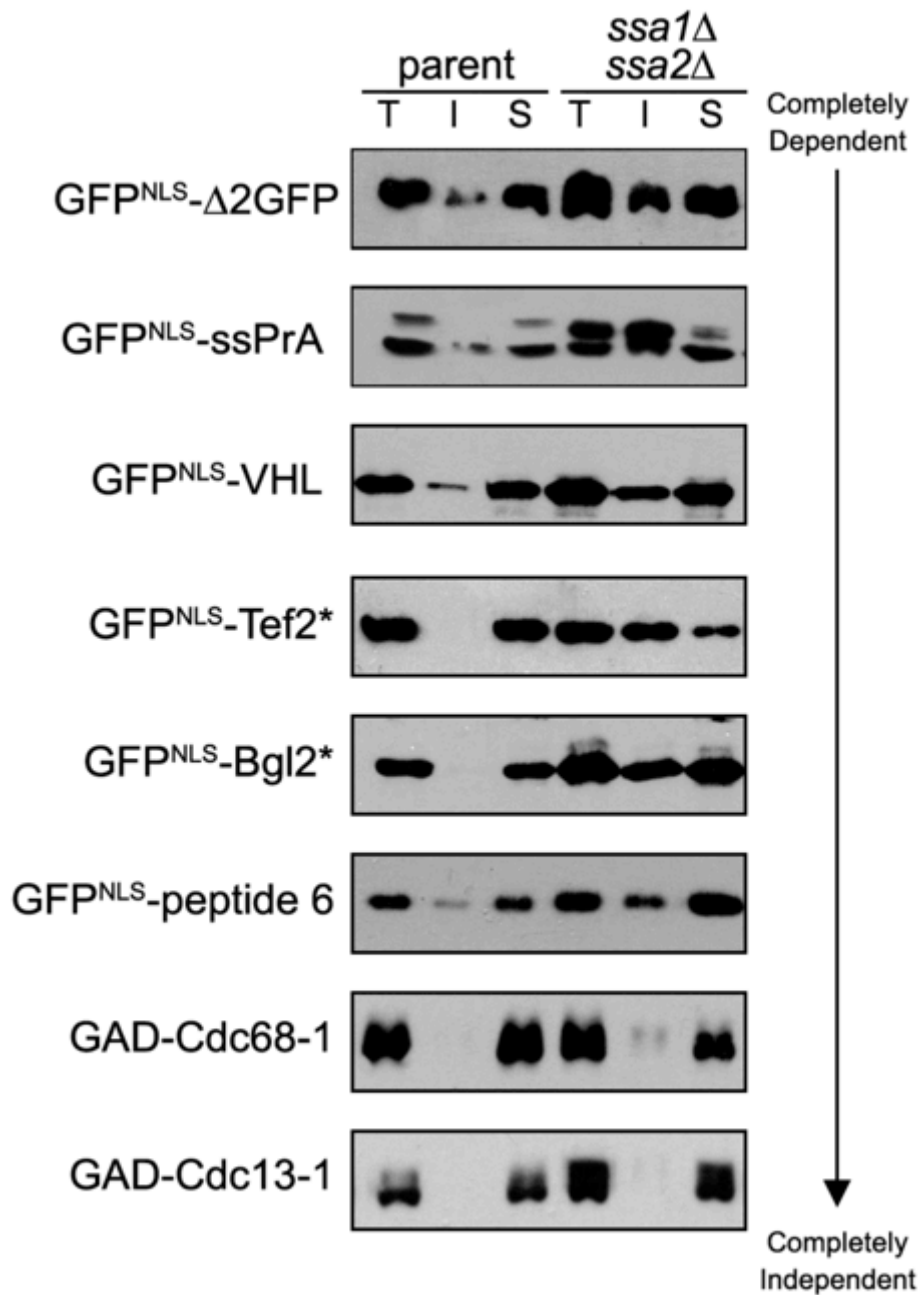


Figure 3.10 Substrate insolubility increases in the absence of Ssa1/2. Summary of data from figures 3.7, 3.8 and 3.9. Complete dependence and complete independence refers to dependence on Ssa1 and Ssa2 for degradation as shown in (Figure 3.5).

CHAPTER IV: THE ROLE OF THE SSA FAMILY IN SAN1 MEDIATED DEGRADATION

Summary of Ssa family findings

Thus far I have discussed the role of HSP70 isoforms Ssa1 and Ssa2 in San1 mediated proteasomal degradation. I showed that HSP70 is differentially required and that the requirement seems to correlate with the solubility state of the substrate of interest. The general correlation is that substrates that partition to the more insoluble fraction seem to require HSP70 the most, while those that reside mostly in the soluble fraction seem to be degraded independently of Ssa1 and Ssa2. In all cases where HSP70 was required, we saw a shift in solubility state towards the insoluble fraction when *SSA1* and *SSA2* were deleted from the cell.

***ssa1-45* strain effect on substrate degradation**

I want to spend some time discussing the use of the previously characterized *ssa1-45 Saccharomyces cerevisiae* strain. This strain has a genetic background where the *SSA2*, *SSA3* and *SSA4* genes have all been deleted and *SSA1* expression occurs from a plasmid-based gene that is temperature-sensitive (Becker et al., 1996). In this strain, as the restrictive temperature is approached, Ssa1 becomes inactivated and no longer functions. Historically, this strain has been used in studies to query the involvement of the HSP70 Ssa family in cell function. One study shows that in *ssa1-45* cells the substrate VHL is stabilized (McClellan et al., 2005). My data suggests that GFP^{NLS}-VHL requires, Ssa1 and Ssa2, specifically for degradation (**Figure 3.2**). Because these data do not delineate potential redundant Ssa function, I tested whether a substrate that I determined to not require HSP70 for degradation, GAD-Cdc68-1, would acquire

dependency in the *ssa1-45* cell line. In *ssa1/2Δ* cells GAD-Cdc68-1 was not stabilized, but in *ssa1-45* cells as the cells approached the restrictive temperature GAD-Cdc68-1 became more stabilized (**Figure 4.1**). I determine that this change in degradation patterns was not due simply to temperature (**Figure 4.2**) and that I would caution interpretation of data when using the *ssa1-45* strain in HSP70 studies.

***ssa1-45* cells increase insoluble fraction shift at restrictive temperature**

I speculated that as the restrictive temperature of 37°C is approached; *ssa1-45* cells have no functional Ssa family HSP70s. The chronic misfolded protein burden associated with the genotype of *ssa1-45* cells may become so high that proteins that previously did not require chaperones for their degradation in the case of *ssa1/2Δ* cells now acquired the need for chaperones in *ssa1-45* cells. If true I would expect to see an increased level GAD-Cdc68-1 in the insoluble fraction in *ssa1-45* cells compared to *ssa1/2Δ* cells. After performing sedimentation assays in *ssa1/2Δ* cells and *ssa1-45* cells expressing GAD-Cdc68-1; I observed an increased shift of GAD-Cdc68-1 from the soluble to insoluble fraction in *ssa1-45* cells (**Figure 4.3**)(**Figure 4.4**). Although this finding doesn't fully support my speculation it sheds light on the possibility of differential interpretations using *ssa1-45* cells to query HSP70 involvement. These results present the possibility that there may be some redundancy in the Ssa family of HSP70s. A summary of these findings can be found in (**Figure 4.5**). To determine if there truly is redundancy, or if the differences we see in *ssa1/2Δ* cells vs *ssa1-45* cells are due to the state of *ssa1-45* cell line we devised a way to test HSP70 inhibition acutely.

Treatment with HSP70 inhibitor, pifithrin- μ and Ssa family redundancy

At this point we didn't know why we saw these differences in *ssa1/2 Δ* vs *ssa1-45* cells. We speculated that due to the fact that *ssa1-45* cells have no functioning Ssa proteins at the restrictive temperature it's possible that the misfolded protein burden becomes more overwhelming and thus leads to PQC degradation machinery overload. This could in turn render misfolded proteins, increasingly aggregation prone. To determine if we could decipher this problem we went to the literature.

Upon a search for compounds I came across 2-phenylethanesulfonamide (PES). 2-phenylethanesulfonamide or Pifithrin- μ is a known broad HSP70 inhibitor (Leu et al., 2009; Sekihara et al., 2013; Monma et al., 2013). It works by disrupting the substrate-binding domain of HSP70 preventing it from interacting with substrates as well as known binding partners such as J domain proteins (Mayer and Bukau, 2005; Leu et al., 2009; Schlecht et al., 2013). Pifithrin- μ inhibits all HSP70s in all cell lines tested thus far (Leu et al., 2011). Therefore I wanted to determine if pifithrin- μ could be used as an acute treatment to inhibit HSP70s in *Saccharomyces cerevisiae*.

Based on my previous results with *ssa1/2 Δ* cells vs *ssa1-45* cells and their inconsistencies, I wanted to remove all speculation associated with using either cell line to study HSP70s involvement in the San1 pathway. Therefore, I acutely treated wild type cells expressing GAD-Cdc68-1 and GFP^{NLS}-VHL with pifithrin- μ at 20 μ M for 1 hour to determine if acute inhibition of the Ssa family and all HSP70s would help us discover redundancies. In addition, we wanted to determine if acute inhibition of HSP70s recapitulated the results in the chronic deletion condition we see in the case of *ssa1-45* cells.

GAD-Cdc68-1 was degraded independently of HSP70 function in *ssa1/2Δ* cells, but stabilized in *ssa1-45* cells at the restrictive temperature. To test if acute treatment with a broad HSP70 inhibitor revealed any consistencies; cycloheximide chase assays with pifithrin- μ treatment were performed as described in **Appendix II**. When I examined the effects of using pifithrin- μ in parent cells; I obtained preliminary data that does not agree with the comparative result in *ssa1-45* cells (**Figure 4.6**). I found that treatment with pifithrin- μ did not expose any redundancies in the case of GAD-Cdc68-1. I also found that GAD-Cdc68-1 was degraded in wild type, vehicle treated and drug treated conditions (**Figure 4.6**). GFP^{NLS}-VHL behaved as expected because it is dependent on Ssa1 and Ssa2 for degradation (**Figure 3.3**) (**Figure 4.6**). These results suggest that it is possible that there are some redundant functions between members of the Ssa family members. However, we did not observe this phenomenon via this acute drug treatment assay.

Although the data in (**Figure 4.6**) suggests there is no redundancy in the Ssa family; based on the lack of stabilization in the case of GAD-Cdc68-1. There are some caveats to this data. These results are preliminary, and the yeast strain background that this experiment was performed in is a cell background that has all of the drug efflux transporters intact. Thus it is possible that the lack of effect we see in the case of GAD-Cdc68-1 when treated with pifithrin- μ , could be due to drug efflux. We acknowledge this and are working on repeating this experiment in yeast cells lines that lack drug efflux pumps to uncover degradation patterns for GAD-Cdc68-1 and GFP^{NLS}-VHL.

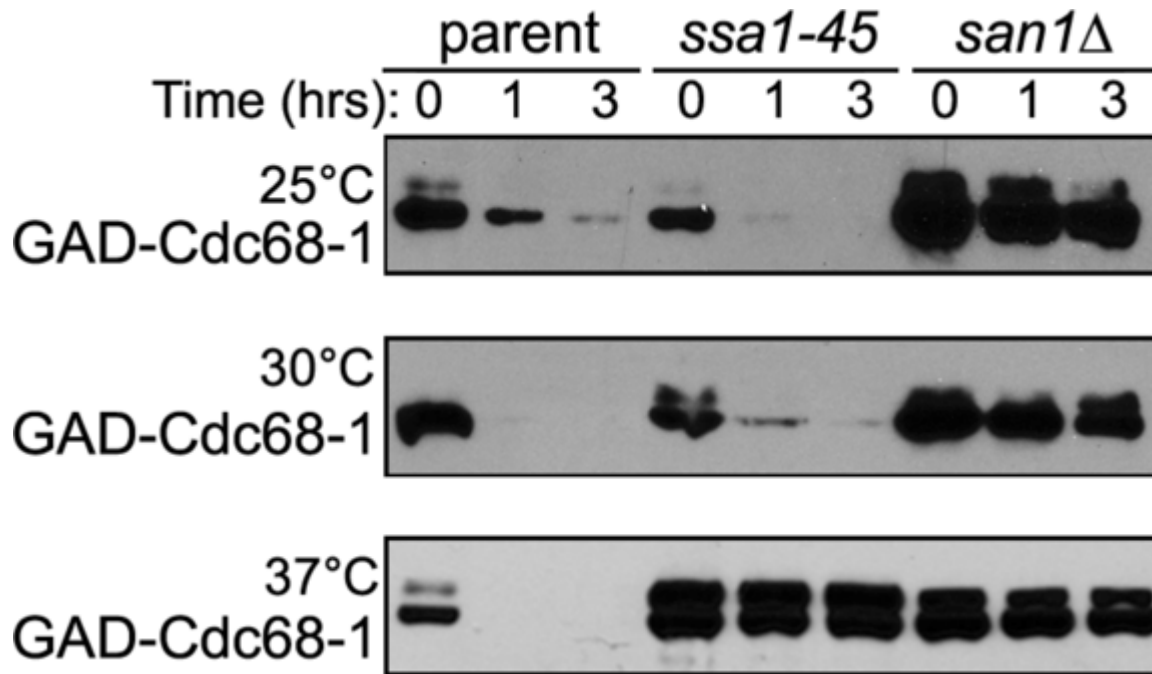


Figure 4.1 Substrate formerly thought to be independent of Ssa1 and 2 shows stabilization in *ssa1-45* cells as restrictive temperature is approached.

Cycloheximide-case degradation assays were performed on parent, *ssa1-45* and *san1*Δ cells to assess the stability of GAD-Cdc68-1 in *ssa1-45* cells. Expression was constitutive before addition of cycloheximide. Cell cultures were shifted from 25°C to 30°C or 37°C for 1 hour prior to harvesting. Anti-GAD antibodies were used to detect the substrate tested.

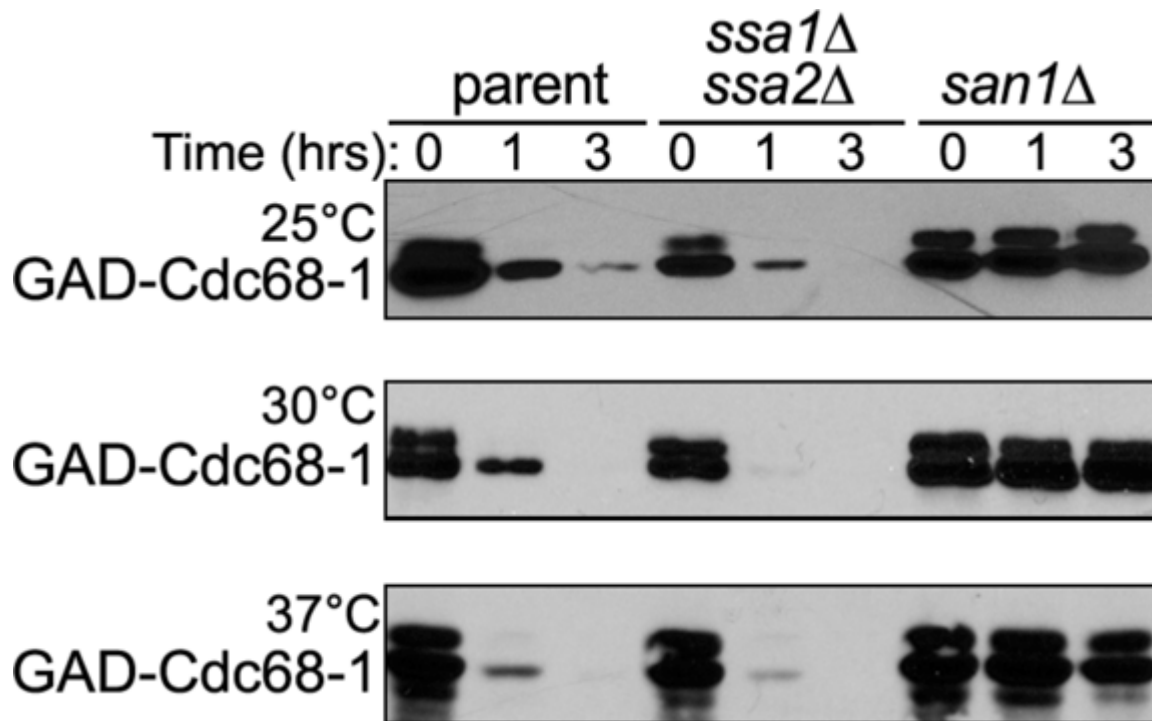


Figure 4.2 GAD-Cdc68-1 remains independent of Ssa1/2 at 37°C. Cycloheximide-case degradation assays were performed on parent, *ssa1/2* Δ and *san1* Δ cells to assess the stability of GAD-Cdc68-1 in *ssa1/2* Δ cells as temperature controls for Figure 4.1. Expression was constitutive before addition of cycloheximide. Cell cultures were shifted from 25°C to 30°C or 37°C for 1 hour prior to harvesting. Anti-GAD antibodies were used to detect the substrate tested.

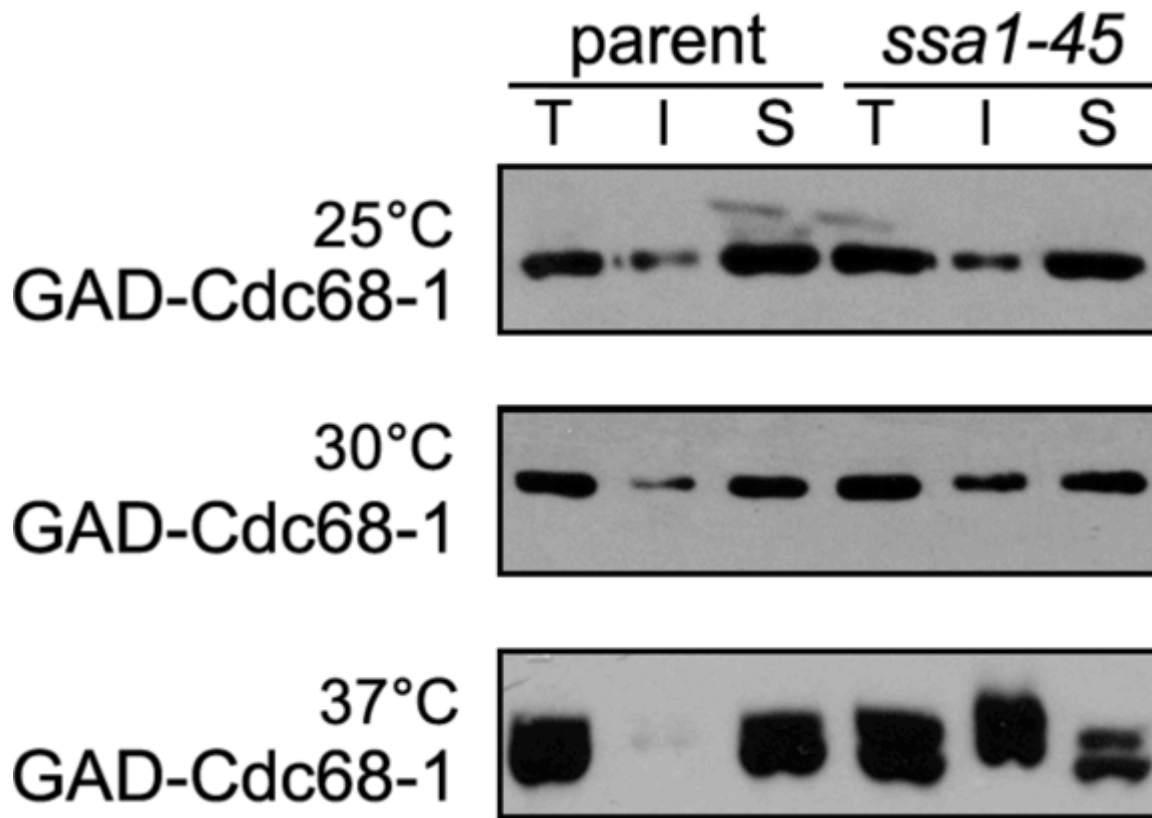


Figure 4.3 *ssa1-45* cells result in increased insolubility as the restrictive temperature is approached. Sedimentation assays were performed to determine how GAD-Cdc68-1 partitions between the insoluble pellet fraction (I) and the soluble supernatant (S) in parent and *ssa1-45* cells. Total lysate (T) indicates the total amount of GAD in cell lysates. Anti-GAD antibodies were used to detect the substrate.

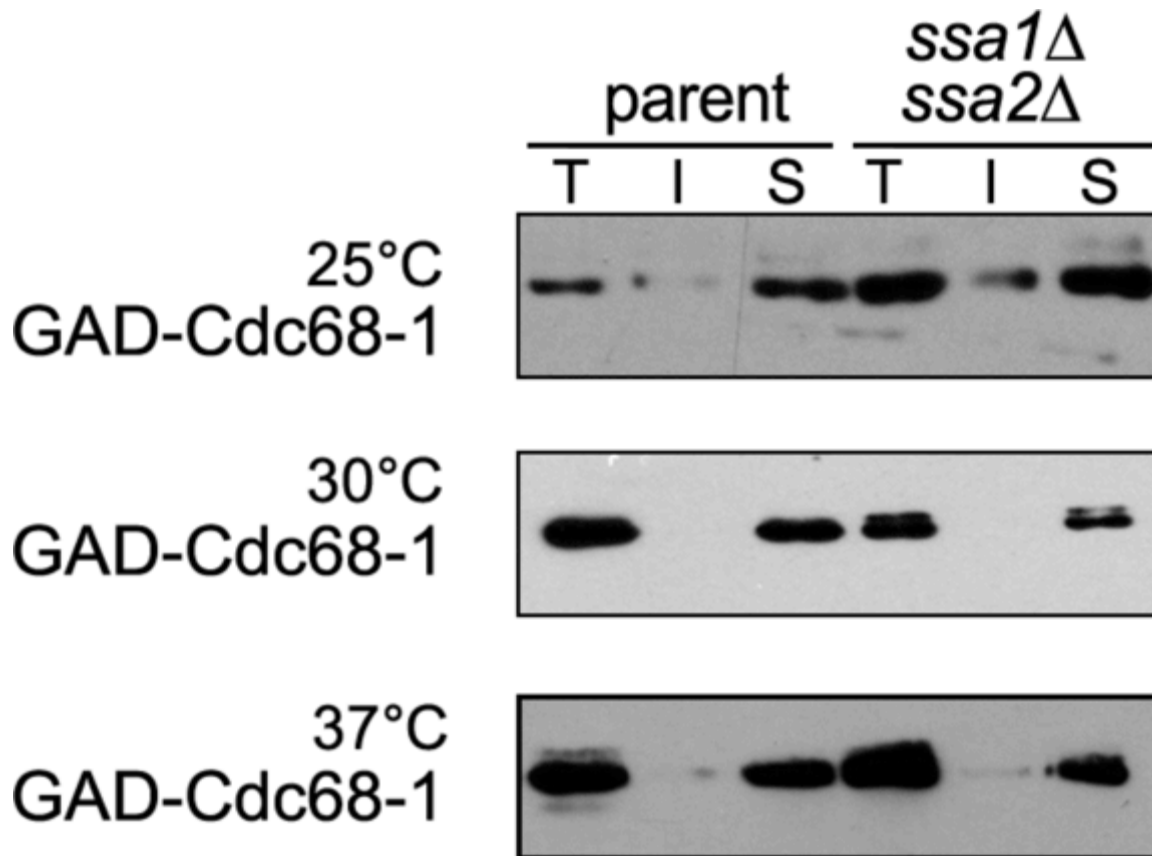


Figure 4.4 *ssa1/2* Δ cells result in little to no change in solubility at 37°C. Sedimentation assays were performed to determine how GAD-Cdc68-1 partitions between the insoluble pellet fraction (I) and the soluble supernatant (S) in parent and *ssa1/2* Δ cells. Total lysate (T) indicates the total amount of GAD in cell lysates. Anti-GAD antibodies were used to detect the substrate.

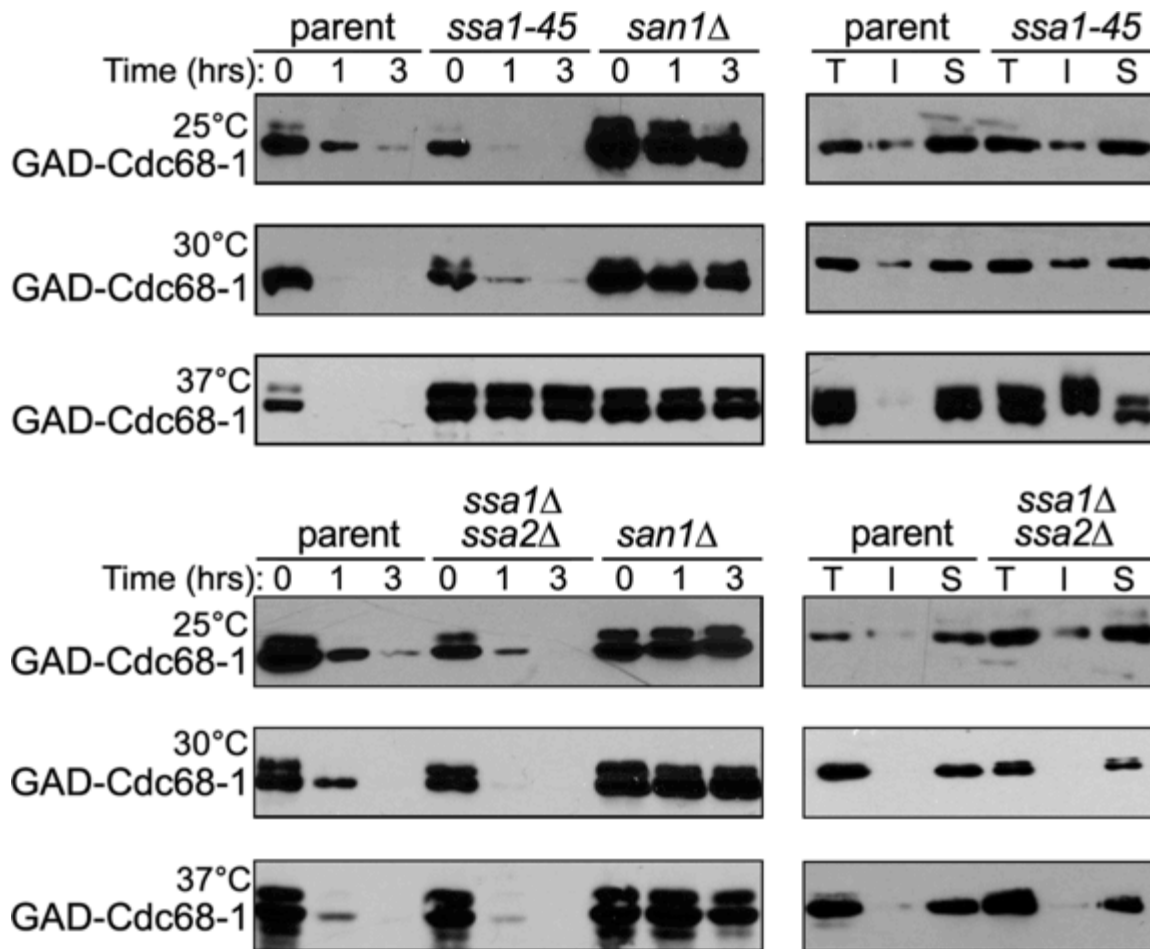


Figure 4.5 Temperature alone is not responsible for insoluble fraction shift in *ssa1-45* cells. Summary of data in Figures 4.1, 4.2, 4.3 and 4.4.

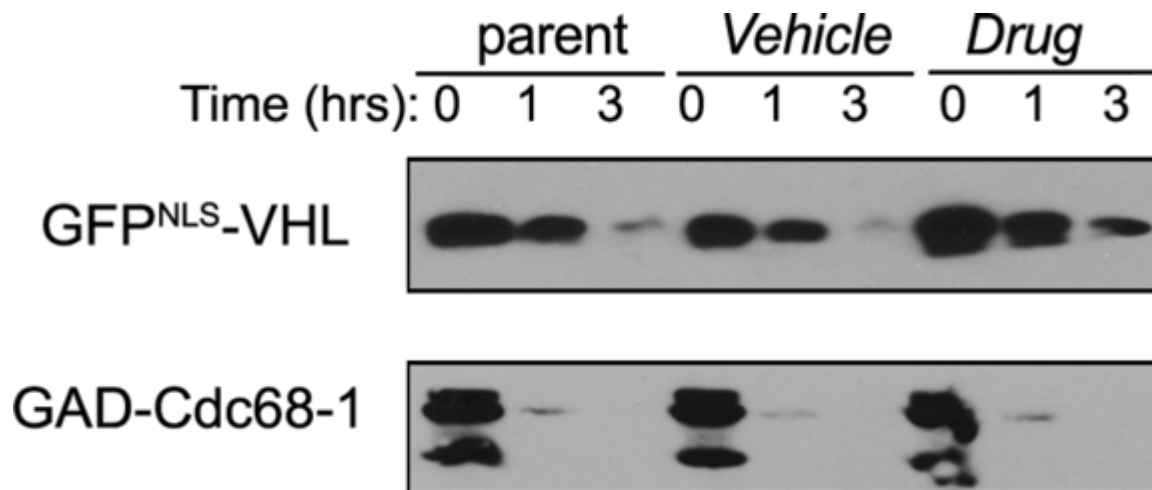


Figure 4.6 Pharmacological HSP70 inhibition and San1 substrates Cycloheximide-case degradation assays were performed on parent cells treated with pifithrin- μ at 20 μ M for 1 hour before lysate collection and addition of cycloheximide. If the substrate required expression from the GAL1 promoter, a 3 hour induction with galactose was performed prior to addition of pifithrin- μ to assess the stability of GAD-Cdc68-1 and GFP^{NLS}-VHL. In the case of GAD-Cdc68-1 expression was constitutive before addition of cycloheximide. Cell cultures were grown at 30°C. Anti-GAD or Anti-GFP antibodies were used to detect the substrate tested.

San1 interacts with substrates but not Ssa1

To this point my data has suggested that Ssa1 and Ssa2 are the major chaperones in this San1 pathway for the substrates queried in this dissertation. However, it was important to determine if there is a direct interaction between San1 and Ssa1 that is necessary for degradation. A previous lab member, Joel Rosenbaum, discovered in a coimmunoprecipitation (coIP) tandem mass spectrometry experiment that San1 showed high levels of enrichment for interactions with substrates but not chaperones (Rosenbaum et al., 2011). This finding supported the idea that chaperones and San1 do not directly interact but we wanted to test this further using the yeast 2-hybrid system.

The yeast 2-hybrid (Y2H) system is a molecular biology technique used to screen for protein-protein interactions. Y2Hs utilize a reporter gene whose activation is contingent on the binding of two transcription factors. The transcription factors can be split; one being attached to a bait protein and one being attached to a prey protein. In cells expressing both the bait and prey proteins, tagged with the appropriate transcription factors, the reporter gene does not get transcribed unless the prey and bait proteins interact.. To conduct the Y2H, we chose Ssa1 because it is the dominant functioning Ssa between Ssa1 and Ssa2. For our bait protein we used San1^{C279S} fused to GBD from our previous work (Gardner et al., 2005). GBD-San1^{C279S} was used because we needed to use a catalytically dead version of San1 to prevent it from tagging potential substrates for degradation, but that would still interact with substrates. We then co-expressed GBD-San1^{C279S} as the bait protein with three San1 substrates, GAD-Tef2*, GAD-Bgl2* and GAD-Cdc13-1 individually, we found from previous work (Rosenbaum et al., 2011) as the prey proteins. We also tested one chaperone from the

mass spectrometry screen that was shown to not interact with San1, (Rosenbaum et al., 2011) GAD-Ssa1 to determine potential interactions. Using this yeast 2 hybrid system, I show preliminary data that we obtained (**Figure 4.7**) suggesting that San1 interacts directly with our test substrate, but not Ssa1. These findings were based on growth or lack thereof on media that required our reporter gene to be activated for cell growth. These results are consistent with our previous work (Rosenbaum et al., 2011). We acknowledge that yeast 2 hybrid assays may potentially lead to false negatives for interaction due to possible weak interactions that yeast 2 hybrid assays are not sensitive enough to detect. We make our conclusions with this assumption in mind. This result taken together with the mass spectrometry data from previous work (Rosenbaum et al., 2011) give us confidence that these interactions with substrates and San1; and null interactions with Ssa1 and San1 are probable. This data along with my previous discussed results suggest that Ssa1 is required to maintain substrate solubility but this maintenance function does not require a direct interaction with San1. This idea fits our model that HSP70 is a facilitator in San1 mediated degradation, but not the key component for the recognition of substrates.

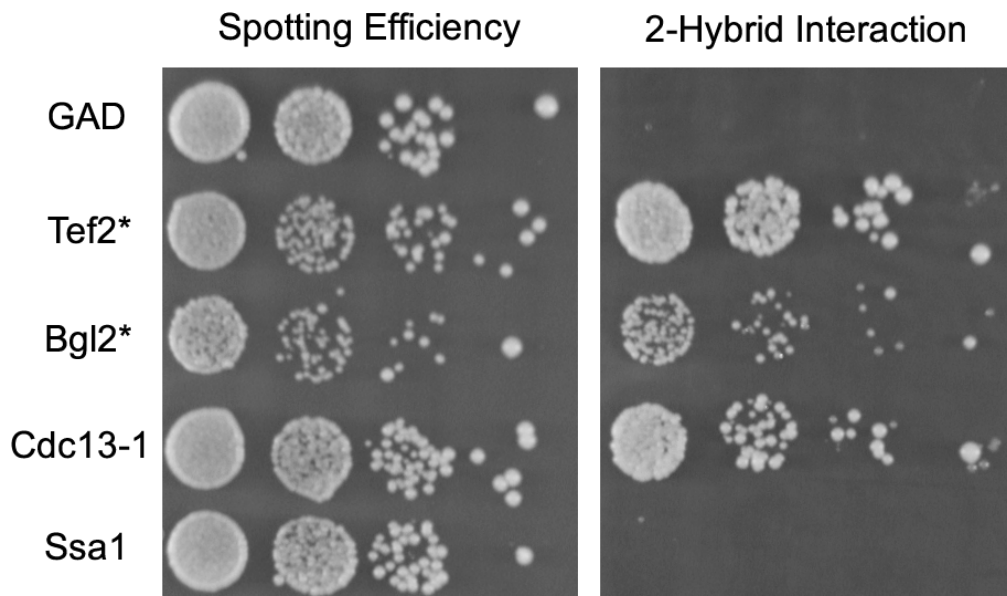


Figure 4.7 San1 interacts with substrates and not Ssa1. Yeast 2 Hybrid assays were performed to determine protein-protein interactions. Interaction was monitored by the methods described in **Appendix II**. GBD-San1^{C279S} was used as the bait protein. GAD, GAD-Tef2*, GAD-Bgl2*, GAD-Cdc13-1 and GAD-Ssa1 were used as prey proteins. Cells were spotted in 10 fold serial dilutions on selective media and grown at 30°C.

Loss of Ssa1 and Ssa2 results in increased nuclear inclusion formation

Based on our results in chapter three we wanted to determine if substrates that seemed to require HSP70 in varying degrees had an increase propensity to form nuclear inclusions. This would support the idea that substrates that suffer from insolubility, due to more exposed hydrophobicity, will display increased levels of aggregation. Nuclear inclusions are generally thought to be sites of sequestration where misfolded proteins are stored in clusters or aggregates to be acted on by PQC machinery later to prevent interactions with other viable proteins. We know that misfolded proteins can influence or disrupt proper folding of other proteins. Thus we would expect in the absence of HSP70 we would observe a shift to more of each substrate forming inclusion deposits because San1 may not be able to recognize them immediately for degradation due to their aggregative state.

We tested three substrates that were either partially or fully dependent on HSP70 in San1-mediated degradation; to determine their propensity to form inclusions in the presence and absence of PQC machinery. In the case for all substrates we see increased inclusion formation in *ssa1/2*Δ cells and *san1*Δ cells in comparison to wild type cells. These data can be found in **(Figure 4.8, Figure 4.9, and Figure 4.10)**. One thing to note is the intensity and size of the inclusions in the case of Tef2*-GFP^{NLS} and VHL-GFP^{NLS} were much more apparent than those in the case of GFP^{NLS}-Bgl2*. We speculated that GFP^{NLS}-Bgl2* may not be sequestered into inclusions as robustly as the other two substrates queried, but inclusion formation kinetics were not monitored in this dissertation. We also acknowledge the possibility that this could be an expression issue but are confident in the data because these results have been replicated in multiple

independent experiments. In addition, we never see issues with levels of expression with GFP^{NLS}-Bgl2* by cycloheximide chase assays (**Figure 3.3**) or solubility assays (**Figure 3.8**).

***ssa1/2*Δ cells display varying levels of cellular toxicity**

Previously we found that some San1 substrates are toxic in *san1*Δ cells (Rosenbaum et al., 2011). Thus we wanted to determine if there were any patterns in toxicity associated with *ssa1/2*Δ cells. In a simple spot test assay with serial 10 fold dilutions in substrate repression and induction conditions we found that in the case of GFP^{NLS}-Tef2*- and GFP^{NLS}- VHL there seemed to be a slight growth defect in the case of *ssa1/2*Δ cells expressing GFP^{NLS}-Tef2* but no defect in *ssa1/2*Δ cells expressing GFP^{NLS}- VHL (**Figure 4.11**) (**Figure 4.12**) when compared to parent cells. However, in the case of GFP^{NLS}-Bgl2* we noticed a considerable defect in cell growth in *ssa1/2*Δ cells (**Figure 4.13**). That data is summarized in (**Figure 4.14**).

The underlying mechanisms of toxicity for aggregation prone, misfolded proteins have yet to be elucidated. There is some speculation that different aggregate species confer toxicity (Yang et al., 2009) but very little is known about what toxicity actually is and how it occurs. More studies are required to understand the mechanisms of toxicity and to determine if/how toxicity and aggregation states are linked and affect the viability of the cell.

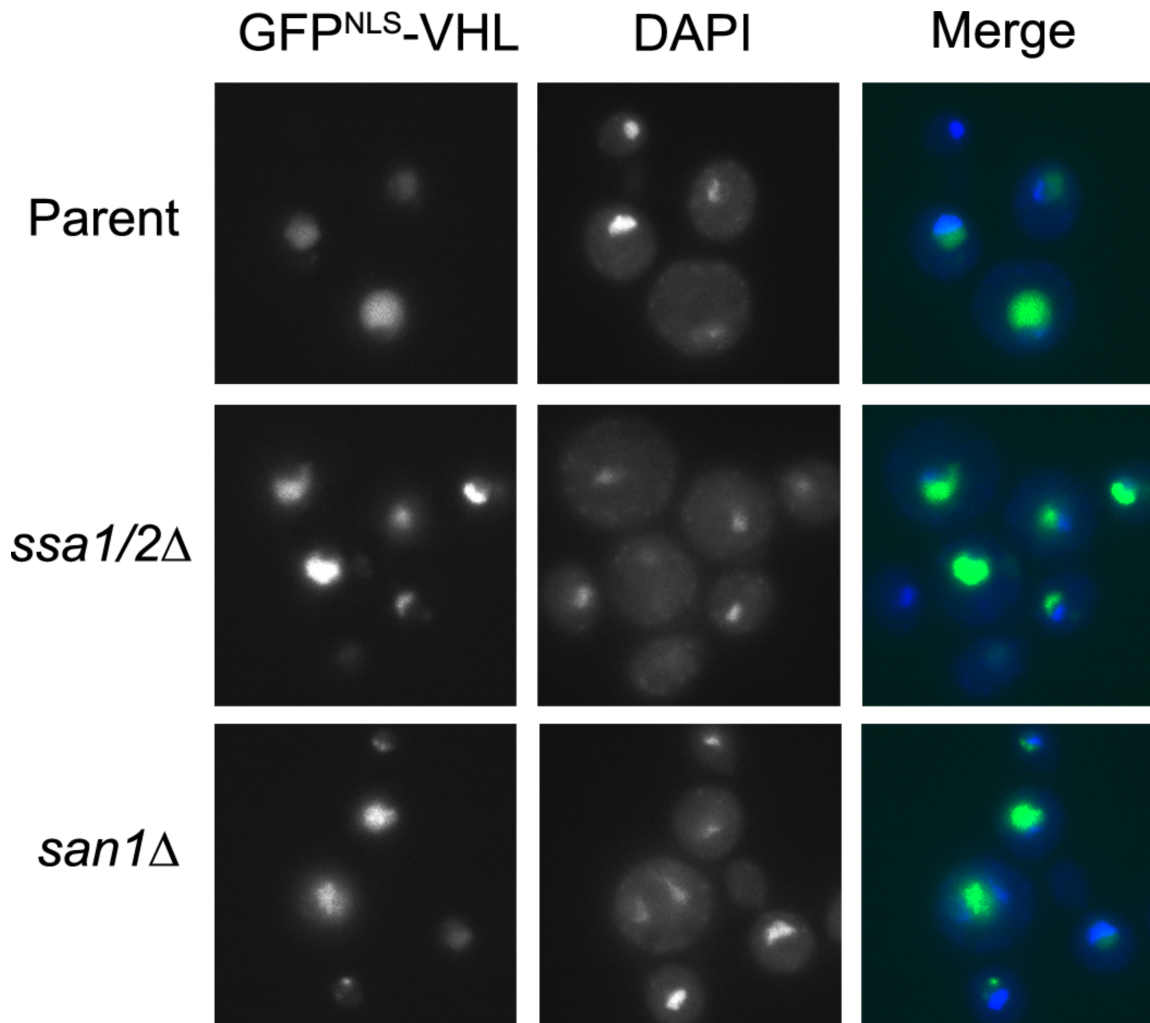


Figure 4.8 Loss of PQC results in increased inclusion formation. Expression of GFP^{NLS}-VHL in parent, *ssa1/2Δ* and *san1Δ* cells was induced by the addition of galactose for 6 hours in log phase. Cells were fixed with paraformaldehyde, stained with DAPI and examined by fluorescence microscopy.

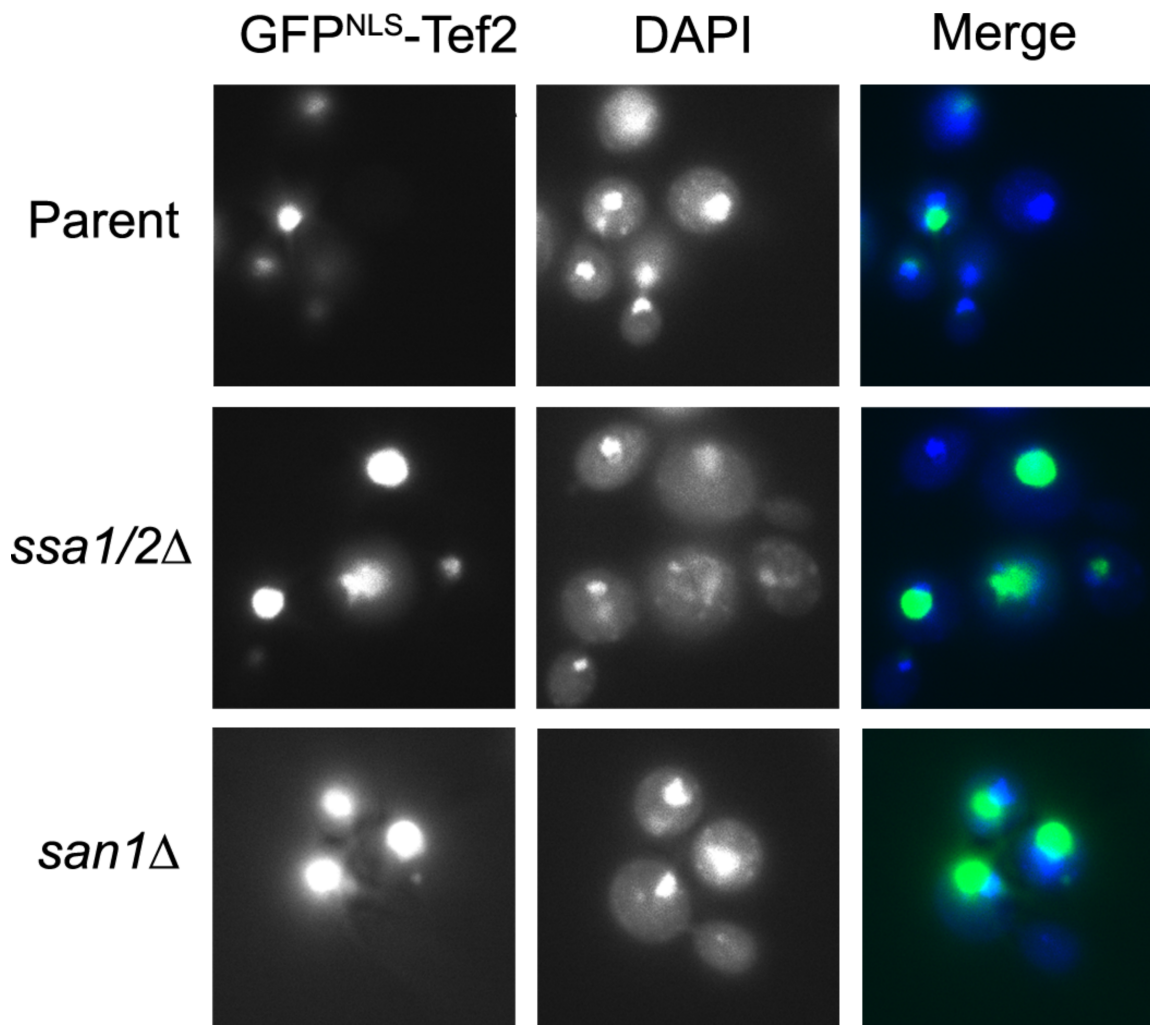


Figure 4.9 Loss of Ssa1/2 results in increased inclusion formation. Expression of GFP^{NLS}-Tef2* in parent, *ssa1/2*Δ and *san1*Δ cells was induced by the addition of galactose for 6 hours in log phase. Cells were fixed with paraformaldehyde, stained with DAPI and examined by fluorescence microscopy.

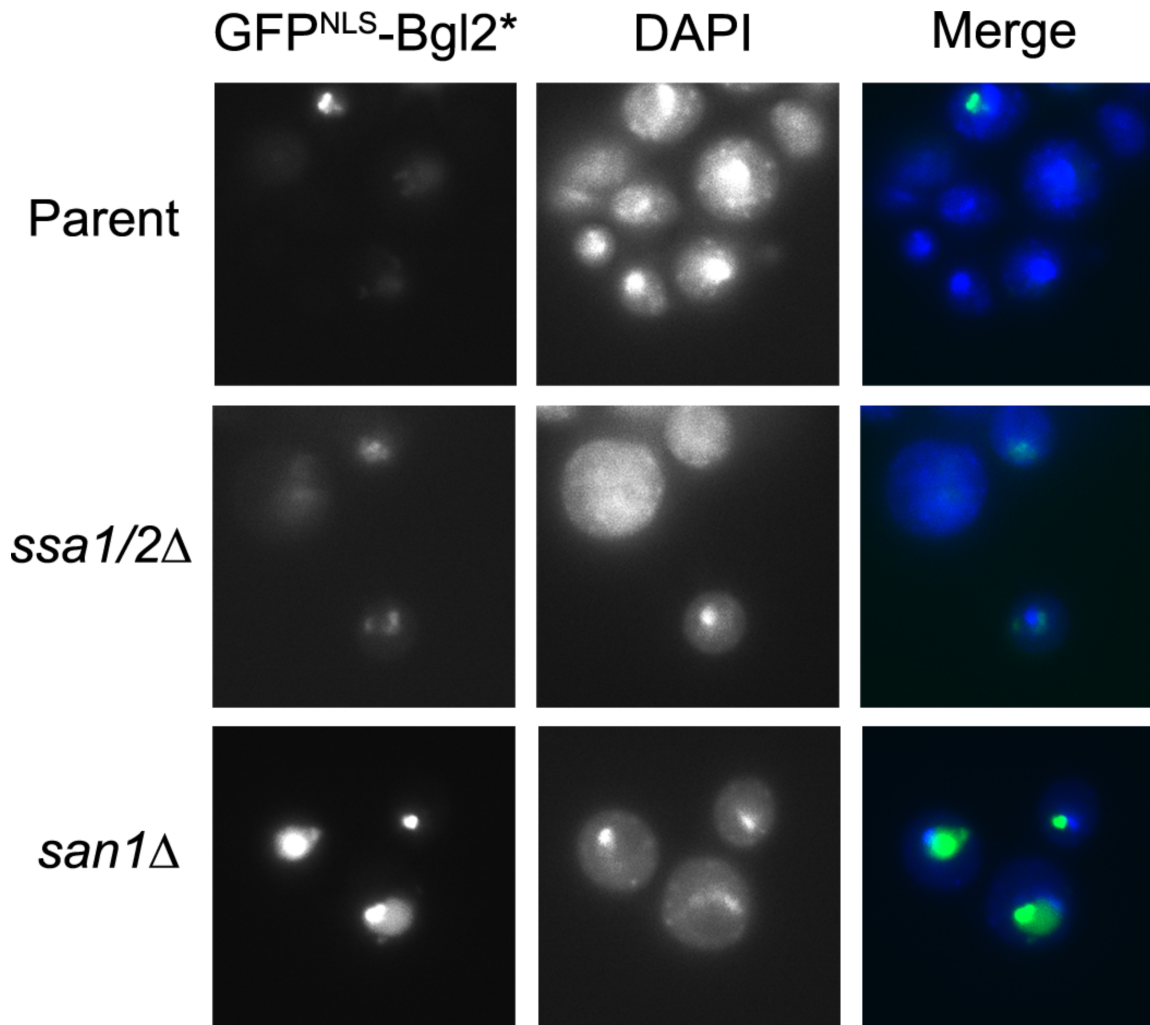


Figure 4.10 Loss of PQC results in increased inclusion formation but not as robust as previously tested substrates. Expression of GFP^{NLS}-Bgl2* in parent, *ssa1/2*Δ and *san1*Δ cells was induced by the addition of galactose for 6 hours in log phase. Cells were fixed with paraformaldehyde, stained with DAPI and examined by fluorescence microscopy.

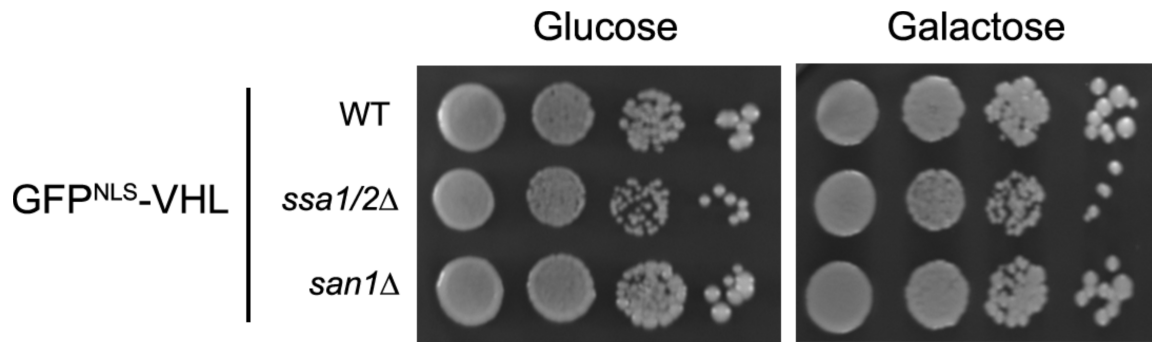


Figure 4.11 GFP^{NLS}-VHL displays no level of toxicity in *ssa1/2Δ* cells. Parent, *ssa1/2Δ* and *san1Δ* cells expressing GFP^{NLS}-VHL were spotted at 10-fold serial dilutions onto media containing glucose to measure spotting efficiency or galactose to induce expression at 30°C.

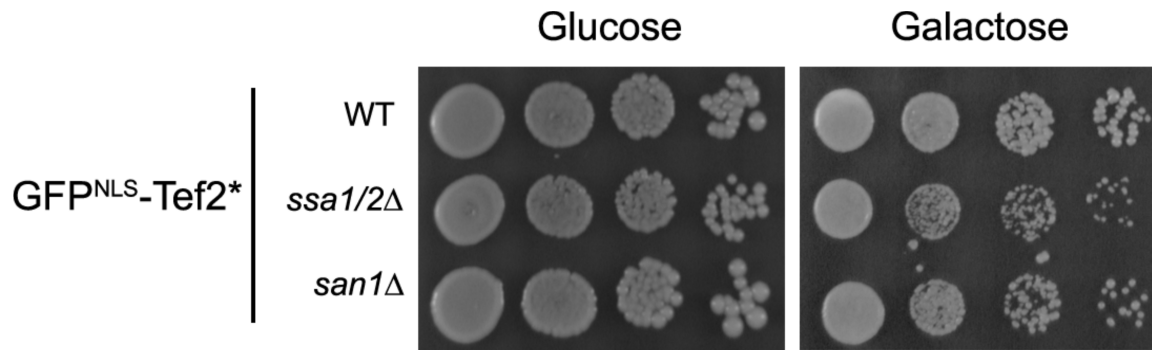


Figure 4.12 GFP^{NLS}-Tef2* displays a slight level of toxicity in *ssa1/2*Δ cells. Parent, *ssa1/2*Δ and *san1*Δ cells expressing GFP^{NLS}-Tef2* were spotted at 10-fold serial dilutions onto media containing glucose to measure spotting efficiency or galactose to induce expression at 30°C.

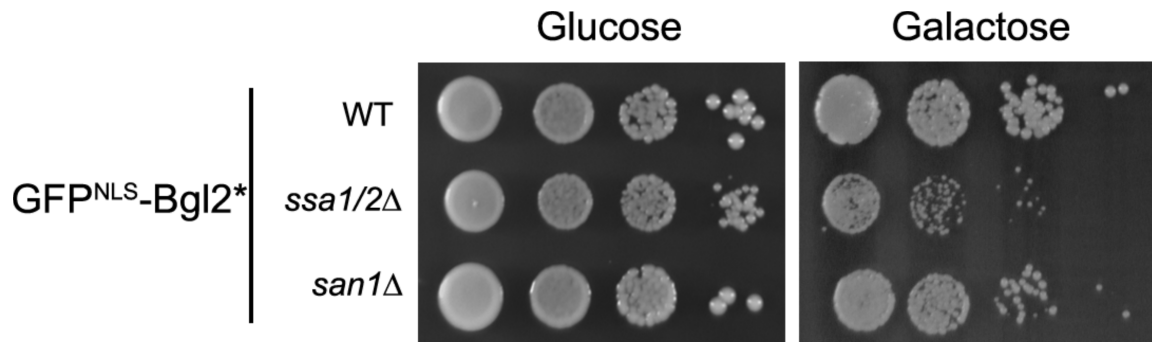


Figure 4.13 GFP^{NLS}-Bgl2* displays considerable toxicity in *ssa1/2*Δ cells. Parent, *ssa1/2*Δ and *san1*Δ cells expressing GFP^{NLS}-Bgl2* were spotted at 10-fold serial dilutions onto media containing glucose to measure spotting efficiency or galactose to induce expression at 30°C.

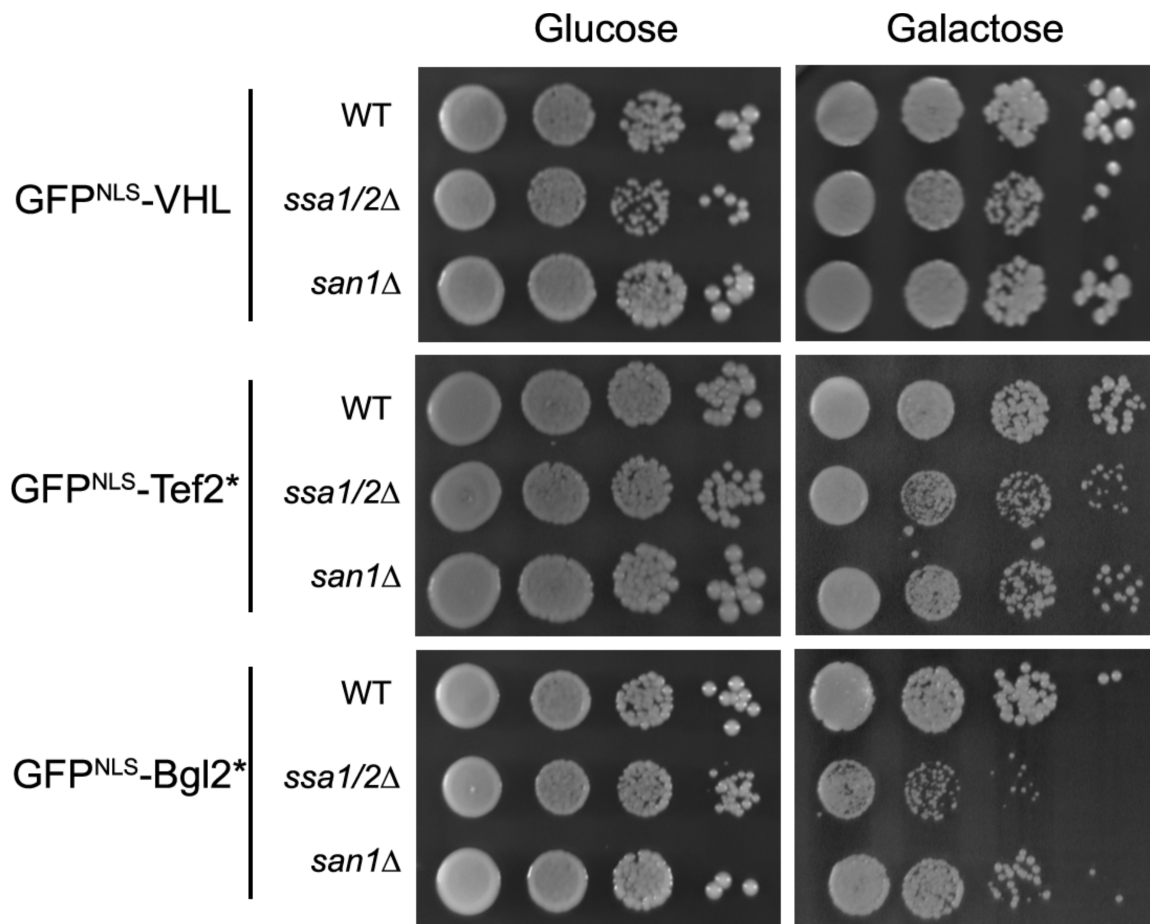


Figure 4.14 Tested substrates have different levels of toxicity. Summary of data in Figures 4.11, 4.12 and 4.13

CHAPTER V: CONCLUSIONS

The results in this dissertation add to the collective of knowledge in the field of protein quality control. In the budding yeast *Saccharomyces cerevisiae*, different HSP70 isoforms have been specifically implicated in protein quality control in every cellular compartment except the nucleus. To this point the nuclear protein quality control mechanisms for HSP70 remained a mystery. My data implicates HSP70 isoforms Ssa1 and Ssa2 in nuclear protein quality control degradation through the action of the nuclear localized, San1 ubiquitin-proteasome system (UPS). Although, Ssa1 and Ssa2 are required for the degradation of some San1 substrates via the San1 UPS; however, this requirement is not universal to all San1 substrates. My findings suggest a shift in the paradigm for HSP70 involvement in PQC. The data presented here supports the ideas that instead of HSP70 being required for PQC in all cases, there seems to be a differential requirement for HSP70 from substrate to substrate. I found that the determinant for this differential requirement is the maintenance of substrate solubility. The correlation these data support is that Ssa1 and Ssa2 are required more for substrates that have increased solubility and are required less or not at all for substrates that are soluble.

The data we've collected has helped us uncover a variety of cellular consequences for cells lacking Ssa1 and Ssa2 as well. Deletion of Ssa1 and Ssa2 resulted in increased nuclear inclusion formation for the substrates tested in addition to increased cellular toxicity in some cases. We as a field do not fully understand the underlying mechanisms for cellular toxicity and how those mechanisms may overlap with nuclear inclusions or sites of aggregation. From our data we can infer that HSP70 isoforms

Ssa1 and Ssa2 are important for preventing toxicity and formation of nuclear inclusions but our interpretations for the mechanisms by which this is occurring are not definitive. Based on our results we speculate that Ssa1 and Ssa2 may partake in a phenomenon known as kinetic partitioning. Kinetic partitioning is best described as a process by which chaperone proteins, in this case HSP70, participate in quality control, when necessary, by partitioning aggregative forms or aggregation prone substrates to more soluble states, so that PQC machinery can then recognize, and degrade or repair them as shown in **(Figure 5.1)**. This may be the case because we know that HSP70 has been shown to be involved with the dissolution of prions and some aggregate forms in various cell lines. We also know Ssa1 and Ssa2 are important for maintaining substrate solubility. One open question we still have is how substrates are delivered to HSP70s and what features of a substrate HSP70s bind to. Could it be specific sequences? Could it be a threshold of exposed hydrophobic or hydrophilic residues in a polypeptide? At this point we don't know, but this information will be important to understand as we look to HSP70s as therapeutic targets for aggregation disorders in the future.

It is important to acknowledge our data on Ssa family redundancy. As I previously mentioned, the members of the Ssa family of HSP70s are very similar and are expressed in different cellular stress conditions. Our use of the historical *ssa1-45* cell line and the conflicting results with the use of *ssa1//2Δ* cell line displayed in this dissertation primes us to provide a caution to field when interpreting broad implications for HSP70s involvement in PQC when using either cell line. We attempted to address some of the speculation between the use of these two cell lines for studying HSP70

involvement in PQC by pharmacological treatment. Our preliminary results support the notion that there isn't a fully functional redundancy shared between all of the Ssa family members. However, at this time we are refraining from making definitive conclusions until all proper precautions and experimentation is conducted to make certain claims.

In the Gardner lab we have revealed a variety of key characteristics and role - players in NucPQC. My work here implicates Ssa1 and Ssa2 in NucPQC and potentially offers insight as to how HSP70 could be working in the nucleus to help facilitate degradation of misfolded proteins that arise there. These processes are important to understand because they could be key to the survival of the cell. Future work will be focused on bettering our understanding of the link and balance between protein aggregation, misfolded protein repair and misfolded protein degradation. Understanding how different arms of PQC, more specifically NucPQC, coordinate will be crucial for understanding how to combat aggregation disease.

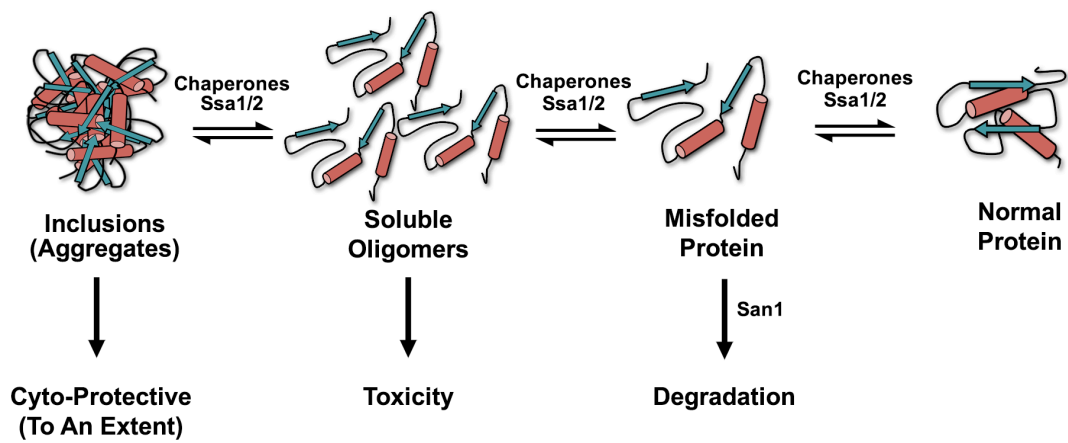


Figure 5.1 A simplified model for Ssa1 and Ssa2 involvement in the San1 UPS

APPENDIX I: STRAINS AND PLASMIDS

Strain Name	Genotype	Parent
RGY2971	<i>ade2-1, MET2, MET15, his3-11, ura3-1, trp1-1, LYS2, leu2-3, can 1-100</i> <i>ssa1Δ::KanMX</i> <i>ssa2Δ::KanMX</i>	W303a
RGY2970	<i>ade2-1, MET2, MET15, his3-11, ura3-1, trp1-1, LYS2, leu2-3, can 1-100</i> <i>W303a</i>	W303a
RGY5113	<i>ade2-1, MET2, MET15, his3-11, ura3-1, trp1-1, LYS2, leu2-3, can 1-100</i> <i>san1Δ::NatMX</i>	W303a
RGY1109	<i>PGAL2-ADE2, met2::PGAL7-lacZ, MET15, his3Δ200, ura3-52, trp1-901, LYS2::PGAL1-HIS3, leu2-3,112</i> <i>gal4Δ</i> <i>gal80Δ</i>	
RGY2175	<i>ADE2, MET2, MET15, his3-11,15, ura3-52, trp1-1, lys2, leu2-3,112</i> <i>Ssa1-45</i> <i>Ssa2-1::LEU2</i> <i>Ssa3-1::TRP1</i> <i>Ssa4-2::LYS2</i>	
RGY2176	<i>ADE2, MET2, MET15, his3-11,15, ura3-52, trp1-1, lys2, leu2-3,112</i> <i>Ssa2-1::LEU2</i> <i>Ssa3-1::TRP1</i> <i>Ssa4-2::LYS2</i>	

RGY2408	ADE2, MET2, MET15, his3-11,15, ura3-52, trp1-1, lys2, leu2-3,112 Ssa2-1::LEU2 Ssa3-1::TRP1 Ssa4-2::LYS2 san1Δ::KanMX	
RGY5867	ade2-1, MET2, MET15, his3-11, ura3-1, trp1-1, LYS2, leu2-3 Ptdh3-GFP ^{NLS} -HA- Δ2GFP	RGY 2970
RGY5868	ade2-1, MET2, MET15, his3-11, ura3-1, trp1-1, LYS2, leu2-3 Ptdh3-GFP ^{NLS} HA- ΔssPrA	RGY 2970
RGY5870	ade2-1, MET2, MET15, his3-11, ura3-1, trp1-1, LYS2, leu2-3 Pgal-GFP ^{NLS} - Peptide6	RGY 2970
RGY5872	ade2-1, MET2, MET15, his3-11, ura3-1, trp1-1, LYS2, leu2-3 Pgal-GFP ^{NLS} -Tef2 (190-458)	RGY 2970
RGY5974	ade2-1, MET2, MET15, his3-11, ura3-1, trp1-1, LYS2, leu2-3 Pgal-GFP ^{NLS} -Bgl2 (20-313)	RGY 2970
RGY5876	ade2-1, MET2, MET15, his3-11, ura3-1, trp1-1, LYS2, leu2-3 Pgal-GFP ^{NLS} -VHL	RGY 2970
RGY5877	ade2-1, MET2, MET15, his3-11,	RGY 2970

	<i>ura3-1, trp1-1, LYS2, leu2-3</i> <i>Padh1-GAD-CDC68-1</i>	
RGY5879	<i>ade2-1, MET2, MET15, his3-11, ura3-1, trp1-1, LYS2, leu2-3, Padh1-GAD-CDC13-1</i>	RGY 2970
RGY5881	<i>ade2-1, MET2, MET15, his3-11, ura3-1, trp1-1, LYS2, leu2-3, san1Δ::NatMX Ptdh3-GFP^{NLS}HA-Δ2GFP</i>	RGY5113
RGY5882	<i>ade2-1, MET2, MET15, his3-11, ura3-1, trp1-1, LYS2, leu2-3, san1Δ::NatMX Ptdh3-GFP^{NLS}HA-ΔssPrA</i>	RGY5113
RGY5884	<i>ade2-1, MET2, MET15, his3-11, ura3-1, trp1-1, LYS2, leu2-3, san1Δ::NatMX Pgal-GFP^{NLS} – Peptide 6</i>	RGY5113
RGY5886	<i>ade2-1, MET2, MET15, his3-11, ura3-1, trp1-1, LYS2, leu2-3, san1Δ::NatMX Pgal-GFP^{NLS} -Tef2 (190-458)</i>	RGY5113
RGY5888	<i>ade2-1, MET2, MET15, his3-11, ura3-1, trp1-1, LYS2, leu2-3, san1Δ::NatMX Pgal-GFP^{NLS} -Bgl2 (20-313)</i>	RGY5113

RGY5890	<i>ade2-1, MET2, MET15, his3-11, ura3-1, trp1-1, LYS2, leu2-3, san1Δ::NatMX Pgal-GFP^{NLS}-VHL</i>	RGY5113
RGY5891	<i>ade2-1, MET2, MET15, his3-11, ura3-1, trp1-1, LYS2, leu2-3, san1Δ::NatMX Padh1-GAD-CDC68-1</i>	RGY5113
RGY5893	<i>ade2-1, MET2, MET15, his3-11, ura3-1, trp1-1, LYS2, leu2-3, san1Δ::NatMX Padh1-GAD-CDC13-1</i>	RGY5113
RGY5895	<i>ade2-1, MET2, MET15, his3-11, ura3-1, trp1-1, LYS2, leu2-3, can 1-100 ssa1Δ::KanMX ssa2Δ::KanMX Ptdh3-GFP^{NLS}HA-Δ2GFP</i>	RGY 2971
RGY5896	<i>ade2-1, MET2, MET15, his3-11, ura3-1, trp1-1, LYS2, leu2-3, can 1-100 ssa1Δ::KanMX ssa2Δ::KanMX Ptdh3-GFP^{NLS}HA-ΔssPrA</i>	RGY 2971
RGY5898	<i>ade2-1, MET2, MET15, his3-11, ura3-1, trp1-1, LYS2, leu2-3, can 1-100 ssa1Δ::KanMX ssa2Δ::KanMX</i>	RGY 2971

	<i>Pgal-GFP^{NLS} – Peptide 6</i>	
RGY5900	<i>ade2-1, MET2, MET15, his3-11, ura3-1, trp1-1, LYS2, leu2-3, can 1-100 ssa1Δ::KanMX ssa2Δ::KanMX Pgal-GFP^{NLS} -Tef2 (190-458)</i>	RGY 2971
RGY5902	<i>ade2-1, MET2, MET15, his3-11, ura3-1, trp1-1, LYS2, leu2-3, can 1-100 ssa1Δ::KanMX ssa2Δ::KanMX Pgal-GFP^{NLS} -Bgl2 (20-313)</i>	RGY 2971
RGY5904	<i>ade2-1, MET2, MET15, his3-11, ura3-1, trp1-1, LYS2, leu2-3, can 1-100 ssa1Δ::KanMX ssa2Δ::KanMX Pgal-GFP^{NLS} -VHL</i>	RGY 2971
RGY5905	<i>ade2-1, MET2, MET15, his3-11, ura3-1, trp1-1, LYS2, leu2-3, can 1-100 ssa1Δ::KanMX ssa2Δ::KanMX Padh1-GAD-CDC68-1</i>	RGY 2971
RGY5907	<i>ade2-1, MET2, MET15, his3-11, ura3-1, trp1-1, LYS2, leu2-3, can 1-100 ssa1Δ::KanMX ssa2Δ::KanMX Padh1-GAD-</i>	RGY 2971

	<i>CDC13-1</i>	
RGY5972	<i>ADE2, MET2, MET15, his3-11,15,ura3-52, trp1-1, lys2, leu2-3,112</i> <i>ssa2-1::LEU2</i> <i>ssa3-1::TRP1</i> <i>ssa4-2::LYS2</i> <i>Padh1-GAD-CDC68-1</i>	RGY2176
RGY5973	<i>ADE2, MET2, MET15, his3-11,15,ura3-52, trp1-1, lys2, leu2-3,112</i> <i>ssa1-45</i> <i>ssa2-1::LEU2</i> <i>ssa3-1::TRP1</i> <i>ssa4-2::LYS2</i> <i>Padh1-GAD-CDC68-1</i>	RGY2175
RGY5974	<i>ADE2, MET2, MET15, his3-11,15,ura3-52, trp1-1, lys2, leu2-3,112</i> <i>ssa2-1::LEU2</i> <i>ssa3-1::TRP1</i> <i>ssa4-2::LYS2</i> <i>san1Δ::KanMX</i> <i>Padh1-GAD-CDC68-1</i>	RGY2408
RGY1110	<i>Pgal2-ADE2, met2::Pgal7-lacZ, MET15, his3Δ200, ura3-52, trp1-901, LYS2::Pgal1-HIS3, leu2-3,112</i> <i>GBD-SAN1-(C279S)-3HSV</i>	RGY1109
RGY1315	<i>Pgal2-ADE2, met2::Pgal7-lacZ, MET15, his3Δ200, ura3-52, trp1-901,</i>	RGY1110

	<i>LYS2::Pgal1-HIS3, leu2-3,112 GAD-no insert GBD-SAN1- (C279S)-3HSV</i>	
RGY1317	<i>Pgal2-ADE2, met2::Pgal7-lacZ, MET15, his3Δ200, ura3-52, trp1-901, LYS2::Pgal1-HIS3, leu2-3,112 GAD-Ura4 GBD-SAN1- (C279S)-3HSV</i>	RGY1110
No RGY Number assigned	<i>Pgal2-ADE2, met2::Pgal7-lacZ, MET15, his3Δ200, ura3-52, trp1-901, LYS2::Pgal1-HIS3, leu2-3,112 GAD-SSA1 GBD-SAN1- (C279S)-3HSV</i>	RGY1110

Plasmids

Plasmid Name	Encoded Protein	Vector
pRG4143	GFP ^{NLS} -Δ2GFP-HA	pRG4137
pRG4142	GFP ^{NLS} -ΔssPrA-HA	pRG4137
pRG1291	GFP ^{NLS} - Peptide6	pRS425
pRG1290	GFP ^{NLS} -Bgl2 (20-313)	pRS425
pRG1289	GFP ^{NLS} -Tef2 (190-458)	pRS425
pRG3027	GFP ^{NLS} -VHL	pRS426
pRG2054	GAL4AD-CDC68-1	pRS425
pRG2056	GAL4AD-CDC13-1	pRS425
pRG3630	GAL4AD-Cdc68-1	pRS426
pRG1056	GAL4AD-Ura4	pRS425
pRG117	GAL4AD	pRS425

pRG935	GAL4BD-San1 (C279S)	pRG119
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APPENDIX II: MATERIALS AND METHODS

Yeast strains

Yeast strains and plasmids are listed in **Appendix I**. This study employed standard yeast genetic methods (Guthrie and Fink, 1991). Plasmids were constructed using standard cloning protocols. Oligonucleotide sequences and plasmid construction details can be provided upon request.

Cycloheximide-chase degradation assays

Cycloheximide-chase degradation assays were performed similarly to previously described (Fredrickson et al., 2011). Cells were grown in liquid synthetic media with 3% raffinose or glucose to $\sim 1 \times 10^7$ cells/ml. Galactose was added to 3% and the cells were incubated 2 hours thereafter where applicable. Cycloheximide was added to 50 μ g/ml and the cells further incubated for 0-3 hours. Cells were lysed at the appropriate time point in 200 μ l SUMEB buffer (8M Urea, 1% SDS, 10mM MOPS, pH6.8, 10mM EDTA, 1mM PMSF, 0.01% bromophenol blue) by vortexing with 100 μ L of 0.5mm acid-washed glass beads (Biospec Products). Proteins were resolved on 8% or 16% SDS-PAGE gels, transferred to nitrocellulose, and immunoblotted with anti-GFP antibodies (Sigma) or anti-Gal4AD (GAD) antibodies (Millipore).

Temperature sensitive Cycloheximide-chase degradation assays

Cycloheximide-chase degradation assays were performed similarly to previously described (Fredrickson et al., 2011). Cells were grown in liquid synthetic Glucose media with to $\sim 1 \times 10^7$ cells/ml at 25°C. Temperature was shifted to 30°C or 37°C depending on the experiment for 1 hour. Cycloheximide was added at 50 μ g/ml and the cells further incubated for 0-3 hours. Cells were lysed at the appropriate time point in

200µl SUMEB buffer (8M Urea, 1% SDS, 10mM MOPS, pH6.8, 10mM EDTA, 1mM PMSF, 0.01% bromophenol blue) by vortexing with 100µL of 0.5mm acid-washed glass beads (Biospec Products). Proteins were resolved on 8% or 16% SDS-PAGE gels, transferred to nitrocellulose, and immunoblotted with anti-GFP antibodies (Sigma) or anti-Gal4AD (GAD) antibodies (Millipore).

Drug cycloheximide chase degradation assays

Cycloheximide-chase degradation assays were performed similarly to previously described (Fredrickson et al., 2011). Cells were grown in liquid synthetic media with 3% raffinose or glucose to $\sim 1 \times 10^7$ cells/ml. Galactose was added to 3% and the cells were incubated 3 hours. Cells were then treated with 20mM pifithrin- μ (Sigma-Aldrich) at 1:1000 for 1 hour. Cycloheximide was added to 50µg/ml and the cells further incubated for 0-3 hours. Cells were lysed at the appropriate time point in 200µl SUMEB buffer (8M Urea, 1% SDS, 10mM MOPS, pH6.8, 10mM EDTA, 1mM PMSF, 0.01% bromophenol blue) by vortexing with 100µL of 0.5mm acid-washed glass beads (Biospec Products). Proteins were resolved on 8% or 16% SDS-PAGE gels, transferred to nitrocellulose, and immunoblotted with anti-GFP antibodies (Sigma) or anti-Gal4AD (GAD) antibodies (Millipore).

Sedimentation assays

Sedimentation assays were adapted from a previously described protocol (Theodoraki et al., 2012). Cells were grown in liquid synthetic media with 3% raffinose to $\sim 1 \times 10^7$ cells/ml. Galactose was added to 3% and the cells were incubated 2 hours thereafter. 5ml of cells were harvested and lysed in Lysis buffer (100 mM Tris pH7.5, 200mM NaCl, 1mM EDTA, 1mM DTT, 5% glycerol, and 0.1% Nonidet P40) + PMSF by

vortexing 5 minutes at 4°C with 100µl of 0.5mm acid-washed glass beads. To remove unlysed cells, lysates were centrifuged at 700x g for 1 minute at 4°C. 50µl lysate, representing the “total lysate”, was removed and added to 50µl SUMEB. 100µL remaining lysate was centrifuged at 12,800x g for 15 minutes at 4°C. 100µl supernatant, representing the “soluble fraction”, was added to 100µl SUMEB. The pellet, representing the “insoluble fraction”, was resuspended in 100µl Lysis buffer and 100µl SUMEB. All samples were incubated at 65°C for 10 minutes and then clarified for 5 minutes by centrifugation at 12,800x g. Proteins were resolved on 8 or 16% SDS-PAGE gels, transferred to nitrocellulose, and immunoblotted with anti-GFP (Sigma) or anti-Gal4AD (GAD) antibodies (Millipore).

Microscopy

Cells were grown in 3% raffinose media to $\sim 0.5 \times 10^7$ cells/ml. Galactose was added to 3% and the cells incubated 6 hours. Cells were harvested, fixed in 4% paraformaldehyde in 0.1 M sucrose for 15 minutes, washed in wash buffer (1.2 M sorbitol, 0.4 M KPO_4), stained with DAPI for 10 minutes in wash buffer plus 2% Triton X-100, and washed two times in wash buffer. Cells were imaged on a Nikon Eclipse 90i with a 100X objective, filters for GFP (HC HiSN Zero Shift filter set with excitation wavelength (450–490 nm), dichroic mirror (495 nm), and emission filter (500–550 nm)) or DAPI (HC HiSN Zero Shift filter set with excitation wavelength (325-375 nm), dichroic mirror (400 nm), and emission filter (435-485 nm)), and a Photometrics Cool Snap HQ2 cooled CCD camera with NIS-Elements acquisition software.

Yeast Two-Hybrid Assays

All interaction tests were performed in duplicate by using two independent isolates. Growth of cells expressing a GAD fusion and a GBD-San1 deletion were used to test for activation of a reporter gene that required express of both the GAD and GBD fusions and their subsequent interaction *in vivo*. Plates were spotted for efficiency and spotted in 10 fold dilutions on selective reporter media. Plates were imaged and processed in photoshop cs6.

Image processing

All western blots were scanned using an Epson Perfection V350 Photo scanner at 300 dpi. All images were processed with a Mac iMac or Pro computer (Apple) using Photoshop CS6 (Adobe).

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Bachelors of Science; Biology; Chemistry Minor

RESEARCH EXPERIENCE

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- Investigation of protein quality control systems in the nucleus in *Saccharomyces cerevisiae*. Advisor: Dr. Richard G. Gardner
- Characterization of HSP70 isoforms; Ssa1 and Ssa2 and their regulatory role in San1 mediated degradation

Laboratory rotations: Various projects

- Studied the interaction between transcription factors TAF1 and TAF7. Spring 2013 Advisor: Edith Wang
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Environmental Health and Safety; Washington State University, 2009-2012

- Lead testing and monitoring
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Undergraduate Research: Washington State University, Department of Biochemistry, Pullman, WA, 2010-2012

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PUBLICATIONS

Jones, Ramon D. and Gardner, Richard G. Structure, July 7th 2015, Volume 23 , Issue 7 , 1151 – 1152

Jones, Ramon D. and Gardner, Richard G. Current Opinions in Cell Biology; COICB, "Nuclear Protein Quality Control Review", 40: 81-89.

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