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Functional Analysis of Cytosolic Hsp70 Nucleotide Exchange Factor Networks in Yeast

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FUNCTIONAL ANALYSIS OF CYTOSOLIC HSP70 NUCLEOTIDE EXCHANGE FACTOR NETWORKS IN YEAST

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A

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Jennifer Lynn Abrams, B.S.

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DEDICATION

I would like to dedicate this thesis to my late Aunt Sally A. Malter. She was always supportive of me advancing my education and encouraged me to keep pushing forward when life was difficult. She will be greatly missed.

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FUNCTIONAL ANALYSIS OF CYTOSOLIC HSP70 NUCLEOTIDE EXCHANGE FACTOR NETWORKS IN YEAST

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The Hsp70 class of molecular chaperones play critical roles in protein homeostasis via an ATP-dependent folding cycle. Cytosolic Hsp70s in the budding yeast *Saccharomyces cerevisiae,* Ssa and Ssb, interact with up to three distinct nucleotide exchange factors (NEFs) homologous to human counterparts; Sse1/Sse2/HSP110, Fes1/HspBP1, and Snl1/Bag1. In an effort to understand the differential functional contributions of the cytosolic NEFs to protein homeostasis ("proteostasis"), I carried out comparative genetic, biochemical and cell biological analyses. For these studies, I developed protocols to monitor protein disaggregation and reactivation in a near real-time coupled assay that revealed the importance of aggregate dynamics in the solubilization of proteins for their refolding. This coupled experimental approach, represents an important step toward developing tools necessary to monitor in vivo mechanisms of proteostasis.

This work determined that the Hsp110, Sse1, is the primary NEF contributing to most Hsp70 functions and also uncovered a unique role for Fes1 in Ssa-mediated regulation of the cytosolic heat shock response, while revealing no significant contributions from Snl1 and Sse2. These findings suggest that NEFs do have overlapping functions, but their distinct associations with the Hsp70s as well as unique structural components could contribute to differential roles in proteostasis.

Additionally, this study uncovered that relative levels of Snl1 and Sse1 are important for optimal growth. To probe this relationship, I exploited the Snl1 overexpression toxicity phenotype exhibited in the absence of Sse1 to examine which unique characteristics of Snl1 are important for its function. I discovered that Snl1 localization to the ER membrane is required for toxicity and that Sse1-mediated alleviation of this phenotype is Ssb-dependent. These results demonstrate a network of interactions that supports a hypothesis where Snl1 plays a role in translation regulation.

This investigation was conducted to gain a better understanding of NEF roles within the Hsp70 chaperone network. Understanding these dynamics is critical to obtain successful treatments that can reverse the debilitating effects of neurodegenerative disorders such as Alzheimer's and Parkinson's diseases. Pharmacological targeting of molecular chaperones and their co-factors, such as the NEFs, is an attractive therapeutic goal that may contribute to improving human health, most notably in the aging population.

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Chapter 1: Introduction

INTRODUCTION AND BACKGROUND

Stress and the maintenance of proteostasis - Cellular viability is at the mercy of the protein homeostasis ("proteostasis") machinery, which includes the protein complexes responsible for maintaining the balance between polypeptide synthesis, transport, modification, and ultimately degradation. Aggregation and loss of function resulting from improper inter- and intramolecular interactions of misfolded and partially folded proteins can lead to a deleterious loss of proteostasis [1]. Challenges in the external environment as well as internal stressors can negatively impact the balance of protein maintenance. For example, temperature, pH, and osmolyte concentration are external factors that once altered beyond physiological range can increase the probability of misfolding and aggregation. In addition, internal factors such as proteome complexity and protein synthetic load can heighten the need for proteome stabilizers such as molecular chaperones. In support of this idea, there is a positive correlation between the complexity of the proteome and the number of conserved chaperone families maintained in an organism [2].

Nascent chain folding is particularly sensitive to stress conditions. Co-translational protein folding is important for longer, more complex, proteins because larger regions of the unfolded polypeptide have to be released from the ribosome in order to reach their native state. The ribosome exit tunnel is 100 Å long x 20 Å in diameter, which is only enough space for smaller intramolecular interactions to take place, limiting folding to simple secondary structures such as α-helices [3-7]. Upon release from the ribosome, the highly crowded, aqueous environment of the cytosol poses a folding challenge to the polypeptide. Exposed hydrophobic stretches will attempt to bury themselves adjacent to other hydrophobic residues, which can lead to improper structural associations [8]. In eukaryotes this is a significant problem because in addition to exposed hydrophobic regions that need to be protected, 30% of the proteins are classified as intrinsically unstructured [9]. It is plausible that high levels of unstructured proteins would be evolutionarily disfavored, but protein function requires a balance of flexibility and thermodynamic potential energy [10,11]. Due to this delicate balance, proteins that fold without

assistance can fall into what is termed a kinetic trap, which is an alternative non-functional fold that has a lower potential energy then the next intermediate fold on the journey to the native active state.

The rate of translation is another factor that can affect nascent protein folding efficiency. The average speed of eukaryotic translation is four amino acids per second compared to 20 amino acids per second in prokaryotes [12]. The mistranslation rate is 1 out of every 10^{-4} residues in eukaryotes, so the rate of translation allows time to correct or eliminate misfolded proteins [13-17]. One way that the cell deals with terminally misfolded proteins is through the ubiquitin proteasome pathway in which 5-15% of nascent chains are ubiquitinated. This number increases upon loss of the chaperone complexes in contact with ribosomes and nascent chains and these complexes include TRiC/CCT and prefoldin, the yeast Hsp70, Ssb, with the ribosome-associated complex (RAC) and the nascent chain associated complex (NAC) and will be discussed in more detail later [18]. All of the above mentioned chaperones are coregulated with the ribosome. As a result their expression is suppressed during stress conditions [19]. These complexes maintain proteostasis at the ribosome. In addition, proteins that have already been folded can be damaged during stress conditions, such as heat shock, and this requires repair mechanisms that involve chaperones, but also usually require global transcriptional responses.

The heat shock response - When the cell senses stress, it activates protective measures in order to prepare for future threats. In yeast, multiple transcription programs including the environmental stress response (ESR), the heat shock response (HSR), the oxidative stress response (OSR), and the unfolded protein response (UPR) allow cells to quickly adapt to changing environments. Stress responses can cross-protect against different stressors, but they each primarily respond to specific stressors. The most general stress response is the ESR and genes upregulated in the HSR are a subset of the ESR-regulated genes [20]. The HSR mounts a global cellular response upon exposure to temperatures greater than 36-37°C

characterized by cell cycle arrest and upregulation of heat shock proteins (HSPs) and other housekeeping proteins.

The master regulator of the HSR is the heat shock transcription factor, Hsf. In mammals there are four different isoforms of this master regulator, HSF1-4, but HSF1 controls the HSR [21]. In yeast there is only one isoform, Hsf1, which is essential for growth at optimal temperatures [22]. Hsf1 consists of three domains: the winged helix-turn-helix DNA binding domain (DBD), which is important for activating gene targets upon initiation of the HSR [23, 24]; the leucine zipper oligomerization domain is important for trimerization, which is necessary prior to binding DNA [25, 26]; and a carboxyl-terminal transactivation domain (CTD), which is important for initial and prolonged transcription activation by the transcription factor [27, 28]. Yeast Hsf1 has an additional amino terminal transactivation domain (NTA), which can initiate transcriptional activity independently or act as a negative regulator modulating CTD initiated activation [28, 29].

In mammals, activation of Hsf1 requires several steps including nuclear localization, trimerization, post-translational modifications, and DNA binding. In yeast, regulation is much simpler in that the Hsf1 is constitutively trimerized, localized to the nucleus, and bound to promoters of many HS genes [30-32]. This is more similar to *Drosophila* than mammalian HSF1 [33]. Activation also results in Hsf1 hyperphosphorylation, which is mediated by multiple kinases that respond to specific stressors. In yeast, it was shown that Snf1 phosphorylates Hsf1 in response to glucose starvation [32], but the kinase responsible for heat shock associated phosphorylation is unknown. In addition to the above-mentioned regulatory mechanisms, Hsf1 is also bound and repressed by the molecular chaperones Hsp70 and Hsp90. This has been shown via genetic evidence in yeast and mammals, and biochemical evidence in humans [34]. Hsp70 cannot completely repress Hsf1 alone; Hsp90 and co-chaperones are also necessary for complete repression [35]. Exposure to heat shock is thought to titrate the HSPs away from Hsf1 allowing activation. Once the HSPs have restored

proteostasis, they reestablish interaction with Hsf1 to attenuate the response. In yeast, there is currently genetic evidence, but no biochemical evidence to support this hypothesis.

Molecular chaperones - In order to maintain proteostasis, cells employ the help of molecular chaperones. All organisms maintain multiple families of chaperones, but not every family. Chaperones are named based on their molecular weights, and many of them are referred to as heat shock proteins (HSPs) due to their induction during heat shock. One of the most highly conserved families is the 70 kilodalton (kd) heat shock protein or Hsp70, which is present in all eukaryotes and bacteria currently identified, but not in all Archaea [36]. There is a roughly linear relationship between the number of genes maintained by an organism and the number of canonical HSPs, with exception of the Hsp100s. There is approximately 1 Hsp70 gene, 5-6 Hsp40 genes, 1 sHsp gene, and 1 Hsp60 gene for every 2000 genes and 1 Hsp90 gene per 6000 genes [2]. It is unclear why this relationship exists since many of these chaperones do not have a high level of specificity, but it appears that throughout evolution mutations have resulted in slight adaptations improving survivability of the organisms that code for them in the above mentioned ratios. These minor differences result in high levels of redundancy with the development of some distinct functions. The variation provides more regulatory opportunities to respond to changing environments. A number of molecular chaperones are classified as HSPs because many of them were originally identified in pulse chase experiments, where protein abundance was monitored by incorporation of [^{35}S]-methionine after heat treatments followed by SDS-PAGE [37]. Although many chaperones including some of the Hsp70s and Hsp90s are expressed constitutively, they are also upregulated upon HS and additional stress responsive isoforms are expressed in order to prepare for any additional assaults or to ultimately attenuate the active response. A subset of these chaperones are referred to as housekeeping proteins or “generalists.” The generalists are involved in ensuring a protein successfully transitions from “birth” as nascent nonfunctional polypepetide to a localized, modified, active protein, and then upon reaching the limit of its lifetime, chaperones target it to

one of the degradation pathways for “death”. A well-conserved example of the generalist family is the Hsp70s that bind promiscuously to exposed hydrophobic regions in partially unfolded proteins (Fig. 1.1). There is also a more specific subset of chaperones known as “specialists,” which include the Hsp90s that interact with specific substrates including kinases and hormone receptors, to promote their maturation, translocation, or degradation.

The Hsp70s - As mentioned earlier, the Hsp70s are highly conserved and play an essential role in maintaining protein homeostasis [1, 38]. Hsp70 functions through a nucleotide dependent cycle to protect exposed hydrophobic regions of polypeptides from the aqueous environment of the cytosol, while they fold into their native structure as seen in Figure 1.1 [39]. Hsp70 switches from a low affinity to a high affinity substrate binding conformation using the energy from binding, hydrolysis and release of ATP (Fig. 1.2) [40, 41]. This cycle repeats as necessary to complete protein folding [42]. Hsp70 has a very low innate ATPase activity, 0.04 ATP hydrolyzed min^{-1}, resulting in a low protein folding capacity. To improve folding efficiency, Hsp70 interacts with a myriad of co-chaperones [43]. The conserved Hsp40s or J-domain proteins interact with Hsp70 and can both activate its ATPase activity at least 200-fold as well as deliver substrate to Hsp70 in some cases [44-46]. The folding cycle is further regulated by the nucleotide exchange factors (NEFs) that bind the Hsp70 nucleotide binding domain (NBD) promoting the release of ADP and in turn substrate [47-51].

The architecture of Hsp70 includes a 44 kd nucleotide binding domain (NBD) and a 25 kd carboxyl-terminal substrate binding domain (SBD) [52]. The SBD is further composed of an α-helical lid domain and β-sandwich domain [53]. Substrates interact with the β sandwich domain in the high affinity, ADP bound state, and the α-helical lid closes over the substrate providing optimal protection [54]. The domains communicate nucleotide status through allosteric coupling of the NBD and SBD via an interdomain linker [40, 41, 53, 55]. Structure and co-chaperone interactions have afforded Hsp70 the functional flexibility to be involved in

Figure 1.1. Model of Hsp70-mediated folding. Hsp70 promotes folding into a native active structure and prevents misfolding and aggregation.

Figure 1.1. Model of Hsp70-mediated folding

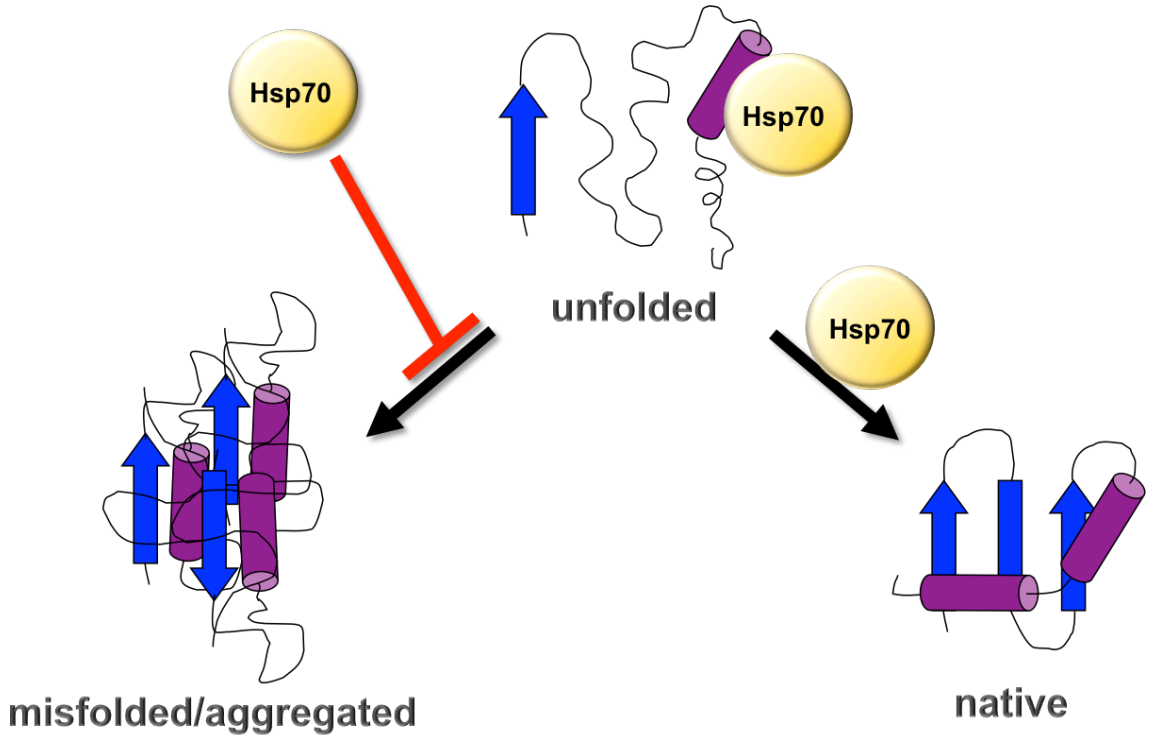
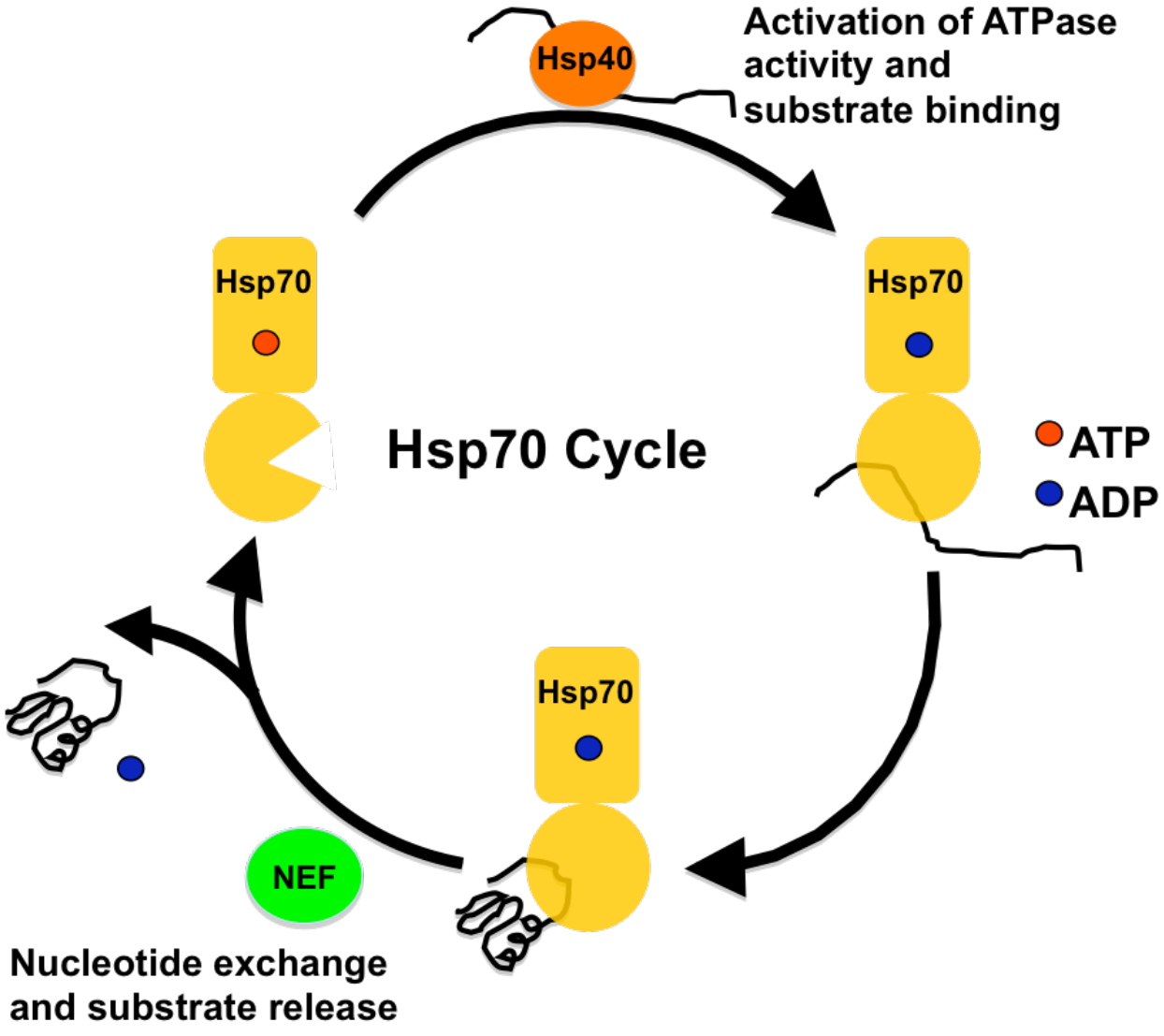


Figure 1.2. Hsp70 Cycle. This schematic depicts the nucleotide dependent mechanism by which Hsp70 binds and releases substrate. In the ATP bound conformation, Hsp70 transiently interacts with polypeptide with low affinity. Interaction with the Hsp40 family of co-chaperones activates Hsp70's innate ATPase activity hydrolyzing ATP to ADP and switching Hsp70 to a high affinity substrate binding conformation. Then the interaction between Hsp70 and the nucleotide exchange factors (NEFs) alters the structure to release ADP and the substrate.

Figure 1.2. Hsp70 Cycle



protein folding/biogenesis, maturation, transport, and degradation [1, 38].

The Hsp70 superfamily includes Ssa, Ssb, Sse, and the atypical Ssz (Stress Seventy A, B, E, and Z) (Table 1.3) [22]. In yeast there are two families of cytosolic Hsp70s, Ssa and Ssb. There are two proteins, Ssb1 and Ssb2, in this non-essential, ribosome-associated family of Hsp70s; they are 99% identical and believed to be functionally redundant [56]. Ssb is a fungal specific Hsp70, not present in metazoans; in mammalian cells, cytosolic Hsp70s are recruited to the ribosome for co-translational protein folding [57, 58]. Ssb1/2 are considered to be functionally distinct from the Ssa family proteins due to the inability of either family to completely complement the absence of the other [22, 59]. In contrast to the stress inducible Ssa family of Hsp70s, Ssb is regulated similar to ribosomal proteins in that its transcription is decreased in stress conditions [60, 61]. Ssb primarily interacts with translating ribosomes in a ribosome-associated complex (RAC) dependent fashion, independent of nascent chain association; however, nascent chain interaction stabilizes the Ssb/ribosome complex [62, 63]. RAC exists as a ribosome-bound heterodimer, which includes the J-domain protein, Zuo1, and the atypical Hsp70, Ssz1, and acts as a co-chaperone for Ssb, activating its ATPase activity [64]. The specific function of Ssz1 is unclear, but it is required for Zuo1 activity. In addition, Zuo1 contains the interaction domain required for RAC association with the ribosomal exit tunnel [64, 65]. Deletion of *SSB, ZUO1,* or *SSZ1* results in identical phenotypes, slow growth, cold sensitivity, and hypersensitivity to aminoglycosides (translation inhibitors) suggesting they act together in one process. Deletion of SSB also results in accumulation of aggregated proteins as well as a decrease in small and large ribosomal subunits, indicating roles in both co-translational protein folding and ribosome biogenesis [19] [66]. In addition to RAC, the highly conserved nascent chain associated complex (NAC) also binds the ribosome at the exit tunnel, but unlike RAC it interacts directly with nascent chains [67]. In yeast, NAC is composed of three subunits, Egd1, Egd2, and Btt1, but the protein is a heterodimer of Egd1 with either Btt1 or Egd2, which allows for differential substrate specificity [68, 69]. NAC has also been

Table 1.1. Yeast Cytosolic Chaperones [22].

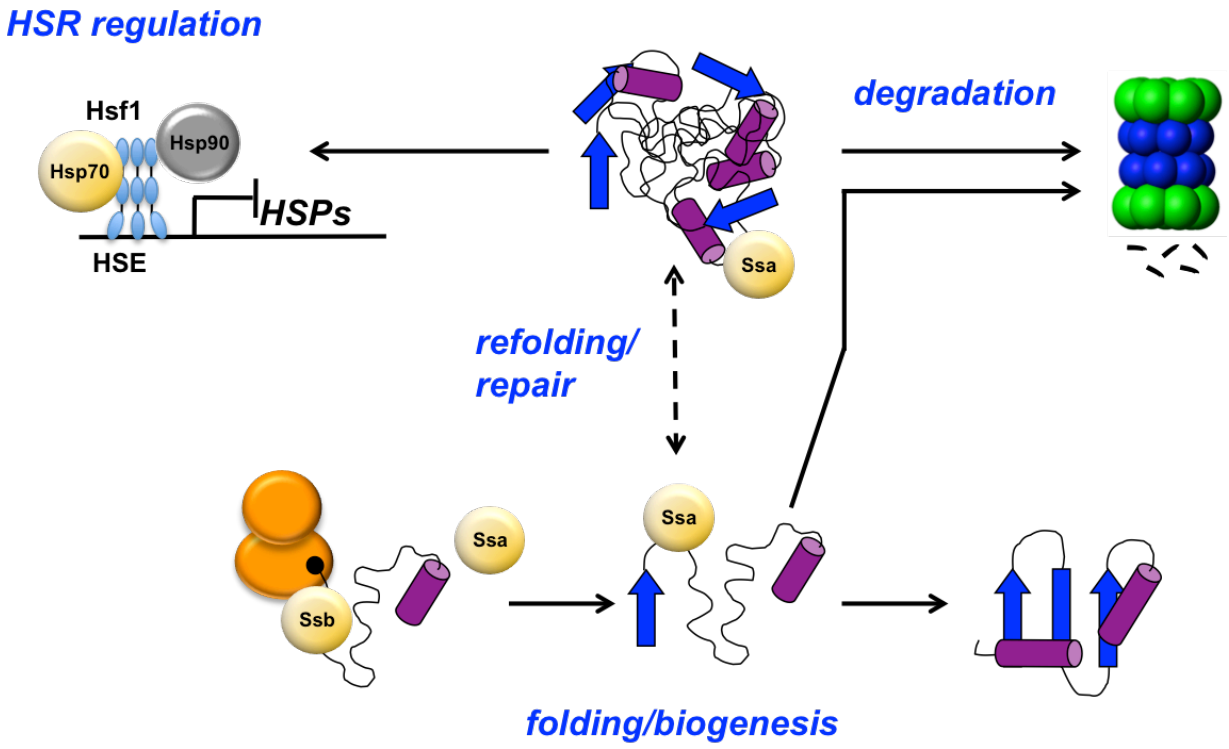
Class	Protein(s)	Function(s)
Hsp70		
Hsp70	Ssa1, Ssa2	Constitutively expressed protein foldase
	Ssa3, Ssa4	Stress inducible protein foldase
	Ssb1, Ssb2	Ribosome localized, constitutively expressed nascent protein foldase
	Ssz1	Atypical Hsp70, involved in ATPase activation of Ssb, part of the ribosome associated complex
Hsp70 co-chaperones		
Hsp70 NEF		
Hsp110	Sse1	Constitutively expressed Hsp70 nucleotide exchange, substrate binding.
	Sse2	Stressed inducible, Hsp70 nucleotide exchange, substrate binding.
HspBP1	Fes1	
Bag-domain	Snl1	ER localized, Hsp70 nucleotide exchange factor
Hsp70 J protein		
	Ydj1	Hsp70 ATPase activator, substrate binding
	Sis1	Hsp70 ATPase activator, substrate binding
	Zuo1	Ribosome localized, Hsp70 ATPase activator, part of the ribosome associated complex
	Jjj1	Hsp70 ATPase activator, substrate binding, ribosome biogenesis
	Swa2	Hsp70 ATPase activator, substrate binding, vesicular transport
Hsp100	Hsp104	Disaggregase, unfoldase
sHsps	Hsp42	Antiaggregase
	Hsp26	Antiaggregase
Chaperonin	TriC/Cct1-Cct8	Protein folding, cytoskeleton substrates

shown to assist in de novo protein folding and ribosome biogenesis and has overlapping substrate interactions with Ssb [66]. Together these proteins affect proteostasis through folding and stabilizing proteins being released from the ribosome during translation.

The Ssa family includes four homologs, two of which are constitutively expressed, Ssa1/2 and the other two of which are expressed only during stress conditions, Ssa3/4. The Ssa family is essential. Upon loss of Ssa1 and Ssa2, the cells exhibit a slow growth defect, heat sensitive phenotype, and derepression of Hsf1, which results in upregulation of the HSR [70, 71]. *SSA3/4* are upregulated in response to loss of Ssa1/2, likely due to derepression of the HSR, but despite high levels of similarity cannot fully complement the loss of Ssa1/2 [71]. Ssa1 and Ssa2 are 98% identical, but still maintain some unique functions, which has been tied to a single residue difference in the NBD [72]. It is thought that the promiscuity of the SBD and the specificity that can be imparted through protein interactions with the NBD allow Hsp70 to contribute to a multitude of distinct functions as seen in Figure 1.3. Elizabeth Craig's laboratory at University of Wisconsin performed work identifying phenotypes associated with the Ssa proteins using an *SSA1* and *SSA2* deletion strain or a strain lacking all four *Ssa* genes and expressing only a temperature sensitive allele (*ssa1-45*), which is inactivated after 30 min at 37°C [73]. Current findings using this construct have to be interpreted with some caution because the results could reflect phenotypes associated with loss of cell viability compared to the loss of the Ssa proteins alone. Many of the in vivo and in vitro folding studies have been performed using well-established model proteins, such as firefly luciferase (FFL) and ornithine transcarbamylase, which have been shown to require Ssa1/2 for both biogenesis and refolding after denaturation [74, 75]. Studies performed in my laboratory and by others have shown that both Ssa1 and its co-chaperones, Ydj1 and Sse1, were necessary for the processing of pre-pro-α factor to the mature form of the mating type specific protein, α factor [76, 77]. Ssa has also been shown to be involved in transport of mitochondrial, vacuolar, and nuclear precursors [78, 79]. Within the cytosol, Ssa is involved in maintaining solubility of immature proteins

Figure 1.3. Hsp70-mediated functions. In yeast the cytosolic Hsp70s are Ssa and Ssb. Both Ssa and Ssb are involved in protein biogenesis, but in some cases misfolding occurs and the Ssa can triage aberrant protein to the ubiquitin proteasome pathway or refold it. Depending on the level of misfolding in the cell, the heat shock response can be triggered, resulting in release of the Hsp70s and Hsp90s from Hsf1 bound to the heat shock element (HSE) in the promoter of heat shock genes allowing for expression of heat shock proteins (HSPs).

Figure 1.3. Hsp70-mediated functions



and delivering them to the endoplasmic reticulum (ER) for translocation and processing. Studies have also been performed relating Hsp70 to degradation of terminally misfolded proteins as well as proteins programmed with short half- lives. In vivo and in vitro studies using well-characterized client proteins such as cystic fibrosis transmembrane conductance regulator (CFTR), von Hippel-Lindau (VHL) protein variants, as well as α-synuclein and poly-Q expanded proteins, have shown that Hsp70 is necessary for efficient protein degradation [81-84]. In mammals the Hsp70 machine along with the NEF, Hsp110, and the J-domain proteins have been shown to act in disaggregation, a process important for clearance of misfolded proteins [85, 86]. In yeast, disaggregation is the primary function of the Hsp104 chaperone, a member of the Hsp100 family that is absent from metazoans, but Hsp70 interacts with Hsp104 to activate disaggregation and promote refolding of the liberated protein [87]. It is interesting that mammalian cells have evolved so Hsp70 and its co-chaperones can perform a function that yeast manages using an alternate family of chaperones. The diversity of co-chaperones establish them as important modulators of Hsp70 function and specificity.

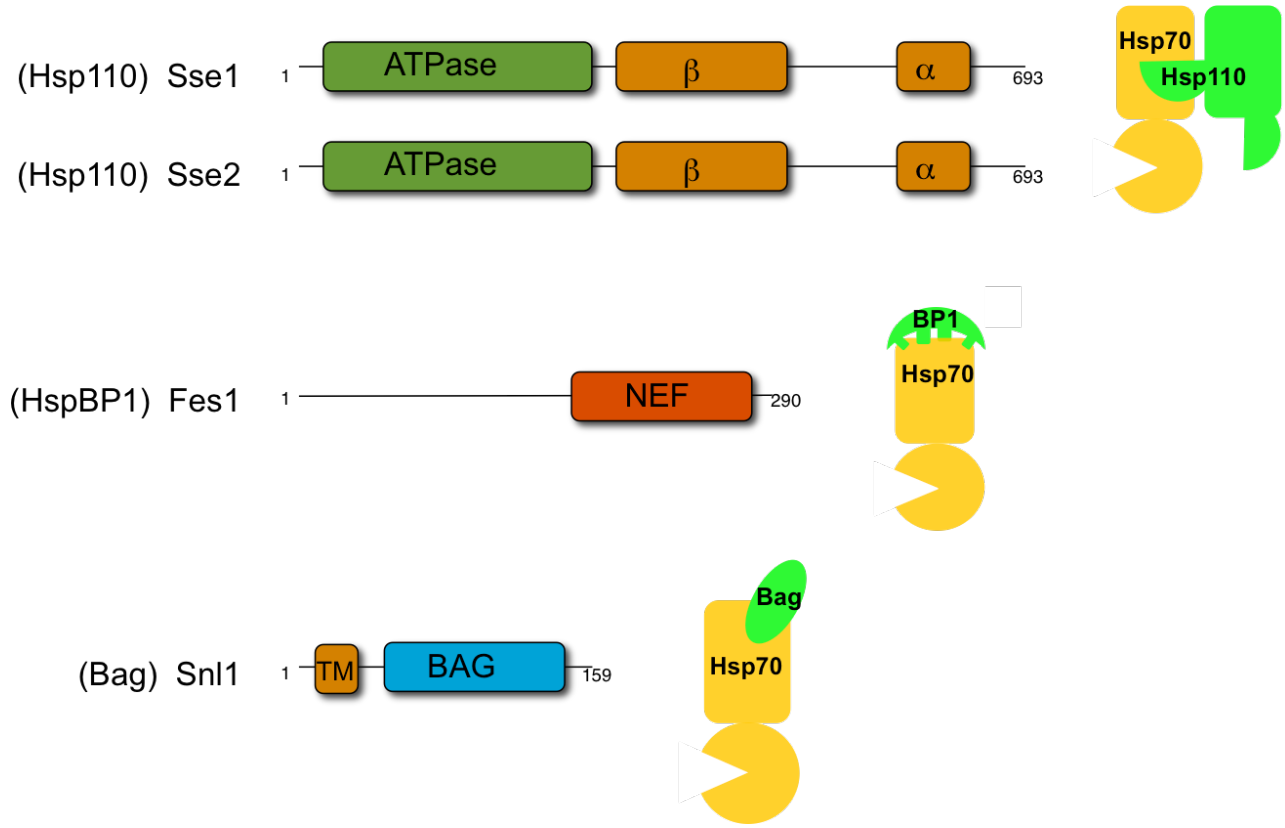
The Hsp40 co-chaperones - Aside from their role in modulating the folding cycle through activation of ATPase activity, the Hsp40 co-chaperones can also recruit Hsp70 for specific functions. The Hsp40s bind to the Hsp70 NBD to enhance the relatively low innate ATPase activity [38]. Yeast cells encode for at least 22 J-domain-containing proteins, or Hsp40s, which vary in substrate binding capabilities and process specificity. Ydj1 is a general Hsp40 and one of the most highly expressed genes in the family. This J-protein is one of the primary co-chaperones involved in Hsp70-mediated protein folding. Jjj1, on the other hand is a highly specialized Hsp40 protein involved in ribosome biogenesis [88]. There are also Hsp40s that have no substrate binding capabilities, such as Zuo1, which is a component of the RAC complex involved in nascent chain folding [38, 89]. In spite of the structural and functional diversity of the J-domain proteins, they all perform the same biochemical function, which is to

activate the ATPase activity of Hsp70. This provides a mechanism by which co-chaperones can delicately modulate a highly promiscuous and ubiquitous chaperone in order to contribute to a plethora of distinct processes.

Cytosolic Hsp70 nucleotide exchange factors - Unlike the Hsp40s, which all have a common J-domain, the NEFs are completely distinct in sequence and structure and are thought to have emerged via convergent evolution (Fig 1.4). The three families that exist in the cytosol of mammalian cells are identified as: Hsp110, HspBP1, and Bag domain-containing proteins [90]. In yeast, the Hsp110 family is represented by the two paralogs, Sse1 and Sse2, which are considered to be atypical members of the Hsp70 family because ATPase activity is limited and not required for their known functions [91]. Analogous to the Hsp70 family, Sse1/2 are composed of an NBD and an SBD. Within the SBD, these proteins also have α and β subdomains important for substrate binding. One striking difference between the Hsp110 and the Hsp70 families is an extended linker region between the two subdomains of the SBD [92]. The extended linker results in a structural change that causes the α domain to play a role in Hsp70 binding and likely plays less of a role in substrate interaction. Due to these structural differences Sse1/2 act independently as holdases, which are proteins that bind to substrate and maintain it in a folding competent conformation, as opposed to foldases, which are proteins that bind and release substrate in iterative cycles to promote folding [93-95]. Co-crystal structures have been solved for the Hsp110/Hsp70 heterodimer, which shows the interaction interface lying between the two NBDs, and the α subdomain of the Hsp110 wraps around the Hsp70 NBD [92, 96]. The structure also shows that the β-sandwich domain of Hsp110 remains exposed and available for substrate binding [77, 92, 96, 97]. HspBP1, or Fes1 in yeast interacts with the NBD of Hsp70 through its armadillo repeat domain, or NEF domain, which distorts the two lobes of the Hsp70 NBD resulting in nucleotide release (Fig. 1.4) [98, 99]. The Bag domain-containing family is made up of six homologs in humans and a single protein in yeast, Snl1, which is ER-membrane bound through its N-terminal transmembrane domain (Fig. 1.4).

Figure 1.4. Cytosolic Hsp70 nucleotide exchange factor (NEF) architectures and interaction interfaces. Schematic representation of the architecture of each of the cytosolic Hsp70 NEFs as well as cartoon figures showing the interaction interfaces between Hsp70 and the NEFs. NEF family classifications are shown in parentheses.

Figure 1.4. Cytosolic Hsp70 nucleotide exchange factor (NEF) architectures and interaction interfaces



The Bag family of proteins interacts with Hsp70 through a triple helical bundle that contacts the Hsp70 NBD, slightly tilting one lobe to release ADP [38, 95, 100]

All of the NEFs are present at different levels in the cytosol: Sse1 is the most abundant NEF, present at 70,000 molecules per cell (compared to approximately 270,000 molecules of the Hsp70, Ssa), which is approximately five times that of Fes1, the next most abundant NEF, and levels of Sse2 and Snl1 are 15-20 times lower than Sse1 [101]. However Sse2 is the only NEF upregulated more than 20 fold in response to a number of stress conditions [61, 102]. Loss of Sse1 results in a slow-growth phenotype exacerbated by temperature stress and deletion of both of the Hsp110s, Sse1 and Sse2, results in a lethal phenotype [102, 103]. *FES1* deletion cells exhibit mild slow growth and heat sensitivity [104]. No growth phenotypes associated with loss of Snl1 or Sse2 alone have been identified.

Similar to the cytosol, the ER also houses two distinct families of Hsp70 NEFs. Kar2, the ER-resident Hsp70, interacts with two proteins Lhs1 and Sil1, that both result in the release of ADP from the Hsp70 NBD. Lhs1 is a member of the Hsp110 family, but there are a few differences between Lhs1 and Sse1. Lhs1 has a more extended linker between the α and β subdomains of the SBD [105]. Furthermore, Kar2 activates the ATPase activity of Lhs1, which is considered to be important for coordinated substrate interaction between the two proteins [106]. This linked chaperone/co-chaperone activity is also observed between the human ER Hsp70 and NEF, BiP and Grp170 [107, 108]; this type of chaperone interaction is thought to be possible between Sse1/2 and the Ssa proteins, but the function of the Sse1 SBD in Hsp70-mediated folding is currently unknown. Other than these two differences, there are many similarities between the Sse1 and Lhs1 including the dual NEF and holdase activities of Lhs1 [77, 109, 110]. Sil1 is thought to be a member of the HspBP1 family, and in humans, a mutation in this protein is associated with the neurodegenerative disorder, Marinesco-Sjögren syndrome [111]. Although Sil1 acts as an equally potent NEF, it does not have completely redundant activities with Lhs1, mirroring the findings that will be later discussed in Chapter 4 between Sse1 and Fes1. Deletion of both *SIL1* and *LHS1* results in synthetic lethality, but loss

of either gene leads to protein folding defects in the ER [112, 113], suggesting these two proteins play unique roles in proteostasis. Lhs1 was also shown to be important in protein translocation into the ER, which is not a function requiring Sil1 [113]. In addition, Lhs1 contributes to Kar2-mediated folding and secretion of the heat denatured substrate, Hsp105Δ-β-lactamase, but was not involved in initial folding of the protein. These findings are similar to the work presented in this thesis, in that the NEFs contribute differentially to Hsp70-mediated functions, and in the finding that ER de novo folding and refolding of heat denatured proteins are distinct processes [114]. The ER-resident NEFs discussed above present a chaperone network very similar to the cytosol and show functional differentiation between two proteins, Lhs1 and Sil1, that perform the same biochemical function to release ADP from the Hsp70, Kar2. This theme will be reiterated throughout the work presented in this thesis.

Several studies have identified a number of individual NEF contributions to cytosolic Hsp70 function. Sse1, the most highly studied yeast cytosolic NEF, has been shown to be important for a number of Hsp70-mediated processes. Sse1 plays a significant role in the flux of nascent substrate through Ssa and Ssb proteins [97, 115]. In addition, Sse1 is necessary for biogenesis of the FFL protein in vivo or refolding in vitro [48]. Multiple experiments have also illustrated Sse1's contribution to Hsp70-dependent co-translational folding, Hsp90-dependent signaling and post-translational translocation of pre-pro-α factor [77, 91, 97, 116]. Both Sse1 and Fes1 have been implicated in prion propagation and curing. Sse1 is essential for [*PSI*$^{+}$] propagation, and cells lacking Sse1 or Fes1 are cured for [*URE3*] [117, 118]. Sse1 and Fes1 also contribute to Hsp70-mediated degradation via the ubiquitin proteasome pathway [119-123]. It was recently shown that Snl1 associates with ribosomes through basic residues located near the Hsp70 binding Bag domain, but the functional ramifications are yet to be determined [124]. Work performed in the last decade has greatly augmented our understanding of the individual contributions of the cytosolic NEFs, but now the functions of these proteins must be studied at the network level to determine their relative contributions in vivo.

In this study I have investigated differential contributions of the cytosolic Hsp70 NEFs to proteostasis, examined the functional significance of Sse1 and Snl1 synthetic growth phenotypes, and developed a coupled assay to address the relationship between protein aggregation and activation. I concluded that Sse1 is the primary NEF involved in most Hsp70 mediated processes. However, it appears that none of the individual NEFs nor the two most abundant NEFs, Sse1 and Fes1, is necessary for protein refolding and repair. The role of Sse1 as the major NEF involved in cellular viability is reflected in the growth assays, which show that an *SSE1* deletion strain exhibits a slow-growth phenotype with temperature sensitivity suggesting it is likely important in protein biogenesis as well as quality control. Loss of Fes1 only showed a mild growth defect and heat sensitivity, suggesting it might be primarily important in protein quality control mechanisms. In addition, cells lacking Fes1 resulted in high de-repression of the HSR. Induction of the HSR also results in an increase in the cellular chaperone load. It is possible that this phenotype could mask Fes1's contributions to Hsp70 function due to the availability of other chaperones. This study did not reveal any unique functions of the Snl1 or Sse2 proteins, but previous studies performed in my laboratory with Snl1, as well as findings in this study, suggest that Snl1 may be involved in regulating some aspect of translation. In addition, it is possible that Sse2 is conditionally needed because it is the only NEF highly induced in response to stress.

In several experiments I compared solubility of a protein to its enzymatic activity or its rate of degradation; these two phenomena are closely connected. In a coupled assay to monitor near real time fluorescence microscopy with automated enzymatic assays, I discovered that the dynamics of an aggregate is directly related to the ease at which the protein is liberated from the aggregate for refolding. In addition, the presence of a disaggregase system is essential for efficient restoration of proteostasis.

Finally, phenotypes identified in my earlier work, as well as the synthetic deletion phenotype identified in the NEF study, led me to examine the functional relationship between Sse1 and Snl1. This line of investigation uncovered that the levels of Snl1 become very important for

viability in the absence of Sse1, which suggests that Sse1 antagonizes the Snl1-mediated activity. Due to the cold sensitivity (known to be associated with translation defects) observed upon deletion of *SSE1* and *SNL1,* the ER localization of Snl1, and the induction of the HSR in the presence of high levels Snl1 in an *SSE1* null strain, I hypothesized that this unknown function of Snl1 is associated with translation regulation. Ultimately, all of these proteins have homology to human cytosolic proteins and therefore these studies can be applied to understand the division of labor among the NEFs in human cells, which can be used to develop treatments to modulate proteostasis.

SIGNIFICANCE OF THIS STUDY

Understanding cytosolic Hsp70 NEFs - Previous findings mentioned above were performed in different strain backgrounds using multiple substrates, which contribute to the understanding of how the NEFs function. However, the NEFs do not function alone in the cell and it is important to investigate how they work within the context of the chaperone network. I have performed comprehensive genetic, biochemical, and cell biological analyses in isogenic single and combinatorial cytosolic NEF deletion strains. This thesis work aims to address a more global question of why have the three families of NEFs been maintained throughout eukaryotic evolution upon converging to perform the same biochemical function, which is to release ADP from Hsp70. All three families have similar levels of NEF activity in vitro but are completely distinct structurally and in sequence. Understanding how these NEFs contribute relatively to the chaperone network is important for two reasons: first, understanding specific functions can lead to the discovery of molecules that modulate Hsp70 function, which is becoming important in treatment for both neurodegenerative diseases and cancer. In addition, there are disorders directly associated with NEF dysfunction such as Marinesco-Sjøgren syndrome, which is an autosomal recessive cerebellar ataxia caused by a mutation in the Fes1 homolog in the ER, Sil1 (BAP) [125]. Second, loss of Hsp110 is associated with tau pathology in a mouse model and huntingtin-related neurodegeneration in a *Drosophila* model [126, 127]. The NEFs provide both direct and indirect targets for the development of drugs to treat a number of diseases.

Human diseases related to proteostasis - Protein misfolding and aggregation in humans have been associated with the formation of highly structured amyloid deposits common to many neurodegenerative disorders including Alzheimer's, Parkinson's, and Huntington's diseases [128]. These neurodegenerative diseases have a common theme in which normally existing proteins with either known or unknown functions adopt an alternative fold. This can lead to the formation of disordered and ordered aggregates that can further develop into highly structured amyloid fibers. Understanding which of these proteins is most toxic and important in

neurodegeneration and what promotes its formation will require many more years of study. In Alzheimer's disease, both accumulation of amyloid beta (Aβ) and hyperphosphorylated tau protein are associated with neuroinflammation and progression of the disease [129]. α-synuclein, the major component of Lewy Bodies, is a neuronal protein that misfolds leading to the formation of oligomers and ultimately fibrils, a mature ordered form of aggregate associated with Parkinson's disease [130-132]. In Huntington's disease and other poly-Q pathologies, disease onset and severity are highly dependent on the number of glutamine residues found in the protein, which have a direct relationship with the tendency toward forming ordered aggregates [133, 135, 136]. There are also a number of neurodegenerative diseases associated with the human prion protein PrP, which switches to the infectious scrapie form (PrP^{SC}) through an unknown mechanism. PrP^{SC} is associated with a number of human diseases including Creutzfeldt-Jakob disease (CJD), Gerstmann-Straussler syndrome (GSS), fatal familial insomnia (FFI), and Kuru [134, 135]. Mechanisms for how the alternate forms of each of these proteins are formed and how they lead to toxicity are mostly unknown. Because protein misfolding seems to be central to all of these human diseases, it is important that the field identifies how chaperone activity can be altered to help correct folding or improve degradation of these aberrant proteins [136].

Treatments and therapies - There are currently no treatments or therapies that slow the progression or reverse the effects of neurodegenerative diseases. Primarily small molecules or genetic modifiers have been identified and tested for their ability to modulate proteostasis [137]. The chief goals for treating these types of diseases are to stabilize proteins to prevent misfolding and aggregation, or once the protein is misfolded, to promote protein refolding or degradation. Both of these can be addressed through activation of stress responses or through direct modulation of molecular chaperones. Hsp90 and Hsf1 have been primary targets for small molecule treatments, but more recently Hsp70 has become a desirable target [137, 138]. Multiple compounds were identified through a high-throughput screen that either increased or

decreased Hsp70 ATPase activity. The group that decreased activity were shown to specifically lower the levels of both total and hyperphosphorylated tau species in HeLa cells, and clear both normal and aberrant forms of the protein from mouse brains [139-141]. In addition, treatment with a compound that increased Hsp70 ATPase activity improved Hsp70-mediated luciferase refolding and suppressed the temperature-dependent slow growth defect observed in *ydjΔ* yeast cells [142]. One major issue with modulating chaperones, such as Hsp70 and Hsp90, is that these proteins act in a wide variety of proteostasis pathways, so it is important to regulate them as specifically as possible. One way to do this is to target the co-chaperones. Although the J-domain proteins are well known to provide substrate and process specificity to Hsp70, this work suggests that the cytosolic NEFs may provide an additional level of control. Gestwicki and associates have identified a family of small molecules that affect the interaction between Hsp70 and the Bag-domain containing family of proteins, and I am now testing these compounds for specificity and efficacy. Targeting distinct families of co-chaperones is promising for developing treatment and therapies of proteostasis diseases.

Chapter 2: Materials and methods

MATERIALS AND METHODS

Strains, plasmids and growth conditions - All strains in these studies are listed in Table 2.1 and are isogenic to BY4741 (MAT*a*/*his3Δ1/leu2Δ0*/*met15Δ0*/*ura3Δ0*), BY4742 (MAT*α*/*his3Δ1/leu2Δ0*/*lys2Δ0*/*ura3Δ0*), or DS10 (MAT*a/leu2-3/112trp1-1/ura3-52/lys1/lys2/his3-11,15)*. Cells were grown on media containing 2% dextrose. Media used in these studies include the nutrient rich media, yeast extract-peptone-dextrose (YPD) or synthetic complete (SC) media lacking only amino acids necessary for plasmid selection and supplemented with yeast nitrogen base (YNB) at 6.71 mg/ml (Sunrise Science Products, San Diego, CA). In addition, for some experiments additives were used including azetidine-2-carboxylic acid (2 mM) (Sigma Aldrich, St. Louis, MO), cycloheximide (100 µg/ml) (Acros Organics, Geel, Belgium), $CdSO_4$ (200 µM) (Sigma) and diamide (300 µM) (Research Organics, Cleveland, OH).

Plasmids used in these studies are listed in Table 2.2. Deletion strains were constructed using cassettes built in pBluescriptII carrying the marker genes *KANMX4*, *LEU2*, or *HIS3,* flanked upstream and downstream by non-coding regions of *SSE1, SSE2, FES1,* and *SNL1.* Descriptions of the plasmids, including restriction sites used for cloning, are provided in Table 2.2. Folding analysis was done in cells expressing the 425MET25-FFL-GFP–*leu2::URA3* plasmid. The original plasmid, p425MET25-FFL-GFP, was a generous gift from John Glover's laboratory at University of Toronto. The plasmid used in this study was constructed by amplifying the *URA3* gene from p426 [143] using oligonucleotides containing homologous regions of the *LEU2* gene (5'-ATGTCTGCCCCTAAGATCGTCGTTTTGCCAGGTGACTAACTG AGAGTGCACCATACCACAGC-3', 5'-TTAAGCAAGGATTTTCTTAACTTCTTCGGCGACAGCA TCACCCACCGCATAGGGTAATAACTG-3'). For recombination of *URA3* into the *LEU2* locus*,* the amplicon and p425*MET25-FFL-GFP* were transformed into BY4741 cells. The construct was rescued from Leu+ Ura- transformants (Geitz laboratory protocol; University of Manitoba) and transformed into *E. coli* for amplification. The plasmid was purified from *E.coli* using standard procedures and confirmed using PCR. The p425*MET25-FFL-GFP*-leu2::URA3

construct was transformed into NEF deletion strains using the rapid yeast transformation protocol [144]. For Hsf1 activity assays, p*SSA3HSE-lacZ*, previously described in [145], was transformed into wild-type cells and each of the single deletion strains. For degradation analysis, strains were constructed using pRH2081 (from Randy Hampton's Laboratory, UC San Diego), which is an integrative plasmid that carries *TDH3*-driven ssCPY*-GFP [120]. The plasmid was linearized using restriction endonuclease Van91I and transformed into wild-type and NEF deletion strains. For immunoprecipitation analyses, yeast cells were transformed with either p413*TEF*-FLAG-*SSE1* or p413*TEF*-FLAG-*FES1*, which were constructed by subcloning the FLAG tagged *SSE1* or *FES1* using SpeI/XhoI restriction sites into the 413*TEF* plasmid as previously described in [124, 143]. For overexpression studies, all *SSE1* and *SNL1* alleles were cloned into parent vectors at SpeI/XhoI restriction sites.

Yeast growth assays - For NEF deletion strain growth analysis, cultures were diluted to A_{600}= 1; 1:10 serial dilutions were spotted on YPD plates. To identify growth phenotypes in both optimal and stress-inducing growth conditions, plated cells were incubated at 15, 25, 30 and 37°C for up to 5 days. To test azetidine-2-carboxylic acid (AZC) toxicity, strains were grown overnight and 1:10 serial dilutions were plated on SC media or SC+2 mM AZC and incubated at 30°C for 3-5 days. For de novo folding analyses, NEF deletion strains containing p425*MET25-FFL-GFP-leu2::URA3* were grown overnight in SC-URA media containing 200 µM additional methionine (addition of methionine represses expression of the FFL-GFP fusion under the *MET25* promoter), subcultured in the same media, and grown to early log phase (A_{600}= 0.4-0.5), then induced in SC-URA-MET media. Refolding assays were performed using NEF deletion strains containing p425*MET25-FFL-GFP-leu2::URA3* and cells were grown overnight in SC-URA and subcultured to log phase A_{600}= 0.8-1.0. Cells were induced in SC-URA-MET for 1 hour at 30°C. Prior to heat shock cells were treated with 100 µg/ml of cycloheximide and then incubated at 42°C for 25 minutes, and recovered for 60 minutes at 30°C. For degradation analysis log phase cells were treated with 100 µg/ml cycloheximide (CHX).

Protein isolation and visualization - For folding and immunoprecipitation analyses, proteins were isolated using glass bead lysis as described in [124]. Western blot analysis was performed using α-Ssa1/2 polyclonal antibody (from M. Ptashne, Sloan Kettering Institute) at a 1:100,000 dilution, α-Ssb1/2 polyclonal antibody (from E. Craig, University of Wisconsin) at a 1:10,000 dilution, α-GFP monoclonal antibody (Roche; Nutley, NJ) at a 1:1000 dilution, or α-FLAG monoclonal antibody (Sigma). For degradation analyses and Hsf1 derepression assays, denaturing extractions were performed. Cells were resuspended in 200 µl SUME buffer (sodium dodecyl sulfate, 1%; urea, 8 M; MOPS, 10 mM, EDTA, 10 mM) + protease inhibitors (aprotinin, 2µg/ml; pepstatin A, 2 µg/ml; leupeptin, 1µg/ml; phenylmethylsulfonyl fluoride, 1mM; chymostatin, 2 µg/ml). Glass beads were added and cells were lysed by vortexing for 3 minutes and then centrifuging cells at approximately 4,600 x g for 5 minutes at room temperature. Supernate was transferred to a new tube and 6x SDS sample buffer (Tris-HCl, pH 6.8, 350 mM; glycerol, 36%(v/v); SDS, 10% (w/v); β-mercaptoethanol, 5% (w/v); bromophenol blue, 0.012% (w/v)) was added and the sample was incubated at 65°C for 10 min. Proteins were separated by 15% SDS-PAGE and transferred to a PVDF membrane (Millipore Corporation, Billerica MA). For degradation analysis α-GFP (Roche; 1:1000) or α-Pgk (Invitrogen, Carlsbad, CA; 1:1000) primary antibodies were used. For Hsf1 derepression assay proteins were incubated with α-Cpr6 (Jill Johnson Laboratory, University of Idaho; 1:1000), αHsp104 (Assay Designs / Stressgen, Farmingdale, NY; 1:1000), α-Sti1 (David Toft laboratory, Mayo Clinic, Rochester, NY; 1:1000), or α-Pgk (Invitrogen; 1:1000). Translocation analysis used anti-pre-pro-α factor (α-ppαF) (Randy Schekman laboratory, University of California Berkeley; 1:2000). To visualize proteins, membranes were exposed to enhanced chemiluminescence reagents and developed on X-ray film using a developer or a LI-COR C-DiGit Blot Scanner and Image Studio Digits software (Lincoln, NE). Coomassie staining was performed by placing the SDS-PAGE gel into Coomassie Brilliant Blue Stain (Coomassie Blue 0.076% (w/v); methanol 45% (v/v); acetic acid 10% (v/v); water 45% (v/v)) for 20 minutes with rocking. Gels were rinsed in water and destain (acetic acid 5% (v/v); methanol 16.5% (v/v);

water 78.5% (v/v)), microwaved for 30 seconds, and allowed to incubate until the desired staining of the protein bands was achieved.

Fluorescence microscopy - Cells were collected and visualized using an Olympus IX81-ZDC inverted microscope as described previously in [146] as well as in Chapter 3. To test steady-state protein solubility, log phase cells were visualized. For refolding analysis, samples were collected prior to heat shock, immediately following heat shock, and 60 minutes post heat shock to be visualized using fluorescence microscopy. To perform degradation assays, samples were collected immediately after cycloheximide treatment and one and two hours post treatment. Quantification was performed by counting approximately 100 cells and dividing the number of cells containing aggregates by the total number of cells.

FFL-GFP activity assay - To test steady-state FFL activity, light unit production was monitored once cells reached log phase exactly as described in Abrams 2013 and Chapter 3. In short, an automated plate reader protocol (BioTek Synergy MX; Winooski, VT) was used to inject 100 µl of cells with 50 µl of D-luciferin reagent (~23 µg) (Sigma) in a 96-well white plate (Greiner; Monroe, NC) followed by mixing and a luminescence reading. For refolding analysis, the activity was measured after cycloheximide treatment and heat shock. In addition, an automated protocol described in Abrams 2013 and Chapter 3 was programmed using the Synergy MX plate reader (BioTek) to measure luminescence immediately after heat shock and at 60 minutes into recovery at 30°C.

Hsf1 de-repression assay - Cells expressing the HSE-*lacZ* reporter construct were grown to log phase. Activity of Hsf1 was determined by adding 50 µl of cells to 50 µl of Beta-Glo reagent (D-luciferin-o-β-galactopyranoside, Promega; Madison, WI) in wells of a white 96-well plate. After a 30 minute incubation at 30°C, the Synergy MX plate reader was used to measure luminescence.

Immunoprecipitation - The in vitro immunoprecipitation (IP) was performed using purified FLAG-Fes1 (3 µg/µl) incubated with no lysate or whole cell lysate at 4.5 µg/µl, or 7.5 µg/µl for two hours. Protein was eluted using 30 µl of M2 FLAG resin (Sigma). For in vivo FLAG-Fes1 IP strains expressing empty vector p413*TEF* or p413*TEF*-FLAG-*FES1* were grown to mid-log phase and the protein was isolated using glass bead lysis. 10 µl of supernate was mixed with 10 µl of 2X SDS-PAGE sample buffer and incubated at 65°C for 10 minutes. For the IP, the remaining supernate was transferred to a new tube and 30 µl of FLAG resin was added along with 700 µl of TEGN (Tris-HCl, pH 7.9, 20mM; ethylenediaminetetraacedic acid (EDTA), 0.5 mM; glycerol 10%(v/v); NaCl, 50 mM) + PI. The IP was incubated for 2 hours at 4°C with rocking, followed by 8 washes with 500 µl of TEGN + PI. After the beads were washed, 40 µl of FLAG peptide solution (7 µg total) was added and the mixture was incubated at 37°C for 25 minutes. Protein solution was centrifuged at 4,600 x g and 40 µl of the supernate was transferred to a new tube, SDS-PAGE sample buffer was added and samples were incubated at 65°C for 10 minutes.

Statistical analysis – The statistical analysis pertains to Chapter 4 only and was adapted from the publication denoted at the beginning of the chapter. All experiments were performed in triplicate and the results are represented as mean ± SD. Significance determinations were performed using the two-tailed Student t test. P values are represented as follows: *, $p < 0.05$; **, $p < 0.005$; ***, $p < 0.0005$. Differences in data sets were considered to be statistically significant for all comparisons where $p < 0.05$.

Table 2.1. Strains used in these studies

Strains	Genotype	Origin
BY4741	Mat*a//his3Δ1/leu2Δ0/met15Δ0/ura3Δ0*	Open Biosystems/ Thermo Scientific
BY4742	Mat*α//his3Δ1/leu2Δ0/lys2Δ/ura3Δ0*	Open Biosystems/ Thermo Scientific
DS10	Mat*a//leu2-3,112/trp1-1/ura3-52/lys1/lys2/his3-11,15*	[147]
sse1Δ	BY4741 *sse1Δ::G418*^{r}	This study
sse2Δ	BY4741 *sse2Δ::G418*^{r}	This study
fes1Δ	BY4741 *fes1Δ::HIS3*	This study
snl1Δ	BY4741 *snl1Δ::LEU2*	This study
sse1Δfes1Δ	BY4741 *sse1Δ::G418*^{r}*, fes1Δ::HIS3*	This study
sse1Δsnl1Δ	BY4741 *sse1Δ::G418*^{r}*, snl1Δ::LEU2*	This study
fes1Δsse2Δ	BY4741 *fes1Δ::HIS3 sse2Δ::G418*^{r}	This study
fes1Δsnl1Δ	BY4741 *fes1Δ::HIS3 snl1Δ::LEU2*	This study
sse1Δfes1Δsnl1Δ	BY4741 *sse1Δ::G418*^{r} *fes1Δ::HIS3 snl1Δ::LEU2*	This study
sse2Δfes1Δsnl1Δ	BY4741 *sse2Δ::G418*^{r} *fes1Δ::HIS3 snl1Δ::LEU2*	This study
ssz1Δ	BY4741 *ssz1Δ::G418*^{r}	Yeast Knockout Collection
zuo1Δ	BY4741 *zuo1Δ::G418*^{r}	Yeast Knockout Collection
edg1Δ	BY4741 *edg1Δ::G418*^{r}	Yeast Knockout Collection
edg2Δ	BY4741 *edg2Δ::G418*^{r}	Yeast Knockout Collection

ssb1Δssb2Δ	DS10 *ssb1Δ::HIS3 ssb2Δ::LEU2*	[56]
ssb1Δssb2Δsse1Δ	DS10 *ssb1Δ::HIS3 ssb2Δ::LEU2 sse1Δ::G418*^{r}	This study

Table 2.2. Plasmids used in these studies

Plasmid	Description
pBluescriptII – *LEU2*	BluescriptII plasmid with ~2173 bp of the *LEU2* gene cloned with BamHI/SpeI
pBluescriptII – *HIS3*	BluescriptII plasmid with ~1200 bp of the *LEU2* gene cloned with BamHI
pBluescriptII – *KANMX4*	BluescriptII plasmid with ~1440 bp of the *LEU2* gene cloned with EcoRI/BamHI
p413TEF	Low copy; HIS+, constitutive promoter [143]
pRS426	High copy; URA+ [143]
pRH2081TDH3 – ss – CPY* - GFP	Integrative plasmid containing the soluble CPY mutant with a C-terminal GFP tag (Hampton, UC San Diego)
p425MET25-FFL-GFP	High copy; Met repressible promoter containing firefly luciferase fused to green fluorescent protein [148]
p425MET25-FFL-GFP – *leu2::URA3*	Contains a LEU2 deletion with a URA3 inserted through homologous recombination
pSSA3HSE-lacZ	Contains a heat shock response element from the promoter of *SSA3* [145]
p413TEF-FLAG-FES1	N-terminally FLAG-tagged *SSE1* ORF inserted into MCS (SpeI/XhoI)
p413TEF-FLAG-SSE1	N-terminally FLAG-tagged *FES1* ORF inserted into MCS (SpeI/XhoI)
p423GPD	High copy; HIS+ constitutive promoter
p423GPD-*SNL1*-FLAG	C-terminally FLAG-tagged *SNL1* ORF inserted into the MCS (SpeI/XhoI)

p413TEF-*SNL1*-FLAG	C-terminally FLAG-tagged *SNL1* ORF inserted into the MCS (SpeI/XhoI)
p413TEF-Δ40*snl1*-FLAG	C-terminally FLAG-tagged *SNL1* ORF (lacking the first 40 aa; transmembrane domain) inserted into the MCS (SpeI/XhoI)
p413TEF-*SNL1*^{E112A,R141A}-FLAG	C-terminally FLAG-tagged *SNL1* ORF (with two mutations E112A and R141A; Hsp70 binding mutant) inserted into the MCS (SpeI/XhoI)
p413TEF-Δ40*snl1*^{E112A,R141A}-FLAG	C-terminally FLAG-tagged *SNL1* ORF (with two mutations E112A and R141A; Hsp70 binding mutant and lacking the first 40 aa; transmembrane domain) inserted into the MCS (SpeI/XhoI)
p413TEF-*snl1*(5K->A)-FLAG	C-terminally FLAG-tagged *SNL1* ORF (with 5 lys mutation in helix 1 of bag domain; ribosome binding mutant) inserted into the MCS (SpeI/XhoI)
p415CYC	Low copy; LEU+, weak constitutive promoter [143]
p415CYC-FLAG-*SSE1*	N-terminally FLAG-tagged *SSE1* ORF inserted into MCS (SpeI/XhoI)
p415CYC-FLAG-*sse1*^{N281A}	N-terminally FLAG-tagged *SSE1* ORF (with N281A mutation in NBD; binds Ssa only) inserted into MCS (SpeI/XhoI)
p423GPD-FLAG-*SSE1*	N-terminally FLAG-tagged *SSE1* ORF inserted into MCS (SpeI/XhoI)
p423GPD-FLAG-*sse1*^{N281A}	N-terminally FLAG-tagged *SSE1* ORF (with N281A mutation in NBD; binds Ssa only) inserted into MCS (SpeI/XhoI)
p416TEF	Low copy; URA+, strong constitutive promoter
p416TEF-*SNL1*-FLAG	C-terminally FLAG-tagged *SNL1* ORF inserted into the MCS (SpeI/XhoI)
p415TEF-*SNL1*-FLAG	C-terminally FLAG-tagged *SNL1* ORF inserted into the MCS (SpeI/XhoI)
p415TEF-*SNL1*-FLAG	C-terminally FLAG-tagged *SNL1* ORF inserted into the MCS (SpeI/XhoI)

Chapter 3: Coupled assays for monitoring protein refolding in *Saccharomyces cerevisiae*

Note: *This chapter has been derived from work that has been published in The Journal of Visualized Experimentation (JOVE).* Abrams, J. L., Morano, K. A. Coupled Assays for Monitoring Protein Refolding in Saccharomyces cerevisiae. J. Vis. Exp. (77), e50432, doi:10.3791/50432 (2013). *I performed all experiments presented in this chapter. Permission to use this work in my thesis was granted by the Deputy Director of Journal Development, Kira M. Henderson, Ph. D..*

INTRODUCTION

In humans, neurodegenerative disorders including Alzheimer's, Parkinson's, and Huntington's diseases have been linked to protein misfolding and aggregation [149]. Cells employ molecular chaperones to prevent kinetic trapping of cellular proteins into misfolded inactive structures [150]. Chaperones participate in intricate interaction networks within the cell, but it is not completely understood how the sum of these interactions contributes to organismal proteostasis. One of the main chaperones responsible for the majority of cytosolic protein folding is the 70 kilodalton heat shock protein (Hsp70) family [22]. It has been shown that in yeast loss of Hsp70 decreases the ability to fold nascent heterologously expressed firefly luciferase (FFL) and to refold the endogenous protein ornithine transcarbamylase, in vivo [74, 75]. The ability to analyze folding with near-real time resolution will facilitate understanding how additional cellular factors contribute to this Hsp70-dependent process. In addition, folding/refolding reactions may not be obligately dependent on these contributing proteins, so assays must be sensitive enough to detect small changes in kinetics and efficiency.

The yeast cell disaggregase, Hsp104, plays a vital role in repairing aggregated misfolded proteins. Although Hsp104 homologs have been identified in fungi and plants, this family is absent in metazoans. It has been proposed that other chaperones, such as those of the Hsp110 family perform disaggregase activity in mammals [85, 86]. Hsp104 is a AAA+, hexameric protein complex that functions in yeast to remodel protein aggregates, contributing to refolding and repair [151]. Hsp104, along with the yeast Hsp70, Ssa1, and the yeast Hsp40, Ydj1, is required for recovery of denatured FFL in yeast cells [152, 153]. The small heat shock protein, Hsp26, has also been shown to be required for Hsp104-mediated disaggregation of FFL [154].

FFL is a two-domain protein that binds the substrate luciferin in the active site and following a conformational change that requires ATP and oxygen, decarboxylates the substrate releasing oxyluciferin, carbon dioxide (CO_2), adenosine monophosphate (AMP), and light [155-157]. The commercially available FFL substrate, D-luciferin, results in light emission at 550-570 nm that

can be detected using a luminometer [158]. FFL is exquisitely sensitive to denaturation from chemical or heat treatments and aggregates rapidly upon unfolding. When exposed to temperatures at 39-45°C FFL is reversibly unfolded and inactivated [159]. In contrast, GFP and its derivatives are highly resistant to protein unfolding stresses [160]. Therefore fusion of these two proteins allows FFL to function as an experimentally labile moiety capable of targeting functional GFP to intracellular deposits that can be visualized using fluorescence microscopy at both the population and single-cell levels. Application of the enzymatic assay in a semi-automated multimode plate reader coupled with automated microscopy allows unprecedented simultaneous assessment of kinetics and yield of refolding reactions. In addition, the facile molecular genetics of the model eukaryote *Saccharomyces cerevisiae* allows both precise manipulation of the protein quality control network and the opportunity for discovery-based approaches to identify novel players contributing to cellular stress response and proteostasis.

In this study, wild-type (WT) and *HSP104* deletion strains expressing FFL-GFP are subject to protein denaturing heat-shock. FFL-GFP refolding is monitored through both an enzymatic assay and microscopy as a proxy readout for repair of the expressed proteome over a recovery time course. When compared to wild-type cells, I show the Hsp104 deletion strain is ~60% less efficient at refolding FFL-GFP, supporting previous findings establishing a role for Hsp104 in reactivation of denatured FFL [154].

METHODOLOGY

1. Construction of strains containing FFL-GFP plasmid

For this study, *Saccharomyces cerevisiae* strain BY4741 (MATa, *his3Δ1, leu2Δ0, met15Δ0, ura3Δ0*) was used along with an *HSP104* deletion strain from the yeast knockout collection (Open Biosystems/Thermo Scientific). The deletion was confirmed by Western blot analysis using an Hsp104-specific antibody.

FFL-GFP was expressed from p426*MET25-FFL-GFP*, constructed from a LEU2-based source plasmid obtained from the Glover laboratory at the University of Toronto [148]. Expression of the FFL-GFP plasmid fusion protein is controlled by the *MET25* methionine-repressible promoter. The plasmid was transformed into each strain using a protocol adapted from [144]:

1. Centrifuge 25 ml of log phase cells in a 50 ml conical tube at 1,800 x g for 2 minutes, wash with 500 µl of double distilled H_20 (ddH_2O), and discard supernate.
2. Resuspend cells in 250 µl of TE/LiAc (Tris-HCl 10 mM pH=7.5, 1 mM EDTA, 0.1 M lithium acetate). Incubate at 30°C without mixing for 20 minutes.
3. Transfer 50 µl of competent cells to a new tube along with 5 µl (50 µg) of carrier DNA and 5 µl (0.1-5 µg) of plasmid DNA. Add 300 µl of PEG/TE/LiAc (same as TE/LiAc plus 40% polyethylene glycol (4000 g/mol) to this mixture, and incubate cells at 30°C for another 30 minutes.
4. Add 1/10 volume (v/v) DMSO (36 µl) to this mixture and heat shock at 42°C for 6 minutes.
5. Centrifuge mixture at 3,380 x g rpm for 30 seconds and remove supernate. Resuspend cells in 100 µl of sterile ddH_20 and plate cells onto synthetic complete media lacking uracil (SC-URA).

2. Induction of FFL-GFP

FFL-GFP is induced once cells are in log phase so the majority of the protein prior to heat-shock is newly made to avoid proteins that have terminally aggregated over time due to aging-associated cellular inadequacies.

1. Day 1: Incubate cells in 5 ml of SC-URA at 30°C rotating overnight.
2. Day 2: Measure OD_{600} and inoculate fresh cultures in 5 ml of SC-URA OD_{600}~0.2.
3. Incubate cultures with rotation at 30°C until they reach log phase OD_{600}~0.8-1.0.
4. Centrifuge cells in 15 ml conical tubes at 1,800 x g for 3 minutes, decant supernate and resuspend cell pellet in 500 µl of ddH_20; transfer solution to a microcentrifuge tube.
5. Centrifuge at 3,380 x g and discard supernate.
6. Resuspend cells in 5 ml of SC-URA-MET. Cells should be incubated rotating in this media for 1 hour at 30°C.
7. Centrifuge cells and repeat wash in ddH_20 and discard supernate.
8. Resuspend cells in 5ml SC-URA media containing 100 µg/ml of cycloheximide to inhibit protein expression, and proceed immediately to Step 3.

3. Enzymatic FFL-GFP recovery assay

This assay is a quantitative approach to determine the levels of active enzyme in a population.

1. Preparation for this assay:
    i. Measure OD_{600} for each sample.

ii. Prepare necessary amount of D-luciferin needed, based on the number of samples and desired number of replicates plus approximately 1200 µl for the tubing. Final concentration of D-luciferin is ~23 µg per 100 µl of the culture. Dissolve the D-luciferin by following the manufacturer's instructions. Briefly, make a stock solution of at a concentration of 7.5 mg/ml and diluted to 455 µg/ml in SC prior to experiment. In Figure 3.2**,** three replicates for each sample were analyzed resulting in a total of 2.4 ml of D-luciferin being used.

iii. Program plate reader for flash luminescence assay (adapted from BioTek Gen5 "Luminescence Flash Assay with Injection"; for other instruments follow manufacturer's instructions)

a. Set temperature to 30°C

b. Set plate reader to add D-luciferin, shake, and read each well individually.

c. Read luminescence (endpoint reading, 5 second integration time, 180 sensitivity level)

d. For recovery assay between each reading, shake for 5 minutes, delay 20 min, and shake an additional 5 min.

e. Dispense 50 µl of D-luciferin from an injector.

2. Pre-heat-shock luminescence is measured immediately after cells are added to media containing cycloheximide to halt protein synthesis.

i. Transfer 100 µl of cells for each sample with desired number of replicates into a solid white 96 well plate.

ii. Controls should include a water blank and cells containing an empty vector.

iii. Start the read with the specifications listed above.

3. To denature the FFL moiety of FFL-GFP, incubate the 5 ml culture in a glass culture tube at 42°C shaking for 25 minutes.
4. Recovery assay luminescence readings start immediately after cells are removed from the incubator, which is time point zero. Measure luminescence same as described above for the first time point and every 30 minutes for 90 minutes.
5. Normalize samples to preheat-shock luminescence values that have been adjusted based on the OD_{600} (divide the preheat-shock values by OD_{600}).

4. Fluorescence microscopy

This assay is a semi-quantitative method to determine solubilization of aggregates over time in a population of cells.

1. Induction is the same as described in **Section 2, Steps 1-8** of protocols.
2. The same time points are taken as described above (**Section 3, Step 4**), but for microscopy collect 1 ml of culture at pre-heat-shock and each recovery time point, and incubate samples rotating at 30°C in culture tube.
3. Visualization:
    i. Centrifuge 1 ml of culture at 3,380 x g for 30 seconds and remove supernate.
    ii. Resuspend cell pellet in 2 µl of ddH_2O.
    iii. Mix 2 µl of cells plus 2 µl of 2% low melt agarose on the slide and add a cover slip. In order to obtain a single plane of cells lightly press finger down in the center of the cover slip and rotate until the cover slip is difficult to move.

iv. Cells are visualized using 100X oil objective on fluorescent microscope; use DIC to visualize cells and the FITC filter to visualize GFP fluorescence.

v. Take multiple pictures for each strain at each time point for quantitation. Fields for pictures should contain ~15-30 cells for quantitative post-experimental analysis.

vi. To calculate percentage of cells containing aggregates, count 50-100 cells total and divide the number of cells containing aggregates.

5. Single cell microscopy

This method is used to follow the solubilization of individual aggregates in a single cell over time.

1. Induce FFL-GFP expression as described in **Section 2, Steps 1-8**.
2. After heat-shock, centrifuge cells in 15 ml conical tube at 4,400 rpm for 2 minutes.
3. Wash cells with 500 µl of water and resuspend in 10-20 µl of ddH_2O.
4. For each strain, cut out a 5 x 5 mm section of an SC-URA agar plate and using the surface that was in contact with petri dish, pipette 4 µl of cells and spread around surface of agar with pipette tip. Let stand for ~1 min.
5. Use tweezers to place agar cube section face-down on a ~55 mm glass bottom dish No. 0 and press down lightly.
6. Visualization:
    i. See **iv** in **Section 4**.
    ii. Set coordinates for 2-4 focal points for each strain depending on density of culture.

iii. Set camera to take pictures with a Z-stack with appropriate range (-/+ 15μm with a 0.5-1.0 μm slice thickness) every 5 minutes for a 90 minute period.

RESULTS

Yeast is dependent on the disaggregase, Hsp104, to efficiently refold heat-denatured proteins. The activity of FFL-GFP was monitored after a 25 minute heat shock, using a luminescence flash assay (Fig. 3.1). The results of this automated assay shown in Figure 3.2 revealed a stepwise increase in activity over 90 minutes that ultimately led to a >80% recovery in wild-type cells. The *hsp104Δ* strain recovered 19% of the original activity over the same time frame. Moreover, the extent of initial inactivation was much higher in the chaperone mutant strain (26% activity in wild-type and 11% in *hsp104Δ*) suggesting that Hsp104 may be serving a protective role pre-stress, or rapidly associates with denaturing FFL-GFP during the thermal inactivation step to reduce loss of enzyme activity.

Population microscopy corroborated the enzymatic activity assay. Nearly all cells in the *hsp104Δ* mutant strain maintained at least one FFL-GFP aggregate compared to the number of aggregates in the wild-type strain, which decreased by 62% within 90 minutes after heat-shock (Fig. 3.3). While a substantial number of aggregates formed immediately after heat-shock in both strains, the number of wild-type cells containing aggregates decreased rapidly while *hsp104*Δ cells failed to clear the observable puncta. These data corroborate the activity assay, which showed a 52% recovery of activity in wild-type versus a minimal 8% recovery in *hsp104Δ* strain.

Single cell automated microscopy revealed that in wild-type cells versus the *hsp104Δ* strain, FFL-GFP was solubilized at a higher rate (representative still images are shown in Figure 3.4**;** supplemental movies for time-lapse series of FFL-GFP re-solubilization available in [150]. In addition, the dynamics of the protein aggregates were very different; in the wild-type cells aggregates tended to fuse before being solubilized, and this was not observed in the *hsp104Δ* strain. This assay not only supports the results from the other two methods, but also provides insight into a possible mechanism for how the aggregated protein is solubilized and refolded.

Figure 3.1. Schematic of FFL-GFP heat-shock recovery assay. FFL-GFP expression is induced in log phase in cells that have been incubated in SC-URA. To induce cells incubate in SC-URA-MET for 1 hour. Centrifuge cells and resuspend in SC-URA containing 100µg/ml cycloheximide (CHX). For heat-shock, incubate cells at 42°C for 25 minutes. Allow cells to recover by incubating at 30°C. Collect samples for a 90 minute time course. For enzymatic assays D-luciferin is added before each reading.

Figure 3.1. Schematic of FFL-GFP heat-shock recovery assay

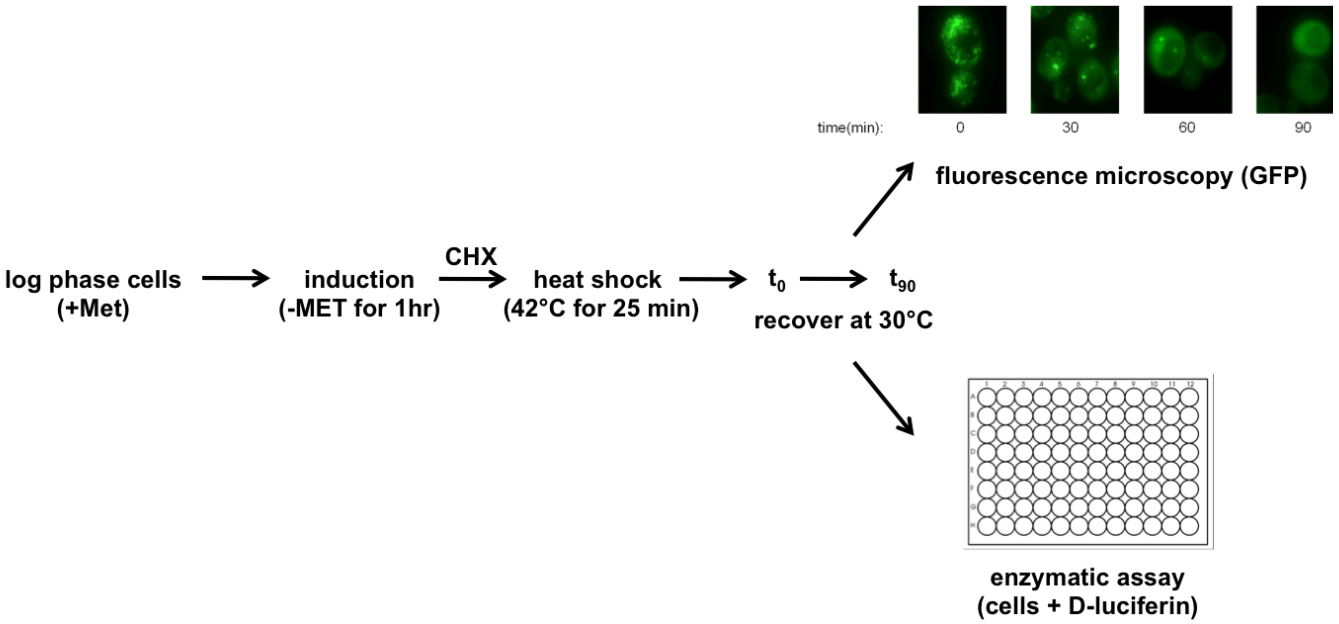


Figure 3.2. FFL-GFP enzymatic refolding/recovery assay. Samples of wild-type (WT) and *hsp104Δ* cells (100 µl) were collected prior to heat shock and immediately after heat-shock for each time point (0, 30, 60, 90 minutes). Three replicates of each sample were aliquoted into wells of a 96-well white plate for each time point. For readings, the plate reader was set to measure luminescence every 30 minutes for 90 minutes at 30 °C.

Figure 3.2. FFL-GFP enzymatic refolding/recovery assay

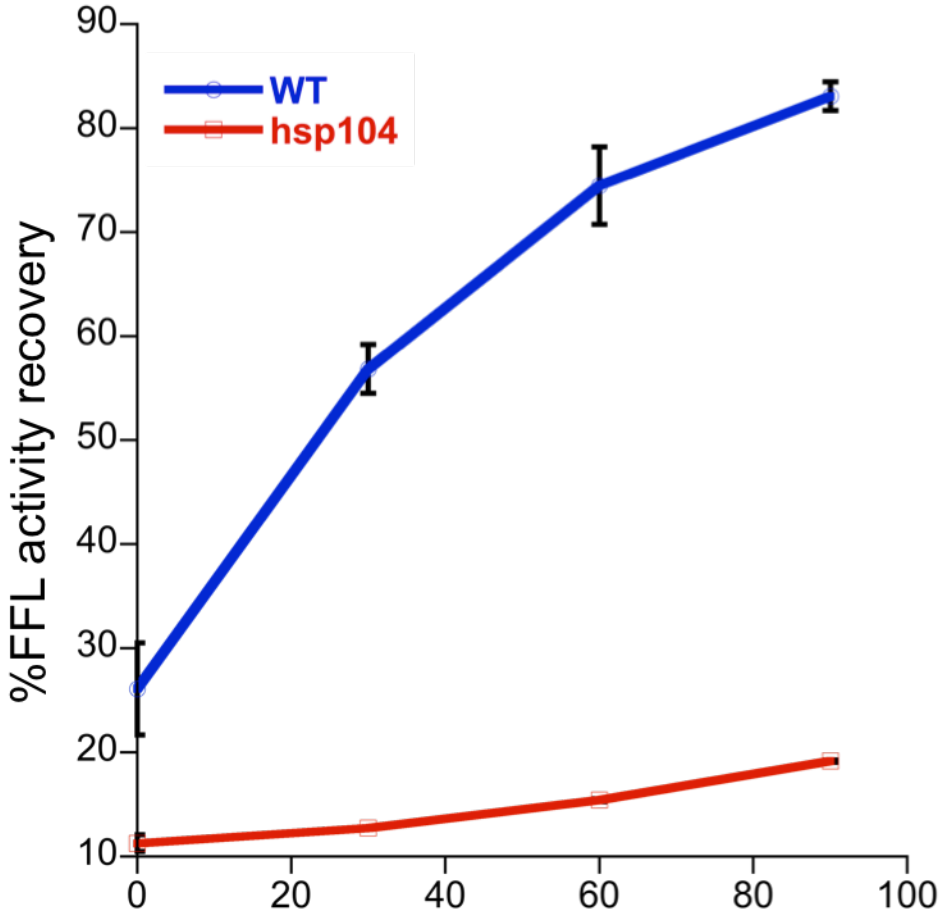


Figure 3.3. FFL-GFP refolding microscopy. 1 ml of cells at each time point (pre-HS, 0, 30, 60, and 90 minutes) were collected and centrifuged. Supernate was aspirated and cells were resuspended in 2 µl of water. Cells were prepared by mixing with 2ul of low melt agarose. Cells were visualized using the 100x oil objective. Representative images of each time point are shown. Quantitation was done by counting cells in 4-5 fields and calculating the percent of cells containing aggregates (n~100).

Figure 3.3. FFL-GFP refolding microscopy

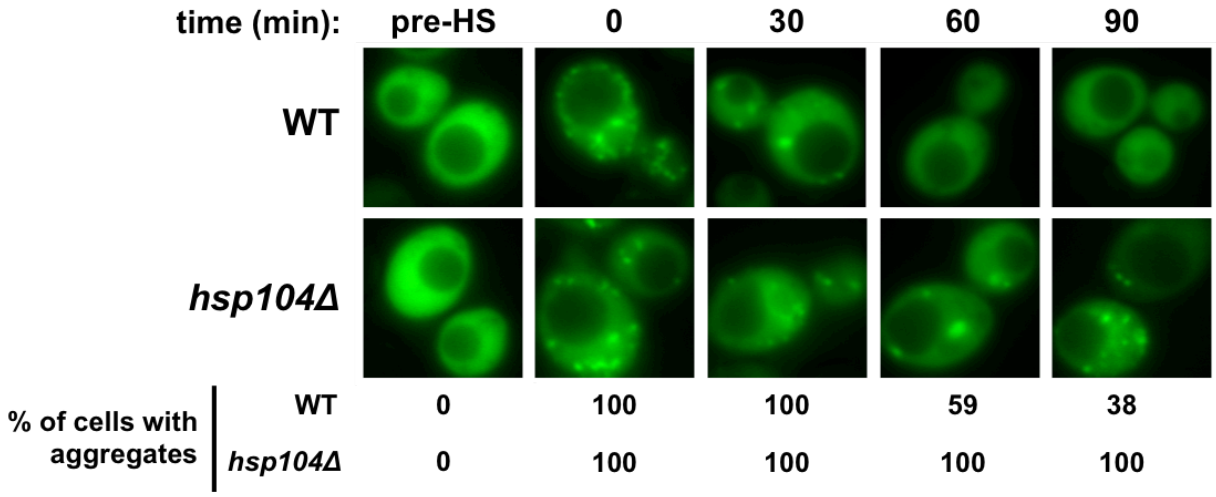
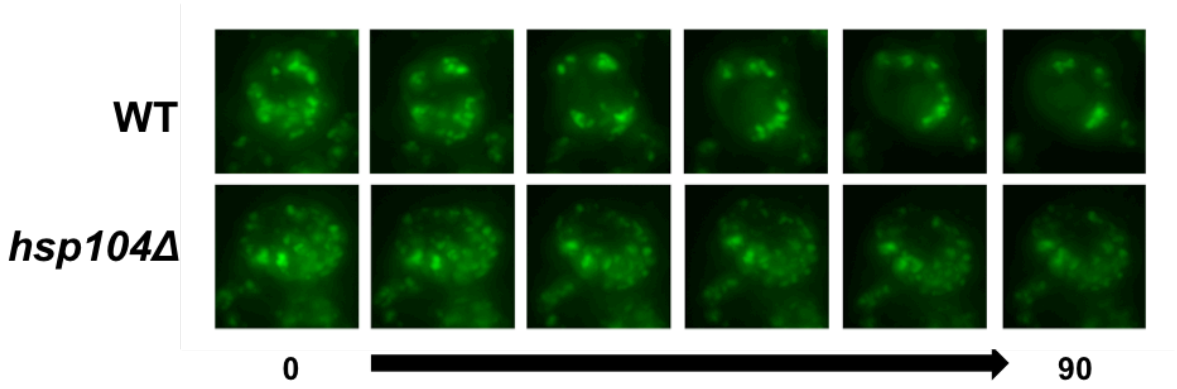


Figure 3.4. Single cell refolding microscopy time course of FFL-GFP. Post heat-shock 5 ml of cells were collected, centrifuged, and resuspended in 20 µl of ddH_2O. 4 µL of cells were placed on a 5 x 5 mm section of SC-URA agar, and visualized using an automated fluorescent microscope over a 90 minute period. The images are projections from a Z-stack taken during the time course.

Figure 3.4. Single cell refolding microscopy time course of FFL-GFP



DISCUSSION

In this article the model protein FFL-GFP was used to show that the yeast disaggregase, Hsp104 contributes to protein re-solubilization and repair. The enzymatic assays and microscopy differentially interrogated the status of the same substrate protein to determine refolding efficiency and yield. Results of the enzymatic recovery assays suggest that not only is the maximal recovery in the *hsp104Δ* mutant strain inefficient, but the initial magnitude of the unfolding stress was greater in the mutant strain (Fig. 3.2). The analysis also shows that while *hsp104Δ* cells appeared to be attempting repair, they were unable to refold the protein as quickly as wild-type cells. This method allowed sensitive and quantitative analysis of FFL-GFP activity and was confirmed by showing the essential role of Hsp104 in repair of this protein.

Microscopy results suggest that the reason for the inefficient repair of denatured FFL enzyme is due to protein trapping within aggregates (Fig. 3.3). The population analysis showed that in cells lacking Hsp104, no cells could completely clear aggregates in 90 minutes. In addition, single cell analysis revealed that in wild-type cells, aggregates fuse over time, suggesting that consolidation of smaller aggregates into larger structures may aid the repair and refolding process. Observation of aggregates over the time course uncovered decreased aggregate fusion in the *hsp104Δ* strain, indicating that these cells do not form the larger structures as efficiently and suggesting that Hsp104 is required for this facet of protein quality control (Fig. 3.4). A further speculation is that cells may solubilize protein more quickly from these larger aggregates, which may additionally contain the disaggregation and repair machinery. Single-cell microscopy can also be used to determine if other chaperones and co-chaperones are present in the larger versus smaller aggregates, and if this residency pattern varies over time.

Together these methods allow both biochemical and cell biological analysis of protein refolding and repair in living cells. Integration of the results from the three methods described affords multi-dimensional insight into the kinetics and efficiency of cellular recovery after

proteotoxic heat-shock. Automation of some or all of the steps in the protocol also allows for greater sample sizes and biological replicates in a given experiment, increasing the robustness and ultimate confidence in the outcome. In addition, these methods theoretically can be extended to use in human cells and not only for genetic analyses, but also to investigate chemicals that alter proteostasis.

Chapter 4: Hierarchical functional specificity of cytosolic Hsp70 nucleotide exchange factors in yeast

Note: *This chapter has been derived from work that has been published in The Journal of Biological Chemistry (JBC).* Abrams, J.L., J. Verghese, P.A. Gibney, and K.A. Morano, *Hierarchical Functional Specificity of Cytosolic Heat Shock Protein 70 (Hsp70) Nucleotide Exchange Factors in Yeast.* J Biol Chem, 2014 [161]. *JBC does not require permission to use published materials in ones thesis: http://www.jbc.org/site/misc/Copyright_Permission.xhtml. I performed all experiments presented in this chapter with exception to panels A&B of Figure 4.6 that were performed by Jacob Verghese Ph.D.*

INTRODUCTION

Cellular viability relies on maintaining protein homeostasis ("proteostasis"), defined as a balance between polypeptide synthesis, transport, modification, and eventual degradation. Exposed hydrophobic regions of proteins resulting from incomplete or improper folding may cause deleterious intra- and intermolecular interactions in both nascent and extant proteins, leading to aggregation and loss of function [1]. In humans, protein misfolding and aggregation have been associated with the formation of amyloid deposits common to many neurodegenerative disorders including Alzheimer's, Parkinson's, and Huntington's diseases [128]. Cells employ molecular chaperones, most notably the highly conserved Hsp70 class, to combat proteotoxic stress. The Hsp70 chaperone functions through a nucleotide dependent cycle to bind and shield short hydrophobic regions of polypeptides from the aqueous environment while the remainder of the protein folds [39]. Hsp70 binds ATP in its amino-terminal nucleotide-binding domain (NBD), which causes conformational shifts in the substrate-binding domain (SBD), allosterically communicated through an interdomain linker, to generate a low-affinity polypeptide binding state [40, 41]. Upon ATP hydrolysis, Hsp70 shifts to a high-affinity substrate binding conformation. Iterative cycles of binding and release ultimately result in promotion of substrate folding to the native state [42]. The intrinsic ATPase rate, and by extension substrate refolding efficiency, of Hsp70 chaperones is quite low, and is accelerated via interaction with co-chaperones [43]. Interaction with an Hsp40 type co-chaperone containing a conserved J domain stimulates Hsp70 ATPase activity [45, 46]. The nucleotide cycle is further enhanced by interaction with nucleotide exchange factors (NEFs), which bind the NBD and cause structural changes that promote release of ADP [47-51]. Co-chaperones also impart specificity by recruiting Hsp70s to distinct cellular processes. For example, yeast cells possess 22 J domain-containing proteins, ranging from those involved in general cytosolic protein folding such as Ydj1, to highly specific factors such as Jjj1, involved in ribosomal subunit biogenesis, and Swa2, required for clathrin-coated vesicle uncoating [38, 88, 89, 162].

Due to their substrate and process specificity, the J proteins provide a model in which Hsp70 participation in various cellular networks is determined by its co-chaperone interactions.

In contrast to the highly conserved core domain architecture of J proteins, three NEF families distinct in both sequence and structure have been identified: Hsp110, HspBP1, and Bag domain-containing proteins [90]. Hsp110 is represented by Sse1 and Sse2 in yeast, and is a divergent relative of Hsp70, with an NBD and SBD, the latter domain lengthened by the presence of an extended linker between the SBDb and SBDa subdomains. Hsp110 proteins bind Hsp70 with high affinity to form a functional heterodimer, with co-crystal structures indicating that the NBDs of Hsp70 and Hsp110 interact, while the extended linker region between SBDb and SBDα allows the α-helical bundle to wrap around the NBD of Hsp70, leaving the Hsp110 β-sandwich domain exposed and in close proximity to the Hsp70 SBD [77, 92, 96, 97]. The structural similarity of these two proteins is reflected in the demonstrated interaction of purified Hsp110s with substrate in a manner that prevents aggregation (holdase activity) but does not result in refolding [93-95]. HspBP1/Fes1 is composed nearly exclusively of armadillo repeats that bind and distort the Hsp70 NBD to promote nucleotide release [98, 99]. The Bag family is composed of six related proteins in humans, with at least two different structural arrangements of a triple helical bundle [38, 95]. A single yeast protein, Snl1, contains a functional Bag domain and additionally is tethered to the endoplasmic reticulum membrane via an amino-terminal transmembrane region [100]. Sse1 is the most abundant of all the NEFs at approximately 70,000 molecules per cell, while Fes1 is present at about one fifth the level of Sse1 and Snl1 and Sse2 15-20-fold lower than Sse1 [101]. Of the four NEF genes, only *SSE2* is significantly induced by stress [62]. Deletion of Sse1 results in slow growth and temperature sensitivity, and Hsp110 is essential in yeast as simultaneous deletion of both *SSE1* and *SSE2* is lethal [102, 103]. *FES1* disruption causes a mild slow growth phenotype exacerbated by heat shock [104]. To date, no phenotypes have been associated with mutations in *SNL1* or *SSE2.*

Functionally, Sse1 and Fes1 have both been shown to be involved in prion formation and curing, as Sse1 is required for [*PSI*+] propagation, and deletion of either *SSE1* or *FES1* blocks

[*URE3*] propagation [117, 118]. Sse1 has been implicated in Hsp70-mediated protein folding at the ribosome, Hsp90 chaperoning of signal transduction and post-translational translocation of pre-pro-α factor [77, 91, 97, 116]. Both Sse1 and Fes1 participate in Hsp70-dependent ubiquitination and degradation of misfolded proteins [119-123]. Snl1 was recently shown to bind intact ribosomes via a polybasic region adjacent to the Hsp70-binding Bag domain, although the consequence of this association is not known [124]. These studies, carried out in different strain backgrounds with different model clients, have contributed in a piecemeal fashion to understanding how the NEFs function individually, but how they are integrated into a comprehensive cellular proteostasis network is still unclear. Additionally, it is not known why Hsp70 NEF function has independently arisen at least three times, given that the relative rates of exchange measured in vitro are approximately equivalent. These are highly relevant considerations, given that human disorders are associated with NEF dysfunction. Marinesco-Sjøgren syndrome is an autosomal recessive cerebellar ataxia caused by a mutation in Sil1 (BAP), an NEF for the ER-resident Hsp70 BiP [125]. Loss of Hsp110 is additionally associated with tau pathology in a mouse model and huntingtin-related neurodegeneration in a *Drosophila* model [126, 127].

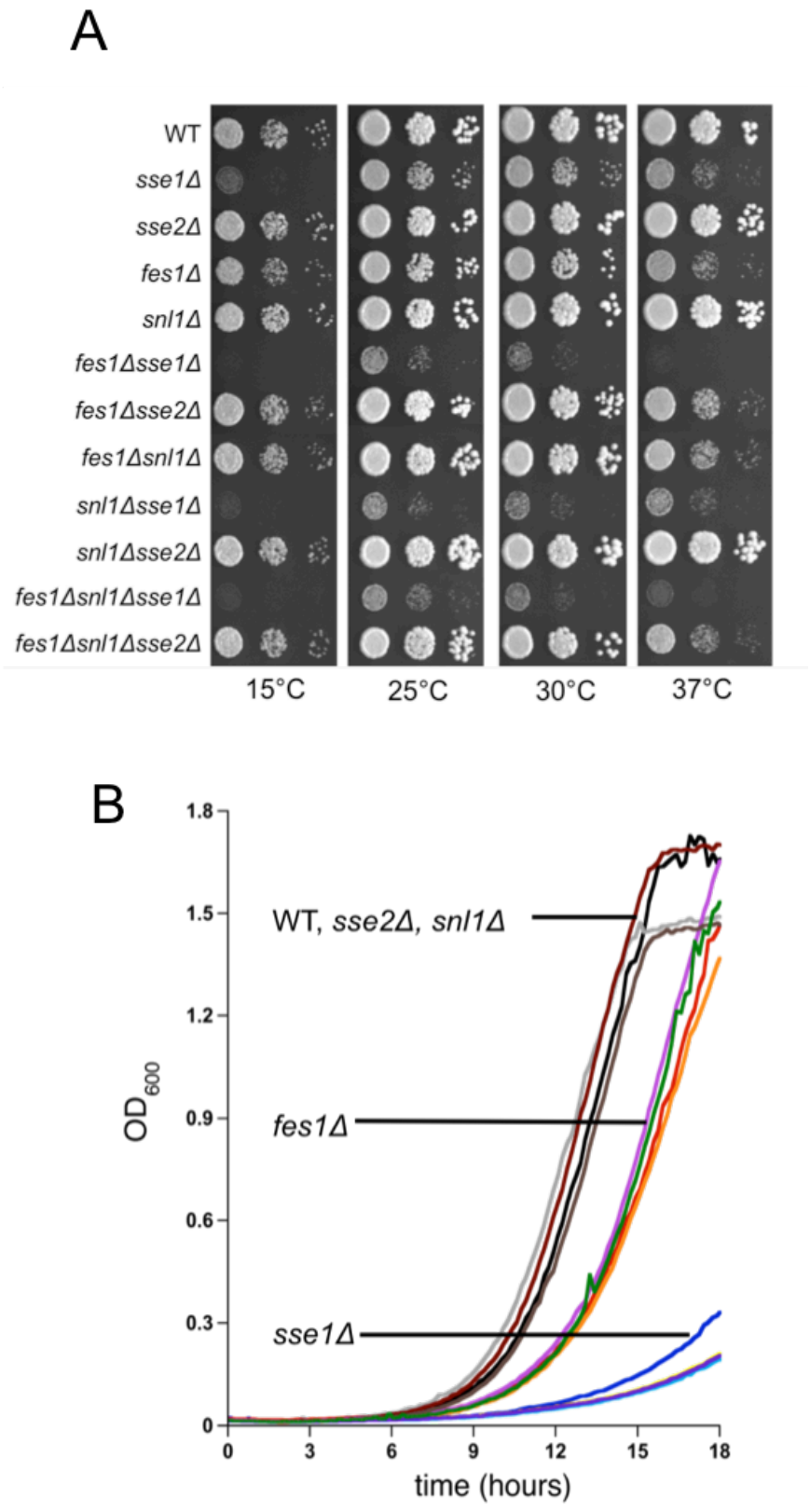
In this study, I undertook a comprehensive genetic and cell biological analysis of cytosolic Hsp70 NEF functions to determine functional specificity. I report that deletion of *SSE1* uniquely results in severe defects in Hsp70-mediated protein biogenesis and quality control while surprisingly, NEFs are not required to assist in refolding of a model misfolded substrate. Deletion of both major soluble NEFs results in constitutive de-repression of the heat shock transcription factor Hsf1, consistent with a role for Sse1 and Fes1 in governing cellular responses to stress through Hsp70. I find that Fes1 associates with the general Hsp70 Ssa1/2, but not the co-translational Hsp70 Ssb1/2 in vivo, in contrast to Sse1 which binds both, providing a possible driver of functional specificity. These findings, along with the absence of consequences for deletion of *SSE2* or *SNL1*, lead us to conclude that Hsp110 may be the principal NEF in yeast and possibly higher eukaryotic cells.

RESULTS

Disruption of genes encoding cytosolic NEFs negatively impacts cell growth in yeast. For example, loss of Sse1 was previously identified to cause a severe growth defect whereas loss of both Hsp110s results in lethality [102, 103]. In addition, *fes1*Δ strains exhibit a moderate growth defect exacerbated by deletion of Sse1 [50]. To comprehensively investigate the contributions of all four yeast NEFs to growth under optimal and stress conditions, a combinatorial and isogenic deletion collection was constructed and dilutions spotted onto YPD plates (Fig. 4.1 A). I confirmed a major slow growth phenotype for *sse1Δ*, a moderate growth defect for *fes1Δ* cells, and an additive severe growth defect for *sse1*Δ *fes1*Δ cells at normal growth temperatures of 25˚C and 30˚C. No growth defects were observed for the *sse2*Δ and *snl1*Δ strains. Interestingly, a similar slow growth phenotype was caused by simultaneous loss of both Sse1 and Snl1, but was not observed in any of the other double knockout strains. Neither of the triple deletion strains showed any synthetic enhancement over the parent double knockouts. Heat shock (37°C) sensitivity is generally associated with protein misfolding/denaturation defects and sensitivity to cold shock (15°C) with defects in translation. Slow growth due to deletion of *SSE1* is intensified during both temperature stresses, with a striking sensitivity to cold shock consistent with previous observations and known roles in protein synthesis [48, 98]. While *fes1Δ* cells exhibited sensitivity to heat stress, only a minor growth reduction was seen at 15˚C. Again, double and triple mutant phenotypes were largely dictated by the presence of *sse1*Δ or *fes1*Δ deletions and no additional synthetic interactions were detected. To further quantify the respective growth phenotypes, microwell automated growth curves were performed and generation times calculated, revealing three distinct classes of growth phenotypes (Fig. 4.1 B). The first group, which included the *snl1*Δ and *sse2*Δ deletion strains, grew at wild-type rates (doubling time (T_D) 1.8-1.9 h). The second group exhibited moderate relative growth defects (T_D 2.0-2.2 h) and are associated with loss of Fes1. The third group displayed severe relative growth retardation (T_D 2.5-3 h), which reflected the absence of Sse1. Together these data indicate that Sse1 is the most important single NEF for

FIGURE 4.1. Growth analysis of wild-type and nucleotide exchange factor deletion strains. A. Serial dilutions of cells were plated onto rich (YPD) media and incubated at the indicated temperatures. Wild-type (WT). All other strains have the indicated genotypes **B.** Automated growth curves in liquid media were generated as described in Experimental Procedures. WT, black; *sse1*Δ, blue; *sse2*Δ, gray; *fes1*Δ, red; *snl1*Δ, maroon; *fes1*Δ *sse1*Δ, yellow; *fes1*Δ *sse2*Δ, orange; *fes1*Δ *snl1*Δ, light purple; *snl1*Δ *sse1*Δ, light blue; *snl1*Δ *sse2*Δ, brown; *fes1*Δ *snl1*Δ *sse1*Δ, violet, *fes1*Δ *snl1*Δ *sse2*Δ, green.

FIGURE 4.1. Growth analysis of wild-type and nucleotide exchange factor deletion strains

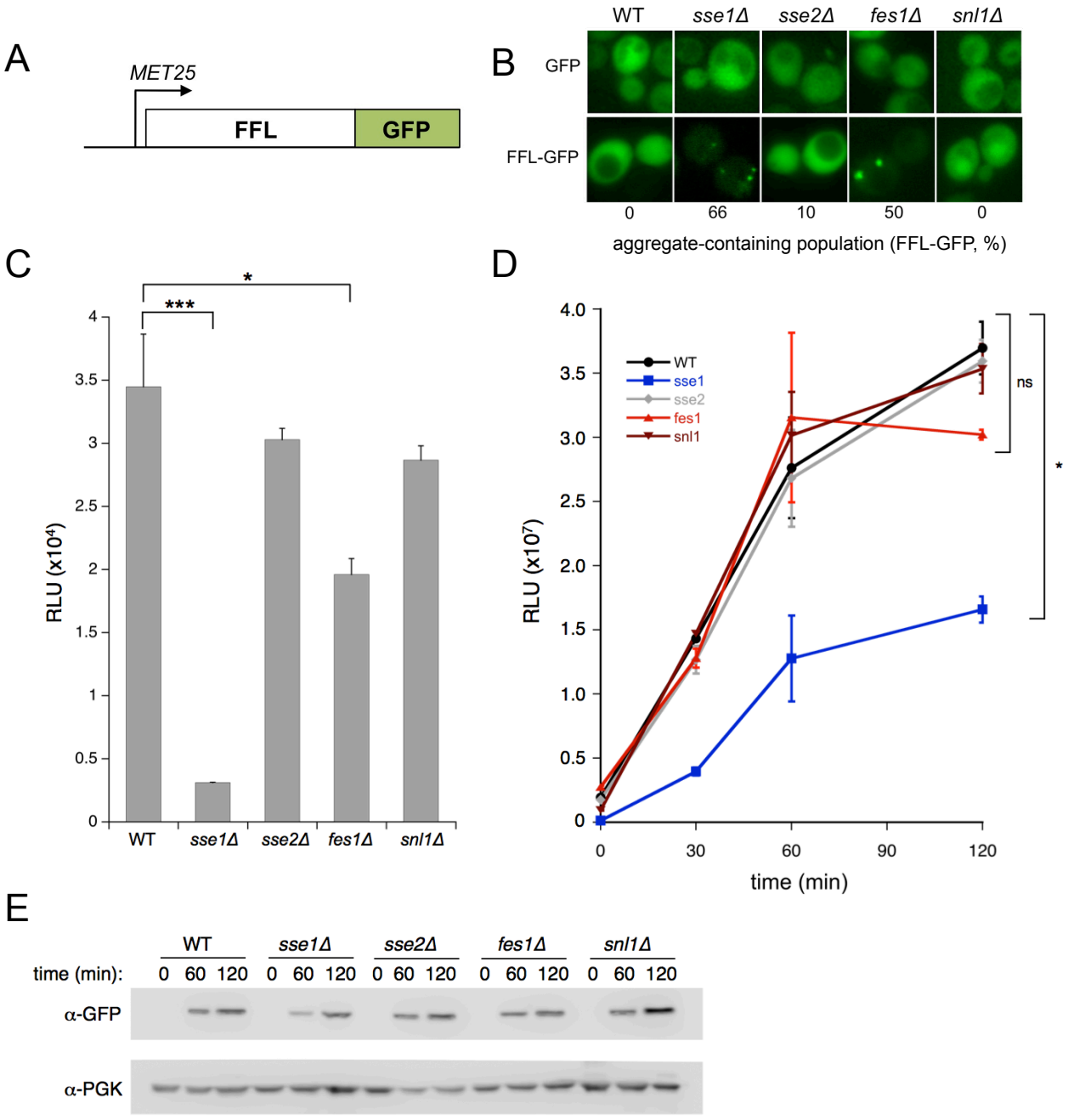


maximal proliferation at all tested temperatures. Fes1 appears to be required for heat tolerance in strains expressing Sse1 or Sse2, suggesting it has unique roles that contribute to survival under these conditions.

The preceding growth assays provided an important top-level analysis of relative NEF contributions to cell viability, paving the way for more in-depth investigation into specific roles each factor plays in critical cellular Hsp70-mediated functions. I first examined whether the NEFs play differential roles in protein biogenesis. Sse1 has been previously shown to have a role in protein refolding in vitro and in vivo and in de novo folding in vivo [48, 49]. Human Hsp105 (Hsp110) has been shown to be important in CFTR folding in vivo [163]. Roles of the other NEFs have not been fully investigated. To identify relative contributions of the NEFs to Hsp70-mediated protein folding, I utilized a well established yeast model folding substrate, firefly luciferase fused to green fluorescent protein (FFL-GFP; Fig. 4.2 A) [148]. This construct allows for enzymatic assay of properly folded luciferase in addition to surveillance of protein solubility via fluorescence microscopy. In addition, expression of the fusion protein is regulated by the methionine-repressible *MET25* promoter, which allows precise control of synthesis initiation and termination. The FFL-GFP plasmid was transformed into wild-type cells and each of the NEF single deletion strains. For steady state analysis, cells harboring FFL-GFP were grown to logarithmic phase without induction or repression resulting in low-level production of the fusion protein as visualized using fluorescence microscopy. Representative images of the population show that the *sse1Δ* and *fes1Δ* strains both contain cytosolic FFL-GFP foci, implying aggregation, while the protein was soluble in wild-type, *sse2*Δ and *snl1*Δ cells. In addition, GFP alone failed to aggregate in any strain, demonstrating that the FFL moiety was serving as a proteostasis sensor (Fig. 4.2 B). As properly folded FFL-GFP should be expected to be enzymatically active, I determined steady state levels of luciferase activity in living cells as shown in Figure 4.2 C. As expressed in arbitrary relative light units, nearly complete loss of

FIGURE 4.2. Nucleotide exchange factor deletions differentially affect firefly luciferase GFP biogenesis. A. Schematic of model folding construct firefly luciferase fused to GFP (FFL-GFP) and controlled by a methionine-repressible promoter. **B.** Representative micrographs showing GFP only control (top panel) or steady state FFL-GFP fluorescence in log phase wild-type or NEF single deletion strains (bottom panel). The FFL-GFP construct is grown in the presence of minimal methionine and is therefore not fully repressed, leading to low level expression. **C.** Steady state FFL activity monitored in the same cells as (**B**) **D.** De novo folding kinetics of wild-type or NEF deletion strains monitored over 120 min. WT, black; sse1Δ, blue; sse2Δ, gray; fes1Δ, red; snl1Δ, maroon. Strains were shifted to methionine-free medium to fully induce FFL-GFP expression. **E.** Western blot of FFL-GFP protein levels from the same cells as in (**D**). Monoclonal antibody against phosphoglycerate kinase (PGK) was used as a load control.

FIGURE 4.2. Nucleotide exchange factor deletions differentially affect firefly luciferase GFP biogenesis



activity relative to wild-type was observed in the *sse1Δ* strain, while a moderate defect was found in *fes1Δ* cells. Cells lacking *SSE2* or *SNL1* displayed essentially wild-type levels of luciferase activity. These data suggest that the aggregation phenotypes observed via microscopy correlate with enzymatically inactive FFL-GFP, and that cells lacking *SSE1* are severely compromised in biogenesis of this model protein. Because the steady state analysis is a function of both protein production and degradation, I probed de novo folding specifically by inducing FFL-GFP expression through methionine withdrawal and measuring luciferase activity over time. The *sse1*Δ deletion strain was impaired in producing enzymatically active protein both in terms of kinetics and total yield (Fig. 4.2 D). In contrast to the steady state analysis, *fes1*Δ mutants showed no discernable defect in luciferase folding. Western blot analysis of the same samples with anti-GFP showed similar levels of overall FFL-GFP synthesis, suggesting that differences in luciferase activity are due to folding and maturation of the enzyme rather than translation (Fig. 4.2 E). In addition, newly synthesized FFL-GFP remained soluble over the entire time course in all strains, as judged by fluorescence microscopy (data not shown). Overall these data indicate that Sse1 and to a lesser extent, Fes1, are required for folding and maintenance of newly translated FFL-GFP, and in their absence a fraction of the total pool aggregates over time. However, the non-aggregated FFL-GFP in *fes1Δ* cells is likely properly folded as indicated by much higher luciferase activity levels relative to *sse1*Δ.

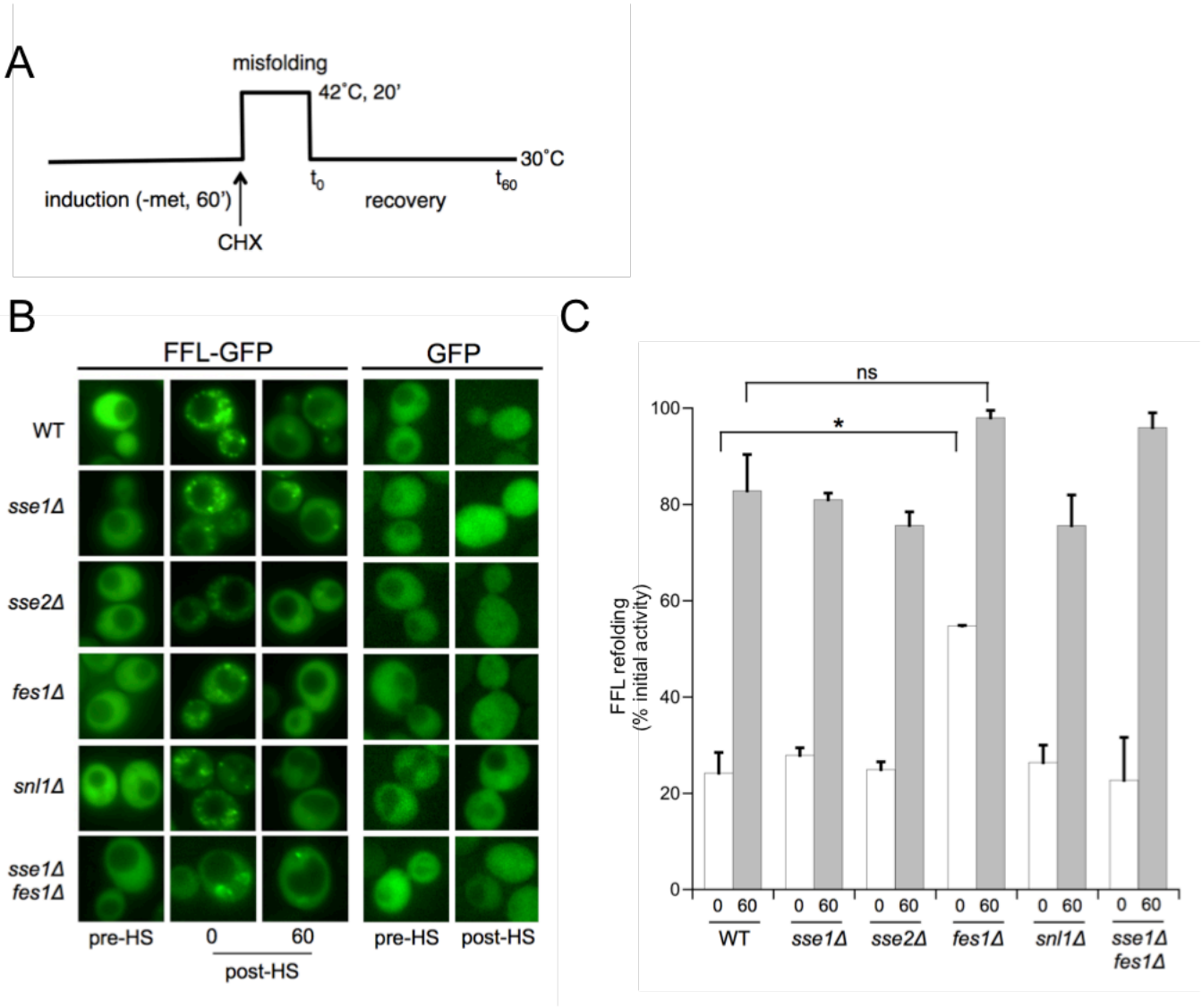
Proteotoxic stress may result in unfolding of both nascent and folded proteins. In addition to other chaperones, Hsp70 is required to stabilize and refold these substrates [152]. Consistently, yeast cells defective in cytosolic Hsp70 (Ssa), or the disaggregase Hsp104, fail to recover activity of model substrates after heat shock [146, 152]. While Sse1 has been shown to be important for refolding of firefly luciferase after temperature inactivation, little is known about the roles of the other NEFs in yeast or higher eukaryotes [48, 49]. I addressed this question by an alternative experimental protocol using the strains described in Figure 4.2. Cells harboring FFL-GFP or GFP alone, were grown in repressing medium then transferred to induction conditions for one hour. Cells were then treated with cycloheximide (CHX) to halt protein

synthesis, and heat shocked at 42°C followed by recovery at 30°C (Fig. 4.3 A). Cells were visualized prior to heat shock (pre HS), immediately after, and 60 min into recovery**.** As shown in Figure 4.3 B, newly synthesized FFL-GFP was completely soluble in all strains. After heat shock FFL-GFP formed multiple aggregates per cell that appeared to be re-solubilized over the 60 min recovery period. GFP alone was insensitive to heat shock. FFL-GFP enzymatic activity was also measured in the same cultures and normalized to the pre-heat shock values. Surprisingly, all the NEF deletion strains recovered activity at least to wild-type levels (Fig. 4.3 C). In this experiment, the *fes1*Δ deletion strain recovered activity to a slightly higher level than the wild-type strain, but also appeared to lose less activity upon heat shock (approximately 45% reduction versus greater than 75% for all other strains). These data suggest that none of the NEFs are individually required for re-solubilization and refolding of an inactivated and aggregated protein in vivo. I therefore tested the *sse1*Δ*fes1*Δ double deletion strain predicted to lack nearly all cytosolic NEF functions and observed that while enzymatic activity was again recovered to wild-type levels, a significant fluorescence signal was retained in cytosolic foci. These results suggest that either refolding does not rely on NEF activity to a significant degree or that Sse2 and Snl1 may contribute enough exchange activity to mask defects in the *sse1*Δ*fes1*Δ double deletion strain. Furthermore, these data suggest that Hsp70-mediated biogenesis and refolding/repair have distinct NEF chaperone requirements.

In eukaryotic cells the heat shock response (HSR) responsible for production of cytoprotective factors including heat shock proteins is primarily regulated by the transcription factor HSF1 [20]. In both yeast and mammalian cells, HSF1 is repressed by the Hsp70/Hsp90 chaperone network in the absence of stress and activates transcription from promoters containing heat shock elements (HSE) bound to DNA as a trimer [34, 164, 165]. Human HSF1 is primarily retained as a monomer in the cytoplasm by the chaperones while yeast Hsf1 is constitutively nuclear and bound to high affinity promoters [21, 32]. It is thought that Hsp70/Hsp90 associates with DNA-bound yeast Hsf1, maintaining it in a transcriptionally inactive state [165].

FIGURE 4.3. Cytosolic nucleotide exchange factors are not required for luciferase refolding in vivo. A. Schematic of refolding assay. **B.** Representative micrographs showing GFP fluorescence for pre-heat shocked cells and cells 0 (white bars) and 60 min (grey bars) post heat shock. GFP only controls are represented in the right panel and they were visualized at 30°C (pre-HS) or immediately after heat shock at 42°C (post-HS). **C.** FFL activity from the same cells as in (**B**). Refolding efficiency is calculated as percent of initial activity pre-heat shock. **, $p<0.005$*; ns, not significant.

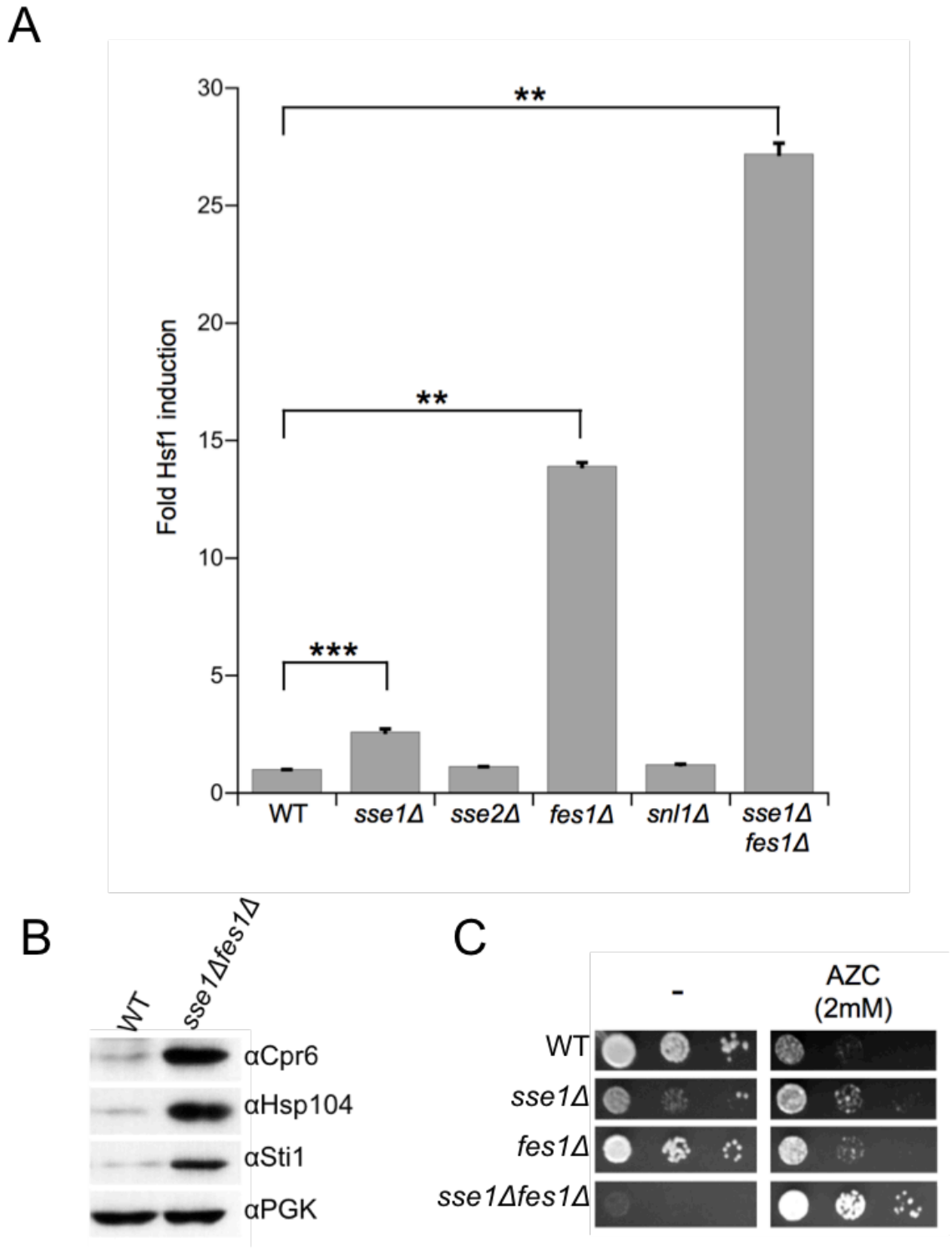
FIGURE 4.3. Cytosolic nucleotide exchange factors are not required for luciferase refolding in vivo



We and others have previously shown that deletion of either *SSE1* or *FES1* results in constitutive HSR up-regulation [116, 123, 166]. To comprehensively determine how the loss of the NEFs affects the HSR I determined Hsf1 activity using a well-documented HSE-lacZ reporter system [167]. Wild-type, *sse2Δ*, and *snl1Δ* strains all maintained Hsf1 in a repressed state at 30˚C, demonstrating a lack of involvement for these NEFs (Fig. 4.4 A). As previously shown, *sse1Δ* cells exhibited approximately two- to three-fold de-repression relative to wild-type. Cells lacking Fes1, on the other hand, showed a dramatic increase (approximately 13-fold) in Hsf1 activity. Moreover, the double deletion strain, *sse1Δfes1Δ,* revealed a striking additive effect, strongly up-regulating the HSE-lacZ reporter by nearly 30-fold. To validate the reporter results, I examined the steady state levels of three heat shock proteins whose expression is controlled by Hsf1 via Western blot analysis, focusing on the up-regulation observed in *sse1Δfes1Δ* cells. As shown in Figure 4.4 B, the Hsp90 co-chaperones Cpr6 and Sti1, and the disaggregase Hsp104 were all produced at much higher levels in the double deletion strain than in wild-type cells in non-stress conditions, confirming global de-repression of the HSR. I predicted that constitutive HSR activation resulting in increased HSP abundance should protect against high levels of protein misfolding. To test this hypothesis, I challenged cells with azetidine-2-carboxylic acid (AZC), a proline analog that incorporates into nascent chains causing protein misfolding [121, 168, 169]. As shown in Figure 4.4 C, all three NEF mutant strains analyzed exhibited varying degrees of AZC resistance consistent with the levels of HSR activity observed in Figure 4.4 A. Strikingly, the *sse1Δfes1Δ* mutant displayed robust growth in the presence of AZC, to the point that the misfolding agent suppressed the severe slow growth defect exhibited by this strain under normal conditions. These results suggest that Sse1 and Fes1 both play major roles in regulating the HSR in the absence of stress, and that Hsf1 hyperactivation in the absence of misfolded proteins may contribute to the observed growth phenotypes of cells lacking both NEFs.

FIGURE 4.4. Sse1 and Fes1 contribute to regulation of the heat shock response through Hsf1. A. Hsf1 de-repression in wild-type, NEF single deletion strains, or the *sse1Δfes1Δ* strain monitored using an HSE-*lacZ* reporter. **B.** Western blot showing differential steady state expression of Hsf1 target proteins Cpr6, Hsp104, and Sti1. The load control is phosphoglycerate kinase (PGK). **C.** Growth analysis of wild-type, *sse1Δ, fes1Δ, and sse1Δfes1Δ* strains in the presence or absence of proteotoxic stress caused by AZC. *, *p<0.0005; ***, p<0.05.*

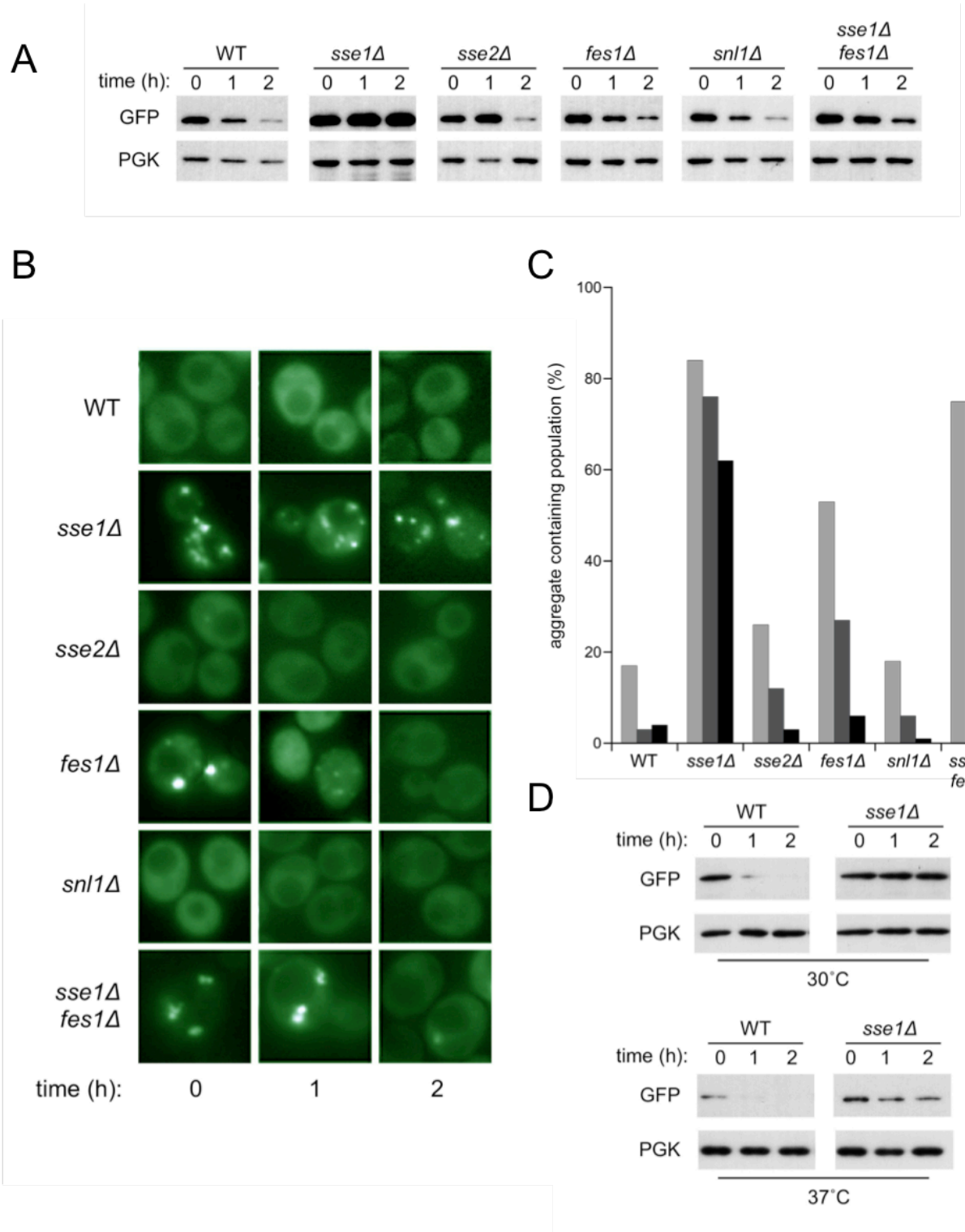
FIGURE 4.4. Sse1 and Fes1 contribute to regulation of the heat shock response through Hsf1



Hsp70 plays a major role in protein degradation through the ubiquitin-proteasome system [170]. In this capacity the chaperone is predicted to stabilize partially folded forms and to perform the "triage" decision whether to continue the folding process or present the substrate to associated ubiquitin ligases (CHIP in mammalian cells, primarily Ubr1 in yeast) to mark for degradation. We and others have implicated NEFs in control over client fate [121, 122]. A variant of the yeast vacuolar protease carboxypeptidase Y (CPY) has been successfully used as a model protein to study chaperone involvement in regulated protein degradation [120]. CPY‡-GFP lacks the ER signal sequence and contains a single destabilizing mutation causing the fusion to misfold in the cytoplasm, but retain GFP fluorescence to enable surveillance via microscopy [171]. The half-life of this fusion is approximately 30-60 min in wild-type cells, and is significantly stabilized in cells compromised for Hsp70 function, including *ssa1^{ts}* and *sse1*Δ strains [120]. I generated strains expressing CPY‡-GFP and followed protein stability via cycloheximide chase and Western blot analysis (Fig. 4.5 A). I confirmed a nearly complete block in CPY‡-GFP degradation in *sse1Δ* cells but noted that all other single NEF deletions and the *sse1Δfes1Δ* strain degraded the fusion with essentially wild-type kinetics. Observation of CPY‡-GFP aggregate formation revealed patterns that closely matched these results (Fig. 4.5 B). Wild-type, *sse2*Δ and *snl1*Δ cells accumulated few detectable aggregates, all of which were cleared, while *sse1*Δ, *fes1*Δ and *sse1Δfes1Δ* cells contained numerous aggregates at the initiation of the cycloheximide chase. In contrast to the *sse1*Δ mutant that failed to resolve and degrade the aggregates, *fes1*Δ and *sse1Δfes1Δ* cells successfully eliminated CPY‡-GFP over the time course, as quantitated in Figure 4.5 C. These results suggest that Fes1 plays essentially no role in degradation of this model substrate, and moreover show that degradation defects in *sse1Δ* cells are suppressed by concomitant deletion of *FES1*. Given that *fes1*Δ and *sse1Δfes1Δ* cells exhibit significant de-repression of the HSR, I reasoned that enhanced production of HSPs and associated factors may accelerate CPY‡-GFP degradation. To test this hypothesis, I attempted to create hypomorphic mutations at the *HSF1* locus in these strain backgrounds, but were unable to do so, perhaps indicative of synthetic lethality. Instead we

FIGURE 4.5. Sse1 uniquely contributes to Hsp70-mediated protein degradation. A. Western blot of CPY‡-GFP degradation over a 2 hr cycloheximide chase period. PGK was used as a load control. **B.** Representative micrographs of wild-type and NEF deletion cells from the time points sampled in (**A**). **C.** Quantitation of aggregate-containing fraction of the total population for each strain from (**B**) at 0 (light grey), 1 (dark grey bars) and 2 hr (black bars) (n~100 cells). **D.** Western blot analysis of CPY‡-GFP degradation in wild-type and *sse1Δ* strains at control (30˚C) or heat shock (37°C) temperatures. **, *p<0.005;* ***, *p<0.05.*

FIGURE 4.5. Sse1 uniquely contributes to Hsp70-mediated protein degradation

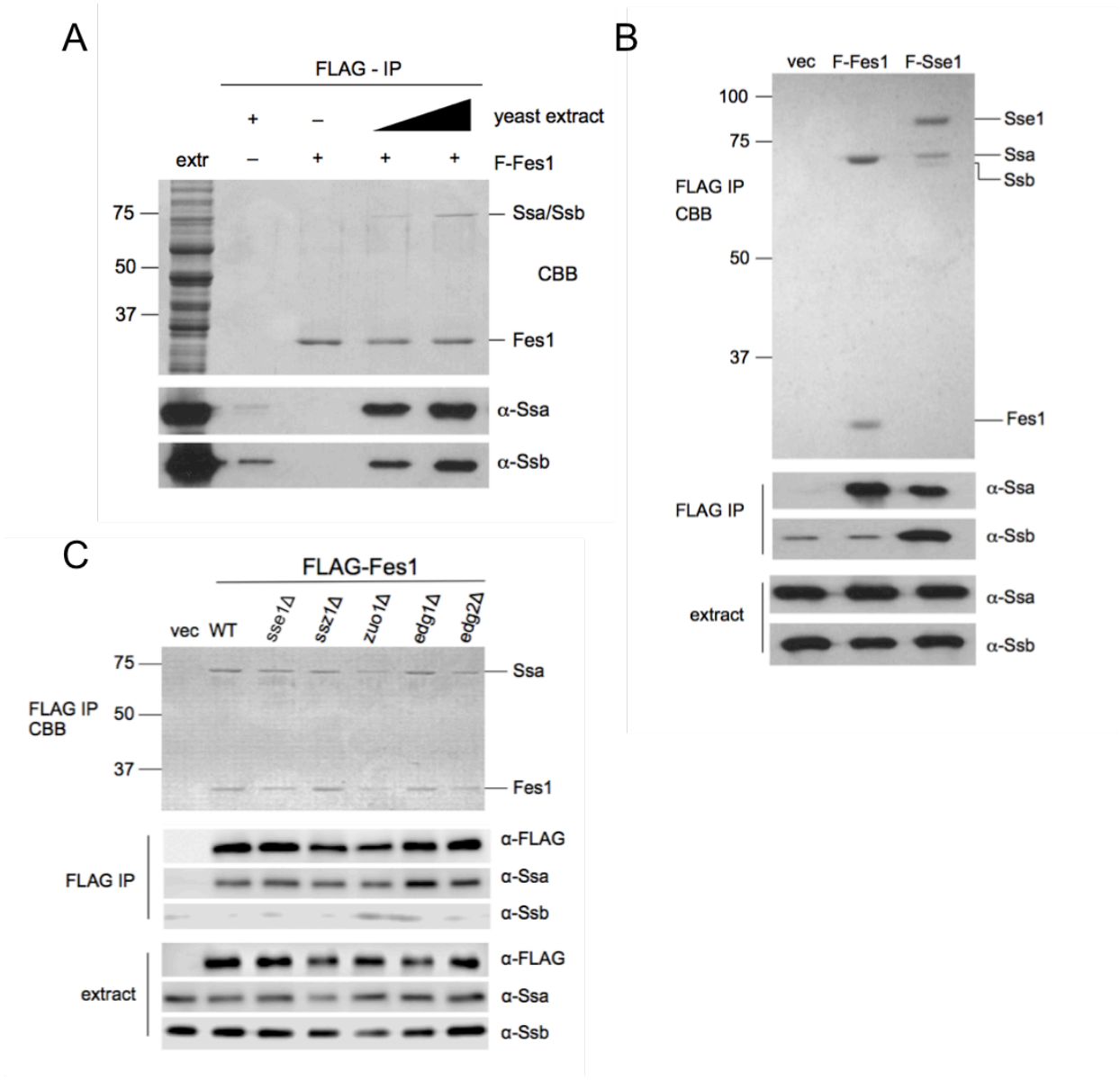


determined whether activation of the HSR via external stress would phenocopy the effects of eliminating the NEFs on Hsf1 regulation. CPY‡-GFP degradation kinetics were determined in wild-type and *sse1*Δ cells exposed to heat shock (37°C) or kept at optimal temperature (30°C) for 30 min prior to initiation of the cycloheximide chase. As shown in Figure 4.5 D, this brief heat shock substantially improved CPY‡-GFP degradation in the *sse1Δ* strain, supporting the possibility that alternative factors induced in the HSR may be substituting for Sse1 to promote CPY‡-GFP degradation. Together, these data suggest that Sse1 is a critical Hsp70 partner for degradation of at least one misfolded protein substrate. In addition my data contrast with a recent report that Fes1 is specifically required for recognition and processing of misfolded substrates as I find no defects in CPY‡-GFP degradation under conditions where *sse1*Δ cells fail to degrade the same protein [123].

My experiments indicated that Sse1 plays roles in protein biogenesis, degradation and Hsf1 regulation, while Fes1 only appeared to contribute significantly to the latter Hsp70-mediated process. In addition Fes1 has been directly implicated in recognition of misfolded proteins during stress conditions. A possible explanation for this distribution of NEF dependency could be differential interaction with the two classes of cytosolic Hsp70 in yeast: Ssa is involved in all the processes I investigated, while Ssb likely only plays a significant role during protein translation, interacting with nascent chains by virtue of its association with the ribosome [115, 172]. To test this hypothesis, I took advantage of previously developed co-immunoprecipitation assays using fully functional FLAG-tagged NEF alleles expressed in yeast. I first performed an in vitro binding analysis using FLAG-Fes1 produced in *E. coli* cells that was mixed with increasing amounts of yeast extract and affinity purified. As shown in Figure 4.6 A, both Ssa and Ssb co-purified with FLAG-Fes1 as demonstrated by Coomassie staining and Western blot. These data are consistent with a previous report that His6-Fes1 produced in *E. coli* likewise binds both Hsp70s [99].

FIGURE 4.6. Fes1 specifically interacts with Ssa chaperone in vivo. A. Coomassie brilliant blue (CBB, top panel) and Western blot (bottom panels) of in vitro immunoprecipitation (IP) of Hsp70 from wild-type cell lysates added at concentrations of 0, 4.5 µg/µl, or 7.5 µg/µl with FLAG-Fes1-bound beads. Western analysis was done using anti-Ssa and anti-Ssb antibodies as indicated. **B.** Coomassie brilliant blue (CBB, top panel) and Western blot (bottom panels) of in vivo FLAG-Fes1 or FLAG-Sse1 immunoprecipitations in wild-type cells. **C.** Coomassie brilliant blue (CBB, top panel) and Western blot (bottom panels) of in vivo FLAG-Fes1 immunoprecipitations from the indicated strains. Panels **A** and **B** were performed by Jacob Verghese.

FIGURE 4.6. Fes1 specifically interacts with Ssa chaperone in vivo



We then expressed FLAG-Fes1, and FLAG-Sse1 as a control, in wild-type yeast cells and immunoprecipitated the tagged NEFs (Fig. 4.6 B). As previously demonstrated, Sse1 strongly interacted with both Hsp70s [77]. In contrast, Fes1 appeared to interact exclusively with Ssa in vivo, with only background amounts of Ssb co-purifying. This striking finding suggested that Fes1 may be unable to bind Ssb in living cells due to other factors. I therefore repeated the immunoprecipitation experiment in strain backgrounds chosen to address this question. To determine if Sse1 outcompetes Fes1 for Ssb binding due to its greater abundance (71,000 versus 13,000 molecules per cell) an *sse1Δ* strain was utilized [101]. To ask whether the ribosome-associated complex (RAC), a potent activator of Ssb, was involved I employed strains lacking Ssz1 and Zuo1, the two RAC components [173, 174]. Lastly, I tested whether another factor associated with polypeptides during synthesis, the nascent chain associated complex (NAC), was involved using cells lacking the ß-NAC protein Edg1 and α-NAC Edg2 [68]. None of the gene deletions altered Fes1 interaction with Ssb, suggesting that competition and occlusion at the ribosome are likely not contributing to the specificity I observed in vivo (Fig. 4.6 C).

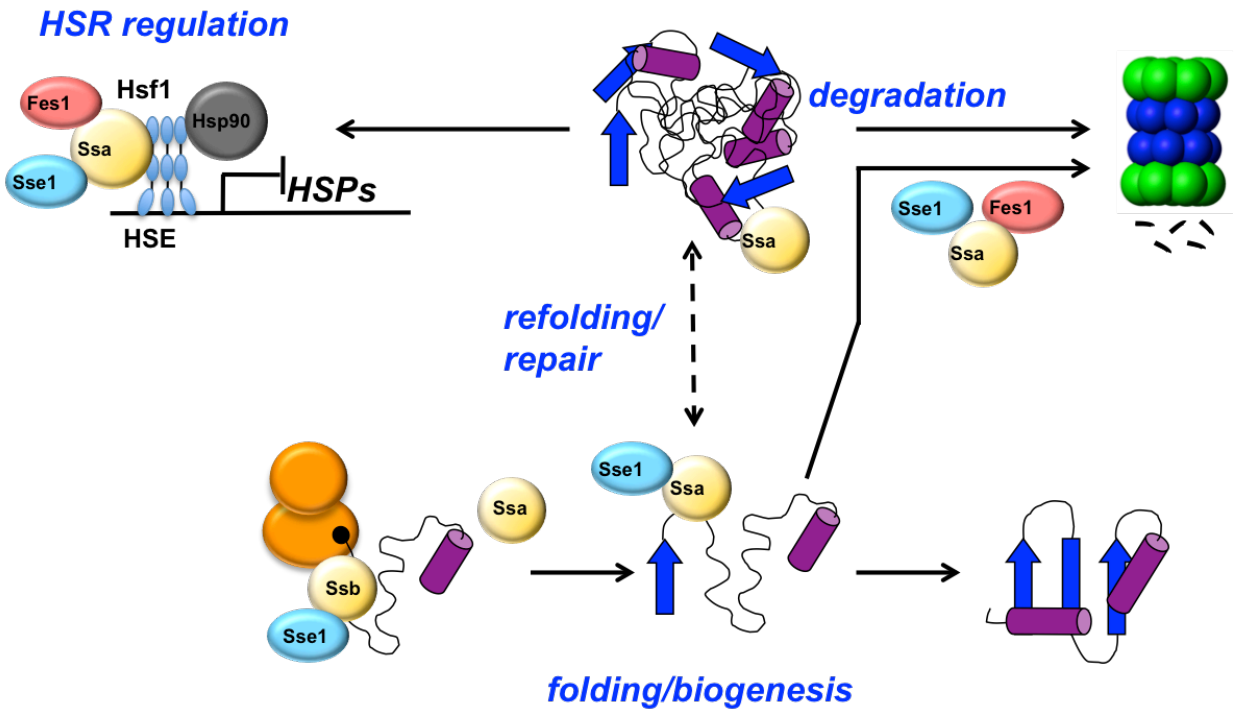
DISCUSSION

The existence of at least three distinct types of eukaryotic nucleotide exchange factor for Hsp70, none of which are related to the bacterial NEF GrpE, suggests significant evolutionary selective pressure to modulate cycling of this critical chaperone. While intense research efforts in the last decade have revealed many features of NEF function in yeast and human cells, most of the work has been focused on individual factors, sometimes leading to conflicting results. For example, deletion of *FES1* led to temperature sensitive growth in two yeast strains (W303-1b and RSY801), and normal growth in another (S1278b) [175]. Simultaneous deletion of *SSE1* and *SSE2* is reported to be viable by one group, and lethal by another [97, 103]. These findings prompted us to generate a collection of combinatorial yeast NEF deletion mutations in a single strain background and to carry out functional assays in strains with significant phenotypes to parse their relative contributions to Hsp70-dependent cellular processes. My results confirmed previous functional analyses and uncovered several previously unappreciated aspects of NEF biology. Most notably, I find that Hsp110 (Sse1) participates in multiple aspects of Hsp70 function in vivo while the HspBP1 homolog Fes1 plays a more restricted role. The heat shock inducible Hsp110 Sse2 as well as the Bag domain-containing protein Snl1 appear to have little to no impact on the processes I analyzed (Fig. 4.7).

To probe NEF roles in protein biogenesis and repair, I utilized a previously generated model substrate consisting of the thermolabile protein firefly luciferase fused to the green fluorescent protein (FFL-GFP). This protein offers multiple advantages as a proxy chaperone substrate: synthesis, solubility and enzyme activity can all be easily assayed and expression controlled in the particular construct I used by a regulatable promoter. Sse1 was found to be required for production of enzymatically active FFL-GFP, but not for its synthesis, while cells lacking Fes1 displayed only minor defects in steady state (non-induced) FFL activity (Fig. 4.2). Interestingly, *sse1*Δ and *fes1*Δ strains both accumulated stable FFL-GFP aggregates, implying either that a subset of the aggregates in *fes1*Δ cells contain active FFL, or that a greater fraction of soluble FFL is active in *fes1*Δ vs. *sse1*Δ mutants. Aggregates were not seen in any NEF deletion strain

FIGURE 4.7. Model of nucleotide exchange factor roles in Hsp70-mediated protein biogenesis and quality control. See text for details. HSE, heat shock element; HSP, heat shock protein. Translating ribosomes are depicted in orange, the proteasome is depicted in blue and green.

FIGURE 4.7. Model of nucleotide exchange factor roles in Hsp70-mediated protein biogenesis and quality control



when FFL-GFP synthesis was induced by withdrawal of methionine from the growth medium and activity and solubility followed over time (Fig. 4.2 and data not shown). These results suggest that FFL-GFP does not aggregate immediately upon synthesis but rather accumulates in the absence of Fes1, while Sse1 is required for both acquisition of enzymatic activity at early stages of biogenesis and stability at later stages. These results fit well with my finding that while Sse1 interacts with both Ssa and Ssb in vivo, Fes1 appears to exclusively associate with Ssa, restricting it to post-translational folding (Fig. 4.6). This binding specificity is not apparent in vitro, with Fes1 produced heterologously in *E. coli*, nor is it due to steric hindrance with the other Ssb-associated factors I tested [99]. These results imply that Fes1 may be modified in yeast, a hypothesis my laboratory is actively pursuing. Interestingly, I observed a modest enhancement of the slow growth and cold-sensitive phenotypes of *sse1*Δ cells and in the *snl1*Δ*sse1*Δ double deletion strain, perhaps indicative of compounded translational alterations. This novel phenotype may be linked to the previous discovery that Snl1 associates with intact and likely translating ribosomes [124]. In addition, stoichiometric balance of the Snl1 and Sse1 proteins may be critical as *sse1*Δ cells are hypersensitive to even moderate overexpression of *SNL1* (Fig 5.1).

The Hsp70 chaperone system is required for refolding of damaged proteins in yeast, in collaboration with the fungal disaggregase Hsp104. It was therefore surprising to find that the NEFs do not appear to be critical for this process (Fig. 4.3). All individual NEF knockout strains lost FFL activity and accumulated FFL-GFP aggregates after heat shock at 42˚C, and most if not all foci were resolved after 60 min of recovery. Moreover, all mutant strains recovered FFL activity similar to wild-type cells. I note that *fes1*Δ cells partially resisted FFL-GFP misfolding in these experiments as evidenced by fewer foci and higher residual post-heat shock enzyme activity. This may be due to hyperactivation of the heat shock response resulting in increased production of HSPs including Hsp104 (see below). Cells lacking both Sse1 and Fes1 likewise exhibited no refolding defects, but accumulated FFL-GFP foci that persisted after 60 min recovery, suggesting that some of the material localized to the aggregates may in fact be

refolded but not mobilized or fully solubilized. My data contrast with those of Bukau and coworkers at Heidelberg University, who found that *fes1*Δ cells recovered less than 50% of initial FFL enzyme activity over a similar time period [49]. These differences may be attributable to the fact that the substrate I used includes the stable GFP moiety fused to FFL. In that report Sse1 was also found to differentially participate in refolding of monomeric FFL as compared to heterodimeric bacterially derived luciferase, raising the possibility that NEF recruitment may be substrate-specific.

In addition to established roles for Hsp70 in protein biogenesis, accumulating evidence places this chaperone at the nexus of the decision to fold or degrade damaged substrates. I examined NEF participation in this process with a permanently misfolded construct, CPY‡-GFP, previously shown to be degraded in an Hsp70-dependent manner (Fig. 4.5) [86]. As with the FFL-GFP construct, the GFP moiety allows simultaneous surveillance of both protein level and aggregation status. As reported, *sse1*Δ cells dramatically stabilized CPY‡-GFP levels as determined by Western blot [120]. I additionally found that this reporter protein accumulated in multiple distinct foci that persisted throughout the cycloheximide chase. Interestingly, cells lacking Fes1 exhibited similar foci that were absent in wild-type, *sse2*Δ and *snl1*Δ cells, yet cleared this material over time as indicated by fluorescence microscopy and Western blot. This result suggests that Fes1 may contribute to processing of misfolded and/or aggregated proteins but not be absolutely required to do so. In a recent study focusing exclusively on the role of Hsp70 NEFs in protein degradation, Gowda and coworkers at Stockholm University, found that both Sse1 and Fes1 contributed to Hsp70-mediated degradation of model misfolded proteins [123]. Because cells lacking Sse1 are also impaired in degradation of Ub_{V76}-Ura3, a ubiquitin-targeted but folded chimeric substrate, it was concluded that Fes1 may specifically target Hsp70 to misfolded substrates to accelerate ubiquitination. However, it is not clear how such specificity is generated, as the soluble Bag domain from Snl1 is fully competent to replace Fes1 in this process, while Sse1 is not [123]. My laboratory also previously demonstrated that only the Hsp110 homolog Sse2, and not the same soluble portion of Snl1 (Snl1ΔN), could efficiently

rescue processing of the Hsp90 substrate Ste11 [121]. The answer may be that both soluble NEFs (Sse1 and Fes1) participate in targeting misfolded proteins for ubiquitination based on as yet undetermined features of a particular substrate.

Remarkably, deletion of *FES1* in the *sse1*Δ background partially restored degradation of CPY‡-GFP in my assays. This double mutant combination also exhibited the highest levels of de-repression of the heat shock response, with corresponding overproduction of HSPs and resistance to the proteotoxic compound AZC (Fig. 4.4). Correspondingly, I demonstrated similar suppression of the *sse1*Δ degradation phenotype when the experiment is conducted at 37˚C. I envision two possible, and not mutually exclusive, explanations to account for activation of the HSR in these cells. Loss of both NEFs may negatively impact Hsp70-mediated folding to an extent that allows for significant accumulation of misfolded proteins, long suspected to be the primary signal for HSR activation via titration of repressing chaperones [176]. Alternatively, general protein folding may not be severely affected, and rather inhibition of Hsf1 transcriptional function by Hsp70, perhaps as part of the Hsp90 super-complex, could be abrogated leading to HSR de-repression. In support of this conjecture, I recently demonstrated that modification of key cysteine residues in Ssa1 is sufficient to induce the HSR [166]. At this time it is not possible to mechanistically deconvolute these two models as both ultimately converge on the same fundamental aspect of Hsp70 function. However, it is worth noting that mutations in the major cytosolic Hsp40 Ydj1 impair protein refolding and degradation, yet do not induce the HSR [116, 177].

Although my current study sheds light on the distribution of labor between the cytosolic NEFs, many questions remain unanswered. Sse1 is the only one of the three that contains a substrate binding domain, yet to date no in vivo role has been directly ascribed to this domain. Interestingly, a mutant *SSE1* allele lacking NEF activity stabilizes and promotes nucleation of the prion-forming domain of Sup35, prompting speculation that the Sse1 SBD is responsible [178]. The lack of a verified mutant in this domain, preferably one that also does not impede NEF activity within the Hsp110/Hsp70 heterodimer, continues to hamper progress in

understanding this important co-chaperone. The recent identification of Hsp70/Hsp110/Hsp40 complexes as functional protein disaggregases that could play a role in clearing amyloid deposits in metazoans that lack Hsp104, further underscores the importance of understanding and perhaps decoupling NEF and chaperone holdase functions [85, 86]. In addition, the high degree of conservation of orthologous Hsp70 NEF families in higher eukaryotes suggests that answers derived from these and future studies in yeast will benefit investigations into human diseases of protein misfolding.

Chapter 5: Snl1 function in proteostasis

INTRODUCTION

Snl1 is an 18 kd cytosolic Hsp70 NEF in the Bag-domain family. In yeast Snl1 is the only homolog in this family identified thus far and it is unique in that it is tethered to the ER membrane through a transmembrane domain (TMD) located at the N-terminus [179]. The TMD is followed by the Bag domain, which is made up of three α-helices; helices 2 and 3 have been shown to interact with the NBD of Hsp70 [51]. When Snl1 binds Hsp70 it causes a 14 Å rotation of the Hsp70 IIB domain that releases the ADP, and this mechanism is conserved in the bacterial DnaK/GrpE interaction, suggesting convergent evolution, as there is no conservation of sequence or structure between Snl1 and GrpE [51, 181]. Snl1 was shown to act as a potent NEF for the Hsp70 family, but unique characteristics and similarity to the Bag family of proteins suggest it also has specific functions.

The Bag family of proteins is known to contain unique sequence motifs associated with distinct roles for each of the isoforms including folding, degradation, signal transduction, apoptosis, and transcription [181-187]. Snl1 was originally identified as a high copy suppressor of the *nup116*C (the carboxyl-terminal portion of nucleoporin 116) overexpression lethality in a *Nup116* deletion strain [179]. Suppression of this phenotype suggests a role for Snl1 in nuclear pore biogenesis and work following this study indicated that suppression was Hsp70-dependent, but the mechanism has yet to be revealed [100]. In a recent study performed in my lab, Snl1 was shown to interact with the ribosome, but no specific function associated with this interaction has been established [124].

For this study, I used genetic and biochemical analysis to attempt to uncover a biological role for the elusive NEF, Snl1. Growth analysis from Chapter 4 showed that deletion of Snl1 alone has no phenotype, but a minor synthetic growth defect and cold sensitivity is present in the absence of both Snl1 and Sse1. Cold temperatures are associated with induction of translation machinery, and sensitivity to this stress suggests an issue with activating translation [188, 189]. Therefore, this phenotype indicates Sse1 and Snl1 work together to maintain balance in a process involving translation. Furthermore, overexpression of Snl1 in an *sse1Δ*

strain, results in toxicity and activation of the HSR. The overexpression phenotype is dependent on Snl1 localization and alleviation of the growth defect was mediated through Sse1's interaction with the Hsp70, SSB. These data support a model where Snl1 provides regulatory activity affecting translation and Sse1 helps to maintain proteostasis by mediating flux of proteins through the Hsp70s, which encourages high efficiency folding kinetics.

RESULTS

Gene deletion is one way to investigate the function of a protein. In addition overexpression can reveal phenotypes that may suggest a role for that protein. Only one major phenotype has been associated with Snl1, which is the suppression of toxicity associated with *nup116C* overexpression. In addition, Snl1 has been shown to interact with the ribosome [124, 179]. Based on the data uncovered in these studies, I wanted to identify additional phenotypes that might illuminate a cellular purpose for Snl1. No growth defect is associated with deletion of Snl1 in wild-type (WT) cells, but analysis of the multiple NEF deletion strains in Chapter 4 (Fig. 4.1), revealed a synthetic growth defect and cold sensitivity upon deletion of *SNL1* and *SSE1*. This suggests that these two proteins work together to contribute to an essential process, which is not deficient upon loss of Snl1 alone. To investigate if other changes in relative levels of these two proteins cause growth defects that may indicate functional roles, I overexpressed *SNL1* using a high copy plasmid with a high expression level promoter (p423*GPD*) in wild-type or *sse1Δ* cells. Spots of serial dilutions were plated at physiological temperature (30°C) and heat shock temperatures (37°C and 39°C) (Fig. 5.1). High levels of Snl1 in an *sse1Δ* strain were severely toxic, suggesting that altering Snl1 levels in an *SSE1* null background possibly leads to additional problems separate from those that already exist in this strain, including defects in protein biogenesis, degradation, protein translocation (Chapter 4, [48, 121]).

This observation suggests that Snl1 could be involved in many different processes. Some unique properties of Snl1 can be exploited to narrow down the possibilities. One distinct attribute of Snl1 is the ER/nuclear membrane localization mediated through an N-terminal transmembrane domain located in the first 40 residues. To test if localization is required for overexpression toxicity, I used a truncated allele of *SNL1* missing the transmembrane domain (Δ40*snl1*), which was previously described in a paper published by my laboratory [124]. This construct and the wild-type allele were placed under control of a high expression promoter and

Figure 5.1. Cells lacking Sse1 are hypersensitive to Snl1 overexpression. Spotted serial dilutions (1:10) of BY4741 (wild-type; WT) or *sse1Δ* cells expressing an empty vector, p423*GPD* (-) or p423*GPD-SNL1*-FLAG (*SNL1*) were plated onto SC-URA plates at physiological temperature (30°C) or heat shock temperatures (37°C or 39°C).

Figure 5.1. Cells lacking Sse1 are hypersensitive to Snl1 overexpression

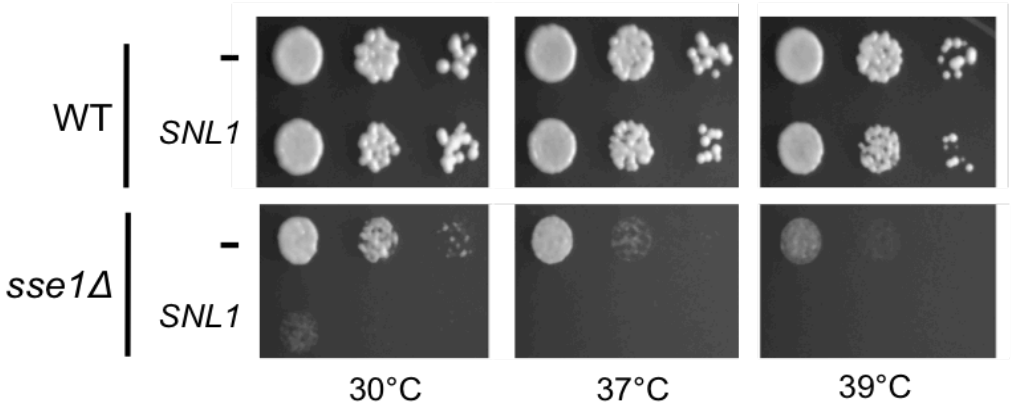
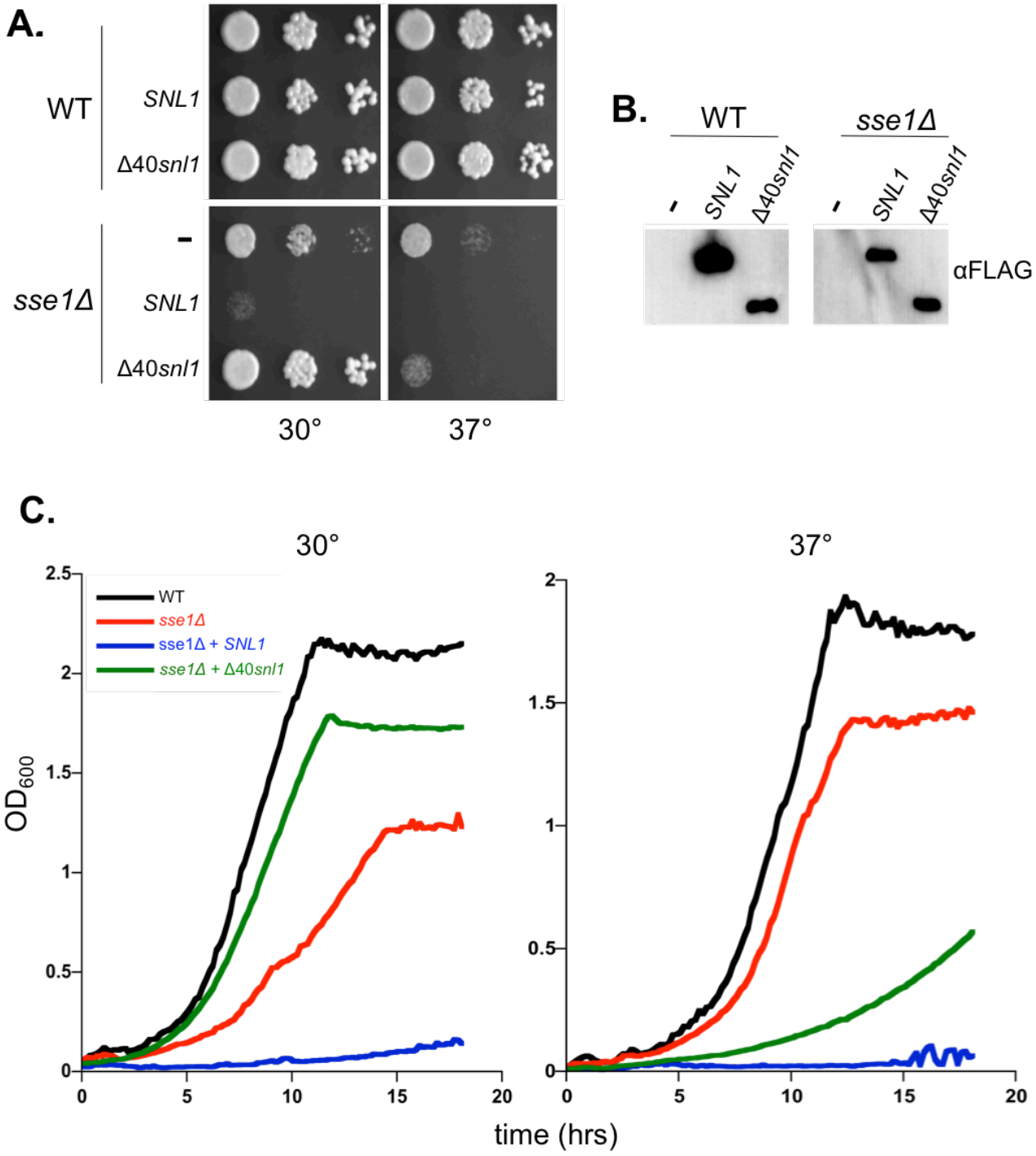


Figure 5.2. Soluble truncation mutants of Snl1 lose toxicity and partially suppress the *sse1Δ* growth defect. A. Spotted serial dilutions (1:10) of BY4741 (WT) or an *sse1Δ* strain expressing empty vector, p413*TEF* (-), p413*TEF*-*SNL1*-FLAG (SNL1), or p413*TEF*-Δ40*snl1*-FLAG (Δ40*snl1*; mutant is soluble due to a 40 aa N-terminal truncation) were plated at physiological (30°C) or heat shock (37°C). **B.** Western blot analysis of Snl1 expression in each strain background. **C.** Liquid growth curves of each strain at 30°C or 37°C.

Figure 5.2. Soluble truncation mutants of Snl1 lose toxicity and partially suppress the *sse1Δ* growth defect



transformed into wild-type or *sse1Δ* cells (Fig. 5.2 A,C). The growth defect observed upon overexpression of wild-type Snl1 was not seen in cells overexpressing the localization mutant. In addition, this mutant suppressed the *sse1Δ* slow growth phenotype at 30°C, but increased temperature sensitivity. Panel B shows that the constructs are being expressed in both strains, so the differences in toxicity are not likely due to differences in protein levels (Fig 5.2 B). This phenotype is also recapitulated in the liquid growth curves, which accentuates the toxicity of the Δ40snl1 protein in the *SSE1* null strain (Fig 5.2 C). These data suggest that Snl1 has to be localized to the ER membrane to cause toxicity when overexpressed in an *sse1Δ* strain. Therefore, it is likely that the process in which these two NEFs work together occurs at the cytoplasmic surface at the ER membrane. These findings also provide insight into Sse1 functions; the soluble version of the Snl1 protein rescues the *sse1Δ* strain at 30°C, but not 37°C, which implies under thermal stress conditions NEF-independent functions of Sse1 are likely important to maintain proteostasis and NEF activity in the absence of this additional function is detrimental. This theory is supported by data in another published study from my laboratory, which showed that even when expressed from a stronger promoter, soluble Snl1 cannot complement loss of *SSE1* at heat shock temperature and this is accompanied by an inability to suppress the *sse1Δ* strain glucocorticoid receptor maturation defect [121].

Snl1 is the only NEF that interacts more robustly with Ssb than Ssa in vivo [124]. This could be due to its ER localization and therefore may play an important role in toxicity observed in the *SSE1* deletion strain. In order to test if Hsp70 interaction is necessary for the Snl1 overexpression phenotype, I employed a published Hsp70 binding mutant [100] that contains two alanine mutations ($snl1^{E112A,\ R141A}$; denoted *snl1***) in residues necessary for Hsp70 interaction. This mutant allele was expressed alone (*snl1***) or in concert with the 40 amino acid N-terminal truncation (Δ40*snl1* **), which produces a soluble form of the Hsp70 binding mutant (Fig. 5.3). The full length Hsp70 binding mutant was slightly little less toxic than the wild-type protein. Furthermore, the soluble Hsp70 binding mutant was a less potent suppressor of the *sse1Δ* growth defect compared to the soluble mutant, suggesting NEF activity is require

Figure 5.3. Snl1 overexpression toxicity is dependent on ER localization but independent of Hsp70 interaction. Spot dilutions (1:10) of BY4741 (wild-type; WT) or an *sse1Δ* strain expressing empty vector, p413*TEF* (-), p413*TEF*-*SNL1*-FLAG, p413*TEF*-*snl1*$^{E112A, R141A}$-FLAG (*snl1**;* mutant does not bind Hsp70), p413*TEF*-Δ40*snl1*-FLAG (Δ40*snl1*; mutant is soluble due to a 40 aa N-terminal truncation), or p413*TEF*-Δ40*snl1*$^{E112A, R141A}$-FLAG (Δ40*snl1**;* mutant is soluble due to a 40 aa truncation and it does not bind to Hsp70) were plated at 30°C or 37°C.

Figure 5.3. Snl1 overexpression toxicity is dependent on ER localization but independent of Hsp70 interaction.

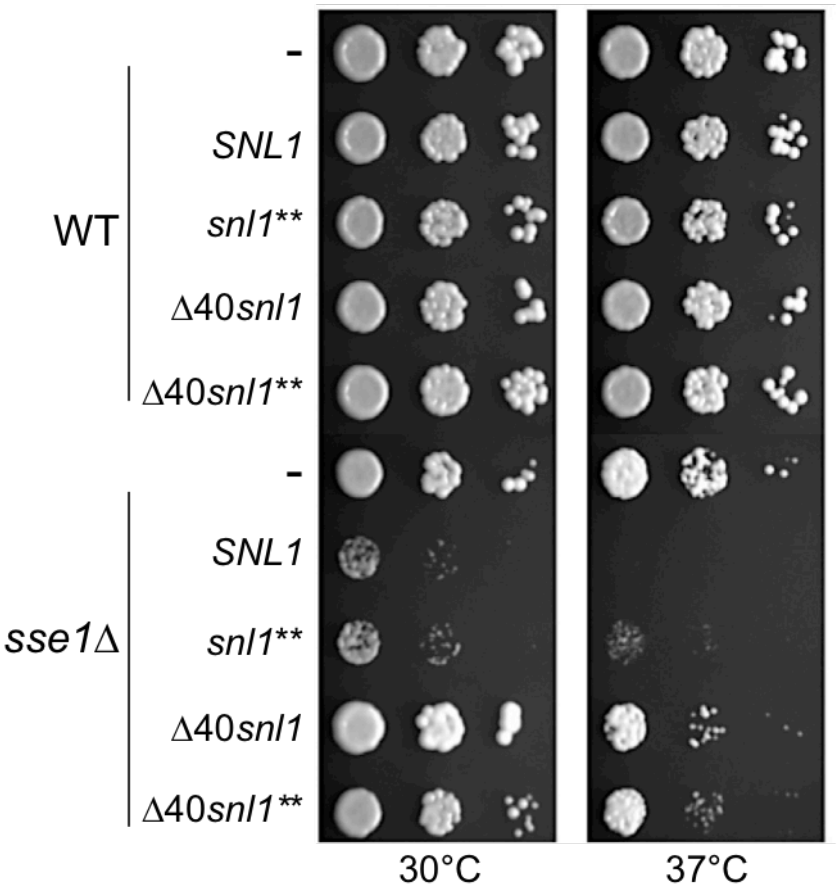
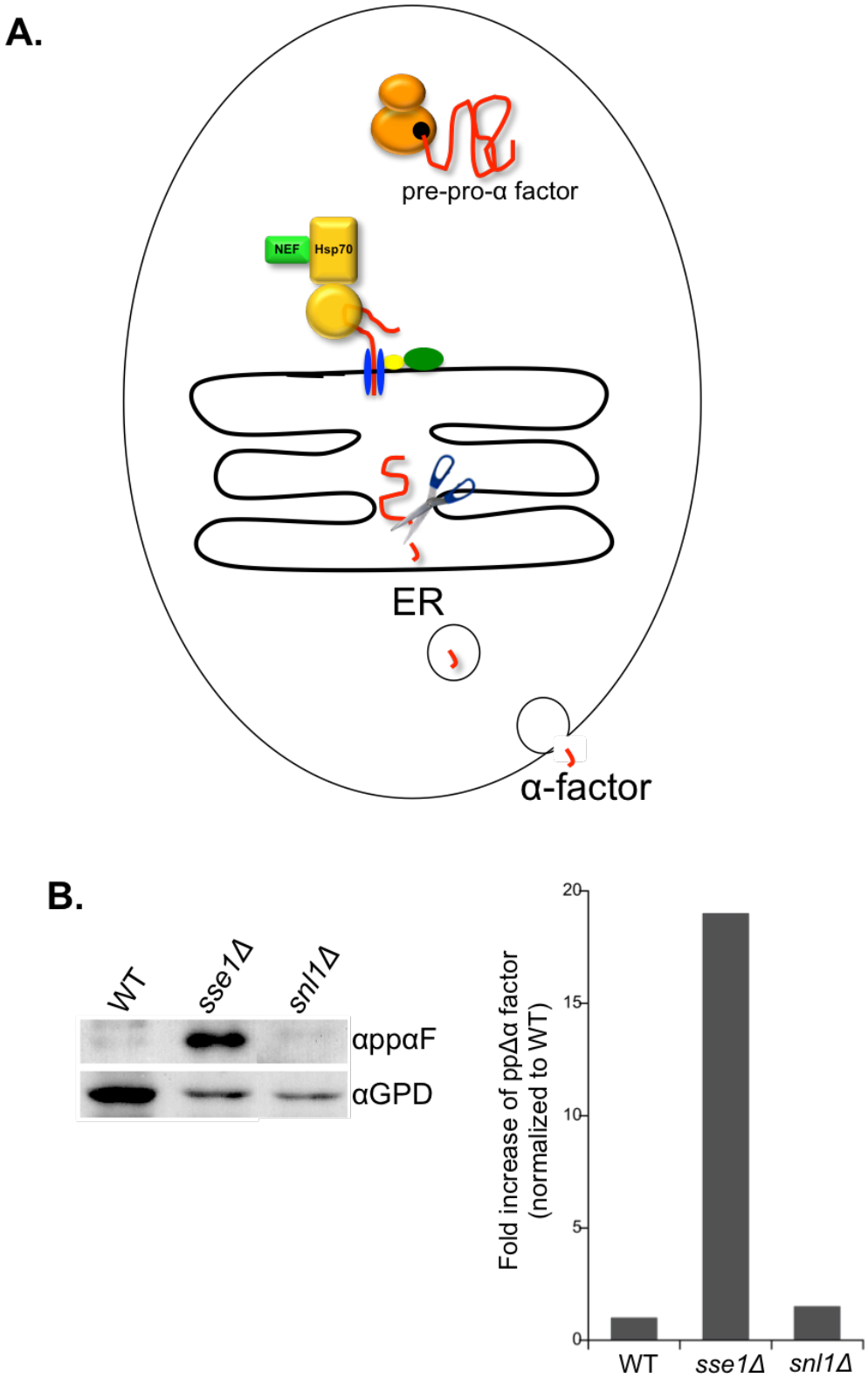


Figure 5.4. Snl1 is not required for post-translational translocation of pre-pro-α factor. **A.** Schematic showing the processing of pre-pro-α factor. The protein is fully translated by the ribosome and then translocated into the ER, assisted by Hsp70 and an NEF. It is processed in the ER prior to being secreted as the mature form of the protein, α factor. **B.** Log phase BY4742 (MATα) (WT), *sse1Δ,* or *snl1Δ* cells were lysed and proteins were visualized through Western blot analysis using α-pre-pro-α factor antibody for the unprocessed protein or using αPGK as a load control with quantitation to the right.

Figure 5.4. Snl1 is not required for post-translational translocation of pre-pro-α factor



for the rescue observed. These data suggest that the primary determinant of toxicity appears to be localization to the ER.

Localization to the ER membrane suggests that Snl1 could contribute to the transport of proteins from the cytosol to the ER lumen. The yeast pheromone, α factor, is one protein known to be post-translationally translocated for ER processing and is ultimately secreted for mating. My laboratory previously showed that translocation of the immature form of the protein was Sse1-dependent [77]. I tested the role of the Sse1 and Snl1 in post-translational translocation using Western blot analysis of the unprocessed form of α factor, pre-pro-α factor (ppαF) (Fig. 5.4). Lack of the unprocessed protein in the *SNL1* deletion strain revealed that Snl1 is not involved in translocation/processing of this secreted protein. One caveat to this experiment is that protein loads are not completely equal, but relative loads of the *sse1Δ* strain and the *snl1Δ* strain are comparable, and loss of Sse1 significantly inhibits processing of ppαF. This suggests that it is unlikely that Snl1 is located at the ER membrane for post-translational protein translocation. Another possibility discussed below is that Snl1 is located at the ER to be in close proximity to translating ribosomes.

This unknown function appears to be independent of NEF activity, the only currently identified function of Snl1, so how toxicity was mediated remained unclear. Snl1 was shown to interact with the ribosome through a five-lysine motif located in helix 1 of the functional three-helix bundle of the BAG domain [124]. It was also shown that mutation of these five lysines to alanine results in loss of interaction with the ribosome. Based on this evidence, I wanted to test if ribosomal interaction was required for Snl1 overexpression toxicity. Growth analysis using the five-lysine mutant (snl1 5K->A) revealed that ribosomal interaction is not necessary for toxicity (Fig. 5.5 A).

The unknown process that involves Sse1 and Snl1 does not require any of the currently known functions or interactions of Snl1. Although, Hsp70 interaction is not required for the Snl1-mediated toxicity, it does interact primarily with the Ssb protein. If Sse1 interaction with

Figure 5.5. Snl1 overexpression toxicity is independent of ribosomal association. Spot dilutions (1:10) of BY4741 (WT) or an *sse1Δ* strain expressing empty vector, p413*TEF* (-), p413*TEF-SNL1*-FLAG (*SNL1*), p413*TEF-snl1*$^{E112A,\ R141A}$-FLAG (*snl1***; mutant does not bind Hsp70), p413*TEF*-Δ40*snl1*-FLAG (Δ40*snl1*; mutant is soluble), or p413*TEF-snl1* (5K->A) (*snl1* 5K->A; mutant does not bind the ribosome) were plated at 30°C, 37°C, or 39°C.

Figure 5.5. Snl1 overexpression toxicity is independent of ribosomal association

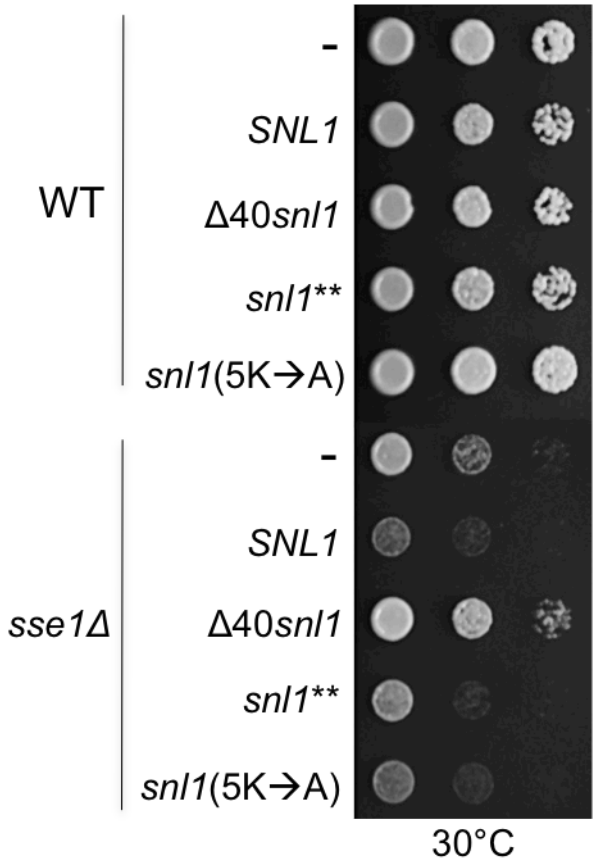
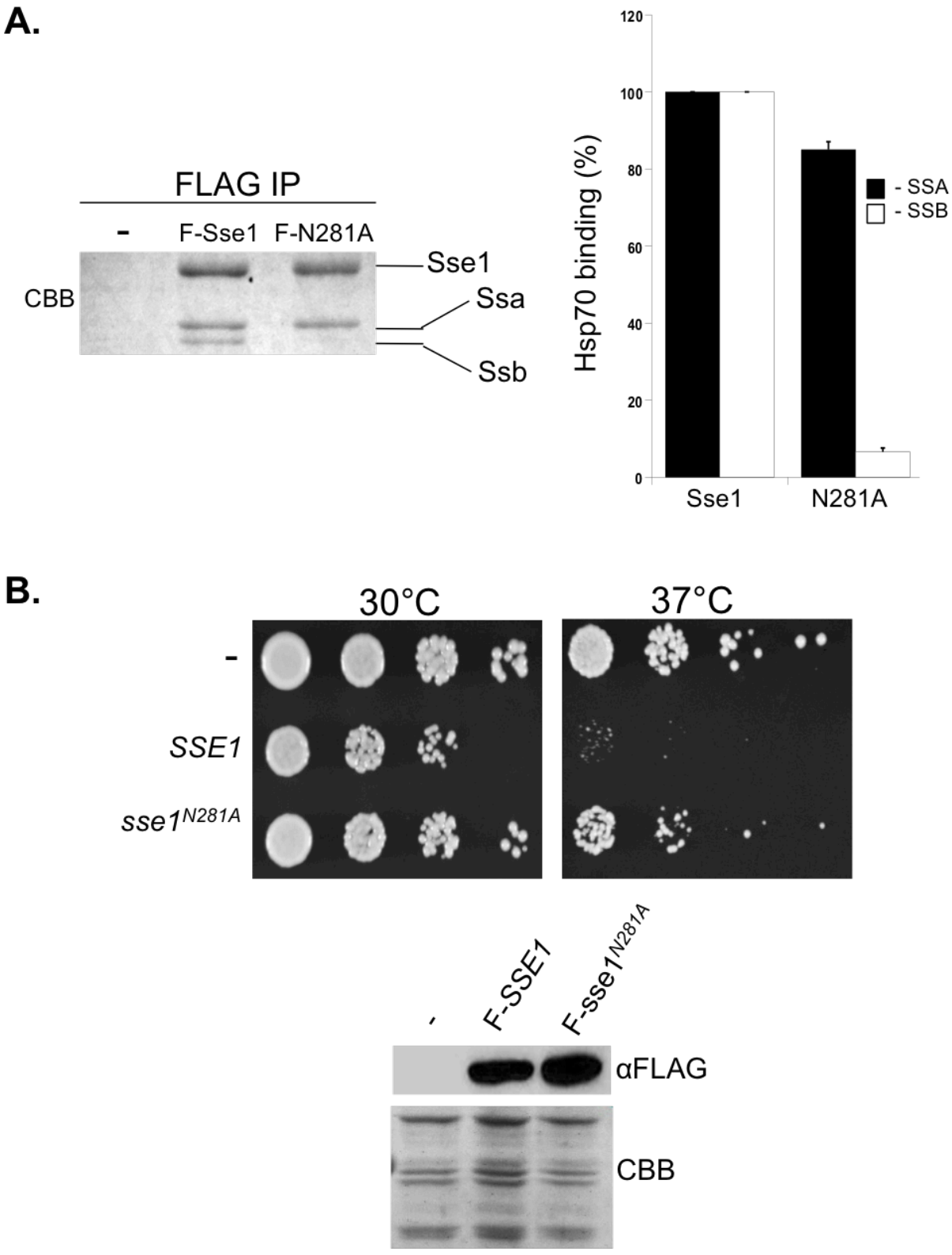


Figure 5.6. The sse1-N281A mutant that interacts with SSA only does not cause overexpression toxicity associated with wild-type Sse1. A. *SSE1* deletion cells expressing empty vector, p415*CYC* (-), p415*CYC*-FLAG-*SSE1*, or p415*CYC*-FLAG-*sse1*^{N281A} were grown to log phase and cell lysate was collected for a FLAG immunoprecipitation (IP). This was visualized by Coomassie stain (CBB) of an SDS-PAGE gel. Bands were quantified using Image J software **B.** Growth analysis of wild-type (BY4741) expressing empty vector, p423GPD (-), p423*GPD*-FLAG-*SSE1* (*SSE1*), or p423*GPD*-FLAG-*sse1*^{N281A} (*sse1-*^{N281A}). Spot dilutions (1:10) were plated at physiological temperature 30°C and heat shock temperature 37°C. Western blot analysis of protein expression in wild-type cells expressing the plasmids above with αFLAG antibody and Coomassie stain (CBB) was used to balance loads.

Figure 5.6. The sse1-N281A mutant that interacts with SSA only, does not cause overexpression toxicity associated with wild-type Sse1



Ssb is required for alleviation of this toxicity, it provides a connection between Sse1 and Snl1. In order to test this, I used a mutant version of Sse1 that has an alanine mutation in the NBD resulting in interaction with only the Ssa family of Hsp70s (*sse1*^{N281A}), which is in contrast to the wild-type protein that interacts with both Ssa and Ssb (*SSE1*) [197]. This is shown in a Coomassie stain of a FLAG immunoprecipitation separated by SDS-PAGE with quantitation in the right panel (Fig. 5.6 A). I performed growth analysis of wild-type cells overexpressing either the wild-type Sse1 or the N281A mutant at 30°C and 37°C (Fig. 5.6 B). This assay showed Sse1 overexpression toxicity, which was previously shown by a former student in my laboratory Lance Shaner, but this growth defect was alleviated by the N281A mutation and the two proteins were expressed at similar levels [190],. It appears that Sse1 interaction with Ssb is required for Sse1-mediated overexpression toxicity in wild-type cells.

To further this investigation, I examined if Sse1 requires Ssb interaction to alleviate Snl1-mediated overexpression toxicity. Snl1 overexpression in wild-type cells does not cause a slow growth defect, suggesting that when Sse1 is present it antagonizes the activity of Snl1 that leads to the growth defect. It is important that we understand how Sse1 deflects the activity of Snl1. Sse1 interacts with both Hsp70s, so I investigated if Sse1 interaction with Ssa or Ssb was specifically required. To test this, *sse1Δ* strains overexpressing Snl1 were complemented with low levels of either wild-type *SSE1* or *sse1*^{N281A} (Fig. 5.7). This assay was analyzed by visualizing growth on plates because many of the strains were very slow growing and spot analysis could give false positive growth due to suppressor mutants. This method provided a larger selection of colonies to assess. Low levels of wild-type Sse1 were only able to partially complement in the presence of high levels of Snl1, which indicates a specific ratio of Sse1 to Snl1 must be present to reestablish proteostasis. The mutant Sse1 not only lacked the ability to complement, but also appeared to cause higher levels of toxicity compared to Snl1 overexpression alone. This suggests that the Sse1 interaction with Ssb and not simply the presence of Sse1 itself is important for blocking the toxic effect of Snl1 overexpression. This also adds a new level of complexity to this mechanism. The increased toxicity due to Sse1

interaction with Ssa implies an Sse1-dependent balance maintained between Ssa and Ssb that also contributes to the Snl1-mediated process.

Since it appeared that Snl1 overexpression toxicity was alleviated through Sse1 interaction with Ssb, I wanted to determine if loss of Ssb would also increase the toxicity in cells lacking Sse1. For this experiment, I overexpressed Snl1 in wild-type, *sse1Δ*, *ssb1Δssb2Δ*, or an *sse1Δssb1Δssb2Δ* strain (Fig. 5.8). I expected to see either complete loss of toxicity due to inability of the Snl1-mediated process to be carried out or increased toxicity in wild-type cells due to inability of Sse1 to restore proteostasis through interaction with Ssb. Loss of Ssb only partially rescues the Snl1 overexpression toxicity in the absence of Sse1. Logically because Snl1 overexpression toxicity does not require Snl1 interaction with Ssb, and alleviation of growth defect is mediated through Sse1 interaction with Ssb, cells lacking Ssb should have an additive growth defect, but this is not what is observed. This indicates that the phenotypes observed are not direct consequences of the protein interactions, but rather the interactions indirectly influence the observed phenotypes.

Because this Snl1-mediated process seems to involve Ssb, I examined if the toxicity is associated with misfolding of nascent proteins. One way to test for the presence of misfolded proteins indirectly is to determine if the heat shock response (HSR) is activated. The HSR is repressed in unstressed cells, but non ideal conditions such as heat shock that results in protein denaturation activates Hsf1, the master regulator of the HSR. It was previously identified in my laboratory that deletion of *SSE1* results in the activation of the HSR and this is thought to be due to the presence of misfolded proteins (Chapter 4, [48, 97]. To test this hypothesis, I used cells expressing a published *lacZ* reporter construct (p*SSA3HSE-lacZ*) to analyze Hsf1 derepression (Fig. 5.9) [145]. As seen previously, the loss of Sse1 activates the heat shock response approximately six-fold, but overexpression of *SNL1* increases the level of Hsf1 derepression approximately 12-fold, a net two-fold increase in the *sse1Δ* background. This suggests that the Snl1-mediated process likely affects nascent protein folding albeit indirectly because toxicity, observed exclusively in *sse1Δ* cells, is independent of Hsp70

Figure 5.7. The sse1-N281A increases Snl1 overexpression toxicity in the *sse1Δ* strain. In an *sse1Δ* strain expressing empty vectors p415*CYC* and p423*GPD* (lanes 1-3,6), *SNL1*-FLAG on p423GPD (*SNL1* o/e) (lanes 4-6), FLAG-*SSE1* (lanes 2 and 4) or the mutant FLAG-*sse1*^{N281A} (lanes 3 and 5) from p415*CYC* (*SSE1* or sse1^{N281A} respectively) were plated onto SC-LEU-HIS and allowed to incubate for three days.

Figure 5.7. The sse1-N281A increases Snl1 overexpression toxicity in the *sse1Δ* strain

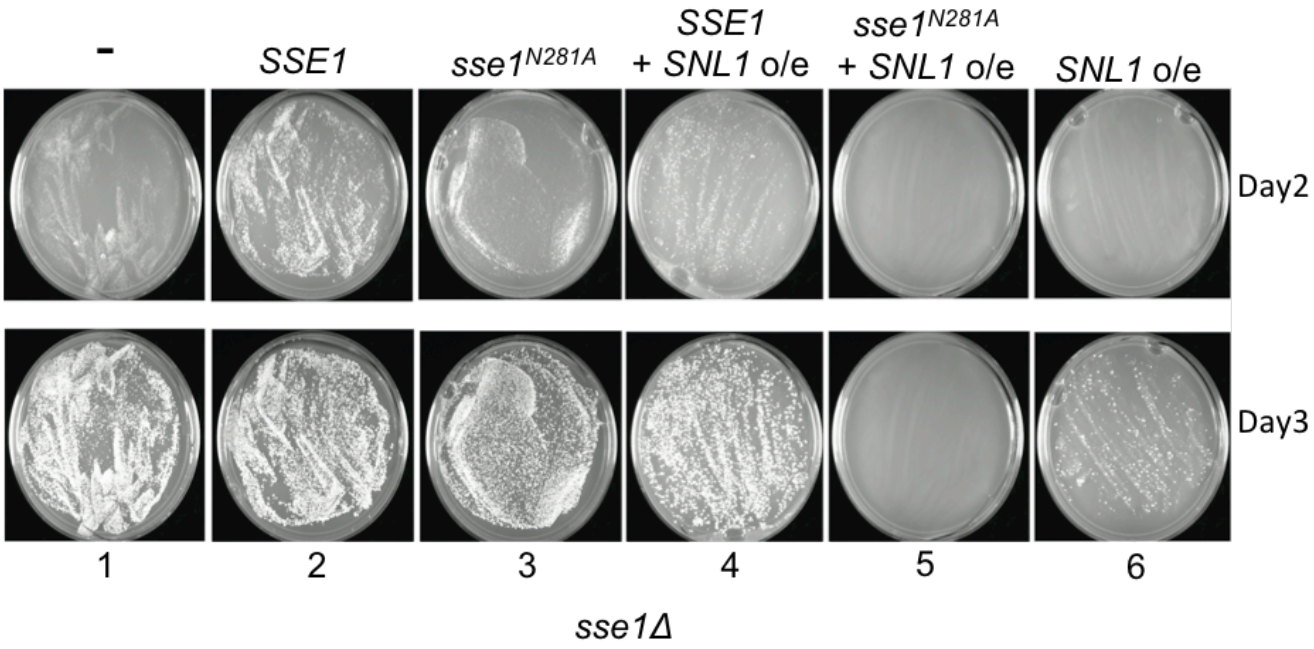


Figure 5.8. Loss of the SSB proteins alleviates Snl1 overexpression toxicity in an *sse1Δ* strain. DS10 (WT), *ssb1Δssb1Δ*, *sse1Δ*, or s*sb1Δssb2Δsse1Δ* expressing either empty vector, p416*TEF* (-), p416TEF-*SNL1*-FLAG were plated onto SC-URA plates at 30°C.

Figure 5.8. Loss of the SSB proteins alleviates Snl1 overexpression toxicity in an *sse1Δ* strain

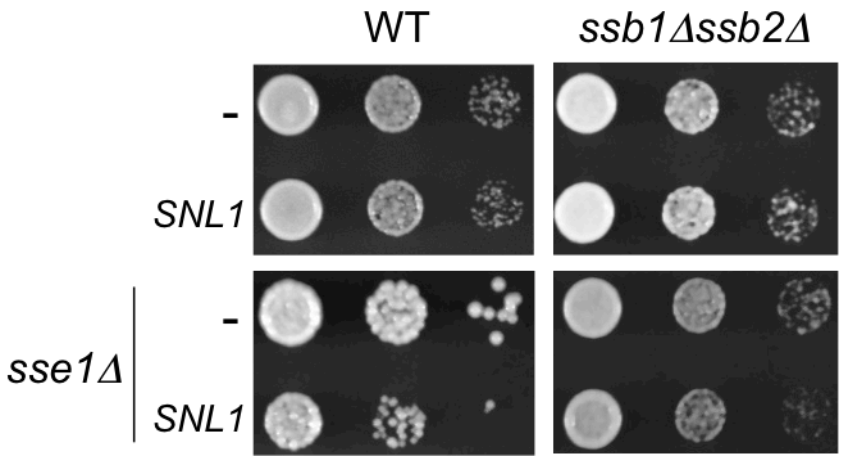
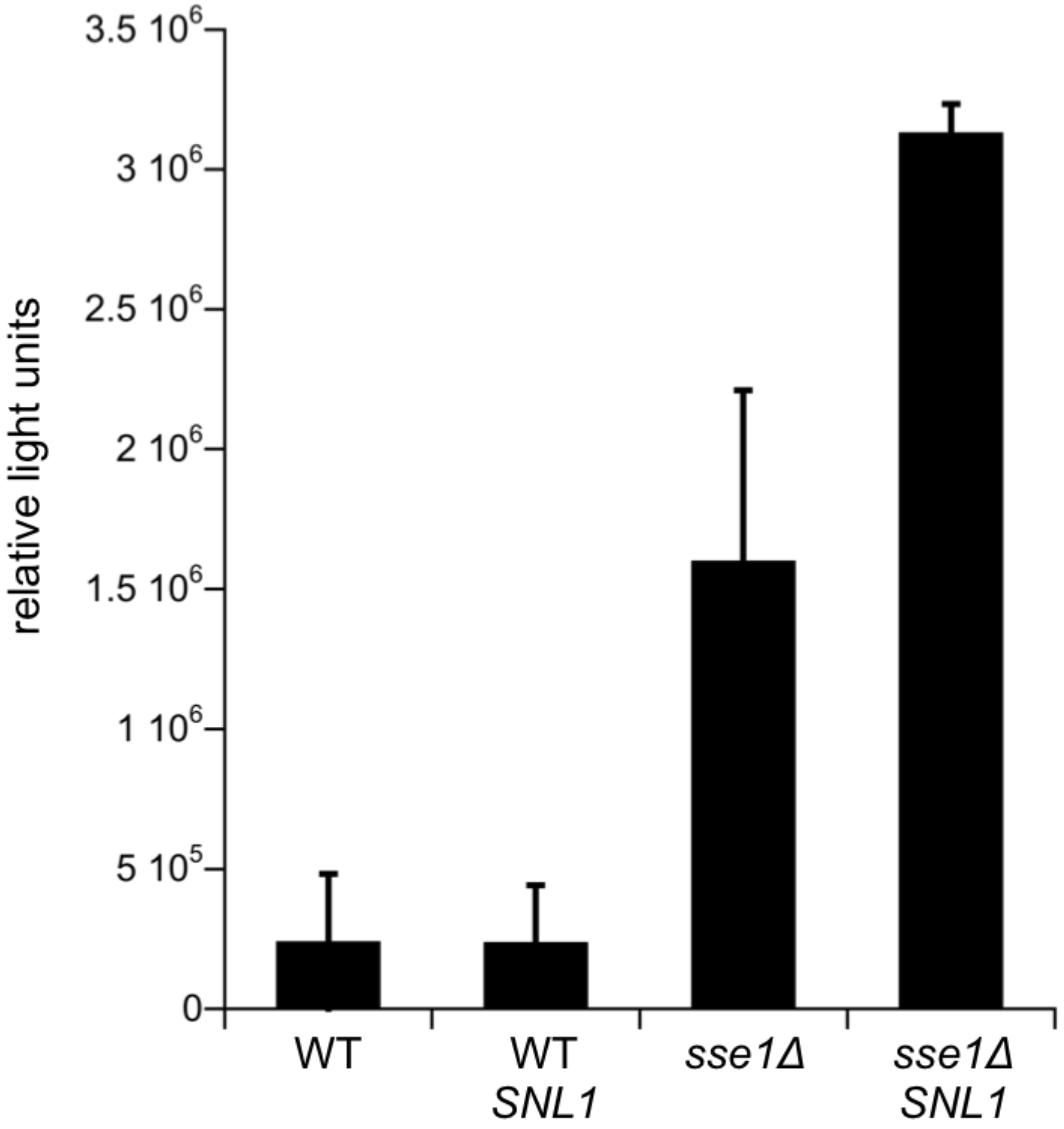


Figure 5.9. HSR activation in an *sse1Δ* strain is stimulated by overexpression of Snl1. wild-type (WT) or *sse1Δ* cells expressing p*SSA3HSE-lacZ* and empty vector, p415*TEF*, or p415*TEF-SNL1*-FLAG. Hsf1 derepression was measured in log phase cells and reported in relative light units (RLUs). Data are representative of two independent experiments.

Figure 5.9. HSR activation in an *sse1Δ* strain is stimulated by overexpression of Snl1



interaction and ribosomal interaction.

Ultimately, these results suggest that Snl1 performs a task that is not essential for viability, but is important for proteostasis in concert with Sse1. This is shown in the growth defects observed in the absence of both Sse1 and Snl1 and in the toxicity exhibited by overexpression of *SNL1* in and *SSE1* deletion strain. In addition, the Hsp70 Ssb appears to play a role in the activity of Sse1 that antagonizes the Snl1 activity. This Snl1-mediated process appears to impact protein folding as indicated by enhanced HSR activation; moreover, altering relative stoichiometry of Sse1 and Snl1 upsets proteostasis resulting in HSR upregulation.

DISCUSSION

Snl1 was originally identified as a high copy suppressor of the *nup116C* overexpression lethality and thus thought to be involved in nuclear pore biogenesis. No further evidence other than this genetic interaction has been reported for this particular function of Snl1 [179]. This protein was then identified as a member of the Bag-domain containing family that acts as an NEF for the cytosolic Hsp70 families [100]. Recently it was shown in my laboratory that Snl1 also interacts with the ribosome independently of Hsp70 [22]. No specific function dependent or independent of NEF activity has been identified for the Snl1 protein. This yeast Bag-domain protein is unique in that it contains a transmembrane domain that tethers it to the ER membrane, which is trait conserved in *Candida albicans* [124], but again no functional link has been attributed to its localization.

To address this problem, I have performed a number of genetic analyses in this chapter to determine a possible role for the Snl1 protein in proteostasis. Initially, a former student in my laboratory, Patrick Gibney and I identified a toxic phenotype exhibited when Snl1 is overexpressed in an *SSE1* deletion strain. This was interesting because in wild-type cells overexpression of Snl1 results in no phenotype. This suggested that there is a functional relationship between Sse1 and Snl1 in vivo and that the relative levels of these proteins are important for maintaining proteostasis. In support of this theory, the genetic screens of the combinatorial deletion strains in Chapter 4 revealed that loss of both Snl1 and Sse1 leads to a synthetic slow growth phenotype and cold sensitivity, whereas the *snl1Δ* strain has no phenotype. This severe slow growth phenotype of the *sse1Δsnl1Δ* strain was found to be worse than the *SSE1* deletion alone. While Sse1 is known to play various roles in protein homeostasis, it is particularly surprising that loss of the least expressed NEF would cause a more exaggerated growth defect.

I examined which activities of Snl1 are necessary to cause the toxicity seen in the *sse1Δ* strain, with the idea that this could possibly help identify the role that Snl1 plays in concert with Sse1. To determine if localization, Hsp70 interaction, or ribosomal interaction was required for

this toxicity, I performed growth analysis in strains overexpressing mutants that were either soluble (Δ40*snl1*), unable to bind Hsp70 (*snl1***), or unable to bind the ribosome (*snl1* 5K->A). The only mutation that impaired toxicity was the Δ40*snl1* mutant. This suggests that localization is the only characteristic of Snl1 necessary for overexpression toxicity. This rules out NEF activity as a possible reason for the negative effect on proteostasis. This finding is interesting because it now leads to a hypothesis in which Snl1 has an NEF-independent function, which could be one reason that it has been conserved as an ER membrane protein in fungi. Interestingly, *Schizosaccharomyces pombe*, the fission yeast, has two Bag family homologs, Bag101 is cytosolic and Bag102 is tethered to the ER membrane respectively [184]. Unlike *S. cerevisiae,* Bag101 contains an N-terminal ubiquitin-like domain, which is more like the human Bag protein and provides an evolutionary link. In addition, the lack of a ubiquitin-like domain suggests that Snl1 may have a function that is not directly associated with the human Bag proteins.

I wanted to examine the chaperone network interactions that were important for Snl1 function. Sse1 interacts with both families of Hsp70s, Ssa and Ssb. A previous student in my laboratory, Lance Shaner, identified Sse1 overexpression toxicity [190], and based on this idea I tested a mutant construct that only interacts with Ssa to determine if this protein also leads to a slow growth defect when overexpressed. It does not, which implies that the Sse1/ Ssb interaction is the primary cause of the cellular damage in the presence of high levels of protein. This is thought to be due to improper regulation of the Hsp70 cycle leading to high rates of nascent substrate release and an increase in nonfunctional proteins present in the cytosol. A study done in 2006 by Ulrich Hartl's laboratory at the Max Planck Institute, supports this claim, as overexpression of Sse1 resulted in a 50% decrease in FFL biogenesis [48], nearly as severe as the phenotype observed previously for the *sse1Δ* strain.

Once it was established that Ssb interaction was necessary for Sse1 overexpression toxicity, I tested whether this interaction was also necessary to alleviate the Snl1 overexpression growth defect. In addition, this experiment was an attempt to establish a more

complete idea of the chaperone network important for this unknown process involving Snl1. The results showed that the Sse1 Ssb interaction was not only necessary, but that the presence of the Sse1 mutant that only interacts with the Ssa family of Hsp70s increased toxicity in strains overexpressing Snl1. This suggests that the Snl1-mediated activity is balanced by Sse1 interaction with Ssb, and upregulation of the HSR upon overexpression of Snl1 further suggests that protein folding is negatively affected by the change in levels of these proteins. This could be due to loss of a regulatory step that involves Snl1 during translation, and if this step is missing, translation is increased and in the absence of Sse1, the increased folding load cannot be handled leading to loss of proteostasis.

More experiments need to be performed to determine whether translation rates are changed in response to the Snl1 levels. Localization appears to be required for the overexpression toxicity phenotype in the *sse1Δ* strain, which could also indicate importance of ER related functions. However, lack of Snl1 involvement in post-translational translocation suggests that the positioning of Snl1 could enhance ribosomal interaction, which would be important if this process affects translation. In addition, because loss of Snl1 is not detrimental, it may only be a certain subset of proteins that relies on Snl1-mediated regulation. Finally, because this process involves one chaperone and two co-chaperones, Ssb, Sse1 and Snl1, it is important to determine if there are other proteins that are part of the network affecting this unknown process.

Chapter 6: Discussion and perspectives

SUMMARY

In this thesis I have presented a comprehensive analysis of the cytosolic Hsp70 NEFs in isogenic strains and performed extensive analysis of the NEFs with unknown cellular functions to determine specificity of these important regulators. In addition, I have developed a novel method to monitor aggregation and protein activity in a coupled assay involving fluorescence microscopy and firefly luciferase (FFL) activity. The goal of this work was to develop a better understanding of the cytosolic Hsp70/NEF network.

Chapter 3 describes a technique I developed using the well-established model folding protein, FFL-GFP, to monitor protein refolding and repair by coupling an enzymatic activity assay and fluorescence microscopy. Hsp104, the yeast disaggregase, must be present in order to release proteins from aggregates post-stress treatment, so in the absence of this chaperone reactivation of denatured proteins cannot occur efficiently. This study revealed that solubilization and reactivation of proteins function together. In addition, the process of liberating proteins from this incompetent state is associated with aggregate dynamics that include fusion of small aggregates into large aggregates prior to dissolution. This observation is supported by observations in a paper published last year that showed that misfolded substrates accumulate into what they termed as Q-bodies [195]. They showed that initially small aggregates formed and then were sequestered into dynamic Q-bodies independent of cytoskeletal components [191].

In Chapter 4, I revealed that Sse1 acts as the primary NEF involved in most Hsp70-mediated processes including protein folding/biogenesis, refolding/repair, translocation and degradation. Although some of the individual findings have been shown in previous studies, they were in various strains and contributions of the other NEFs were not always included [48, 49, 92, 98, 120, 123, 179, 192]. An interesting finding in this study was that none of the individual NEFs nor the two most abundant NEFs, Sse1 and Fes1, were required for misfolded protein repair. This result was unexpected because the NEFs are central to Hsp70 function. Furthermore, for the first time Fes1 was shown to interact with only the Ssa family of Hsp70s,

and to play a primary role in regulation of the HSR. Upon deleting *FES1* the HSR was highly induced; even more than in the absence of the most abundant NEF, Sse1. Because it did not appear that Fes1 played a primary role in protein folding, which would lead to high levels of misfolded protein, it seems more plausible that Fes1 is directly involved in Hsf1 repression. Specific contributions of Snl1, and Sse2 were not identified in this chapter, but Sse2 is the only NEF highly induced upon exposure to high temperatures; this suggests it may be important in stress conditions, which could explain the reason why loss of both Hsp110s is detrimental. Deletion of *SNL1* results in a synthetic growth defect and cold sensitivity in the absence of Sse1. In concert with the previous finding in my laboratory revealing Snl1 interaction with the ribosome, this led to the theory that Snl1 plays a regulatory role in translation.

In Chapter 5, I expanded upon a previously identified finding that overexpression of Snl1 in an *sse1Δ* strain causes toxicity to investigate possible roles of Snl1 in proteostasis [193]. To do this I used a number of Snl1 mutants including an Hsp70 binding mutant, a soluble truncation mutant, and a ribosomal binding mutant to test the importance of each of these characteristics in the observed toxicity. In this study, I uncovered that ER/nuclear localization was required for toxicity and Sse1 alleviated it in an SSB-dependent manner.

DISCUSSION

The role of NEFs in regulating Hsp70 activity – All of the NEFs play relatively equal biochemical roles as Hsp70 cycle regulators. Each of the three families of NEFs, the Hsp110s, HspBP1s, and Bag1-domain containing proteins appears to have evolved separately as indicated by their distinct sequence and structures (Fig. 1.5), but they all perform the same biochemical activity suggesting convergence. This also indicates that Hsp70 regulation is very important for viability and requires some redundancy amongst its regulatory factors. The three NEF families are distinct from one another, but their structural similarities to their mammalian homologs set the premise for each of the NEFs to have unique functions as well. Keeping with this hypothesis, the Hsp40s, or J-domain proteins that regulate Hsp70 ATPase activity, also have structural differences, which allow them to provide specificity to Hsp70. There are 22 identified Hsp40s in yeast, and they include proteins that bind a wide range of substrates (Ydj1, Sis1), proteins that bind very specific substrates (Jjj1, Swa2), and proteins that do not bind substrate (Zuo1, Sec63) [38]. Based on their protein interactions and their protein networks, these co-chaperones contribute to a plethora of Hsp70-dependent and Hsp70-independent processes. This theme is also maintained by the ER-resident Kar2 NEFs, Lhs1 and Sil1, which belong to the Hsp110 and HspBP1 families, but have unique and overlapping functions.

The regulatory architecture described above allows Hsp70, one of the main housekeeping proteins in eukaryotic cells, to be highly promiscuous when the situation demands and highly specific when needed. When considering the NEFs, although there are fewer compare to the Hsp40s, they present some comparable characteristics, such as substrate binding. The Hsp110 family is very similar to the Hsp70s, in that they have nucleotide binding and substrate binding domains (NBD and SBD respectively), but the Hsp110s are thought to function independently of ATPase activity. Two recent studies claim that Hsp110 ATPase activity is required for a novel disaggregase activity in mammalian cells [87,198]. However, another study presented conflicting results [85, 86, 194]. The Hsp110 SBD is distinct from the Hsp70s in that it has an extended linker domain between the α and β subdomains [105]. This results in

the α domain wrapping around the Hsp70 and being involved in Hsp70 binding rather than or in addition to substrate binding. In Hsp70, the α domain acts as a lid over the β domain increasing affinity for the substrate (Fig1.3 B,C). Based on the structure and genetic data showing the α domain is necessary for Hsp70 binding, leaving the β domain free and exposed to interact with substrate wen Hsp110 is in complex with Hsp70 [93]. Several studies have shown that Sse1 in fact does interact with the substrate and that substrate binding activity contributes to preventing accumulation of disease-causing protein isoforms [126, 163]. Substrate interaction is one way that the NEFs can provide specificity to Hsp70 and it has been shown that the binding affinities for different substrates are distinct for Hsp70 and Hsp110 [94, 95]. In addition, Lhs1, the ER Hsp110, binds substrate in a coordinated activity with the Hsp70, Kar2 to promote efficient substrate refolding [114], which also occurs between BiP and Grp170 in humans [107, 108] suggesting this is a well conserved mechanism.

Another method for modulation of Hsp70 specificity is through network interactions and/or localization. The Bag-domain family NEF, Snl1, has an N-terminal transmembrane region that tethers it to the ER membrane. Genetic and biochemical evidence has linked Snl1 to the ribosome; immunoprecipitation experiments show interaction between ribosomal subunits and biochemical evidence shows that it is regulated concurrently with translational machinery [101, 124]. ER localization and association with translation machinery is a possible mechanism by which the NEFs can target Hsp70 for specific processes. In addition, Snl1 has been shown to primarily interact with the Ssb family of Hsp70s, while Fes1, the HspBP1 family NEF, only interacts with Ssa in vivo. Because both of these NEFs have been shown to interact with both Hsp70s in vitro, their specific interactions in vivo could be part of the mechanism by which these cytosolic NEFs specifically regulate Hsp70 [22, 48].

In addition, NEF regulation of the Hsp70s could be conditional. Sse2 is the only NEF highly induced, approximately 20-fold, by stress conditions, which would change the dynamics of the Hsp70 chaperone networks. The Hsp70s induced during stress are Ssa3/4, and although these proteins are very similar to Ssa1/2 slight alterations in the structure could change the

mechanism of function [52, 72, 195]. Ultimately there are many mechanisms by which the NEFs can regulate not only the Hsp70 cycle, but also Hsp70-mediated processes. Although there is some evidence to support these hypotheses, more work needs to be done in vivo to determine if they are valid.

Understanding differential NEF contributions to Hsp70-mediated processes – In order to determine if the three families of cytosolic NEFs differentially contribute to Hsp70 function, I tested four Hsp70 associated processes including protein biogenesis, refolding and repair, regulation, and degradation, in the presence and absence of each of the NEFs alone or in combination with each other. I initiated these studies by developing a collection of NEF deletions strains and performing growth analysis; this was based on the known growth defects previously observed upon loss of Sse1 and Fes1 and the synthetic lethality resulting from deletion of both Hsp110s. In the same strain background I wanted to determine if any of the other NEFs exhibit growth phenotypes, which would suggest roles in vital cellular processes. The only novel growth phenotype was the severe slow growth defect and temperature sensitivity observed at physiological temperature upon deletion of both *SSE1* and *SNL1* (Fig. 4.1). This synthetic growth phenotype was interesting and unexpected because no growth defect is associated with the loss of Snl1 alone. Furthermore, the cold sensitivity implies there is a problem with upregulating translation, which is necessary when cells are exposed to cold temperatures. These studies did not reveal any unique contribution of Snl1 to Hsp70-dependent processes, but I performed more genetic analysis to uncover a possible function for Snl1 in Chapter 5, which I will discuss later. Ultimately these growth assays showed that Sse1 of the Hsp110 family is the most important NEF for cellular viability. Fes1 also appears to play an important role as loss of Fes1 in the presence of Sse1 results in a mild growth defect. Whether the role of Fes1 is distinct from Sse1 could not be determined from this level of analysis because the reason for this defect could be due to an overall decrease of cytosolic NEF activity.

In order to address what could specifically contribute to the various growth phenotypes, I developed a number of assays to determine efficiency of known Hsp70-mediated processes. Folding is one of the most well known activities of the Hsp70s and many studies have been done using a well-established yeast model protein, firefly luciferase, which is a thermolabile protein that unfolds and aggregates when exposed to high temperatures. For these studies I used FFL-GFP [74, 75, 148, 196]. Data collected using both fluorescence microscopy and enzymatic assays uncovered Sse1 as the primary NEF involved in biogenesis of this model protein (Fig. 4.2). Fes1 also appears to play a minor role in maintaining the active form of protein, but not in de novo folding. This fits well with a model where Sse1, that binds both families of Hsp70s, interacts with Ssb to fold nascent chains while Fes1, which only interacts with the Ssa family, is more involved in later stages of biogenesis once the protein is completely released from the ribosome.

One interesting finding was in the refolding and repair experiment; FFL-GFP reactivation after heat denaturation did not require any of the individual NEFs or the two most abundant NEFs, Sse1 and Fes1 (Fig. 4.3). This was surprising specifically because Sse1 and Fes1, which have been shown in this and other studies to play a role in protein folding, do not appear to be particularly important for maintenance after stress. Another NEF that might be considered to be important in this pathway is Sse2 due to upregulated in stress conditions, but I detected no refolding defect in the *sse1Δ* strain. There are two likely explanations as to why I obtained these results; one is that in stress conditions the NEFs could become highly redundant, so unless all of them are absent during recovery, protein repair occurs efficiently. Snl1 is unlikely to fit into the NEF redundancy hypothesis for refolding damaged proteins since the Ssa proteins are important for this process and Snl1 primarily interacts with Ssb. A future experiment would be to test the quadruple deletion mutant that I have constructed whose growth is supported by a temperature sensitive allele of *SSE1.* The temperature sensitive allele of *SSE1* is unique in that it becomes inactive at 30°C, which is the physiological temperature for yeast. This strain will allow us to determine if loss of all the NEFs still supports

refolding of the model protein. These experiments would also have to be interpreted with caution similar to studies done with the temperature sensitive allele of *SSA1 (ssa1-45)* because deletion of both Hsp110s is lethal; therefore, phenotypes could be attributed to cell death versus loss of specific proteins. Another possibility for why I see no specific NEF necessary for protein refolding is that there are other known or unknown factors that contribute to this process during stress to regain proteostasis. For example one of the factors important for reactivation of denatured proteins is the yeast disaggregase, Hsp104. Hsp104 is upregulated in response to stress and it along with Ssa1 and Ydj1 has been shown to refold luciferase without an NEF in vitro, suggesting that the NEF activity may not be necessary in this process in the presence of a disaggregase system [85, 86]. It is known that Hsp104 is necessary for recovery of proteins after heat stress, but the open question is whether it is sufficient [146, 152, 153].

I also performed Hsp70-mediated degradation assays using a soluble mutant allele vacuolar protease carboxypeptidase Y (CPY‡) fused to GFP that is quickly degraded in the cytosol (Fig. 4.5). These data revealed that Sse1 is the only NEF necessary for efficient clearance of this mutant protein; this finding has been corroborated by evidence presented in a number of studies [120, 121]. Surprisingly, the double deletion mutant *sse1Δfes1Δ* exhibited improved degradation kinetics compared to the *sse1Δ* strain. Fluorescence microscopy showed that the CPY‡-GFP protein is highly aggregated in *sse1Δ* cells and these aggregates are maintained throughout the two hour cycloheximide chase. Fluorescence microscopy showed the *fes1Δ* and the *sse1Δfes1Δ* strains initially contain aggregates of the model protein, but the *FES1* deletion strain clears the aggregates to wild-type levels in two hours whereas the double mutant has an intermediate phenotype when compared to the *sse1Δ* and the *fes1Δ* strains. This suggests that inability to clear the mutant protein could be due to inability to liberate the protein from aggregates. A number of scenarios can be envisioned to explain this phenomenon. One possibility is that the proteins are not being ubiquitinated to target them to the proteasome for degradation. This could lead to the mutant protein collecting in cytosolic granules. There is evidence to support this showing that *SSE1* deletion cells are defective in

protein ubiquitination [120, 121], but the Gowda *et.* al. 2013 paper counters this presenting evidence that there is a high level of ubiquitinated protein in the absence of Sse1 that is increased upon exposure to stress [123]. To determine if this is the issue the mutant CPY protein would need to be immunoprecipitated in the absence of proteasome activity in the NEF deletion strains followed by Western blot analysis using antibody specific to ubiquitin. This would reveal if the mutant protein is ubiquitinated in an *SSE1* deletion strain. Alternatively, the issue could be the dissolution of the aggregates. The Hsp110s have been shown to increase reactivation of an aggregated model protein in the presence of Hsp104, but have not been shown to directly affect Hsp104 disaggregation activity [85, 86]. Because these results were presented as differences in FFL activity, disaggregation could not be deconvoluted from refolding. To resolve this issue aggregation assays using spectrophotometric techniques would have to be done in the presence and absence of the NEFs. Aside from the reason for protein accumulation in the *SSE1* deletion strain, there is still the open question as to why loss of Fes1 somehow improved the cells ability to clear CPY-GFP?

The role of Fes1 in the heat shock response – In attempts to explain why the double deletion strain, *sse1Δfes1Δ,* shows suppression of the CPY-GFP[‡] degradation defect seen in *sse1Δ* cells, I turned to previously published data from my laboratory [166] and in Claes Andreasson's laboratory at Stockholm University [123] that showed upregulation of the HSR in a *fes1Δ* strain. The heat shock response is regulated by the heat shock transcription factor, Hsf1. In yeast this protein is trimerized, DNA bound, nuclearly localized, but repressed in unstressed cells [30-32]. There are two main events associated with activation of Hsf1, which include dissociation of heat shock proteins (HSPs) and hyperphosphorylation of Hsf1. Once Hsf1 is active, it upregulates HSPs, which ultimately act in attenuation of this stress response [26]. I tested the activation of the HSR in each of the single NEF deletion strains as well as the *sse1Δfes1Δ* strain (Fig. 4.4). As seen previously in my laboratory, loss of *SSE1* upregulates the HSR two-to-three-fold [127]. Activation of the HSR in the *sse1Δ* strain is thought to be due to increased

levels of misfolded proteins; this is based on the known role of Sse1 in protein folding, but this has only been tested indirectly [48, 66, 92]. In the *fes1Δ* strain, the HSR is activated approximately 13 fold, which is significantly higher than the *sse1Δ* strain. The Gowda *et. al.* 2013 paper suggests that this is likely due to high levels of misfolded protein in the *fes1Δ* strain background. Fes1 has not previously been shown to be necessary for folding of nascent proteins, which would be the primary reason for high levels of misfolded proteins leading to upregulation of the HSR. The work performed in Chapter 4 reveals a minor role for Fes1 in FFL-GFP biogenesis, but the role of Sse1 is more significant and the upregulation of the HSR in the *sse1Δ* strain is one sixth of the *fes1Δ* strain, which makes this hypothesis unlikely. A study in 2006 tied Fes1 to refolding of denatured luciferase [49]; however, in these studies, the cells were heat shocked prior to the refolding assay, and in cells where the HSR is already highly upregulated heat shock could be damaging. This theory fits with the heat sensitivity of the *fes1Δ* strain, and would explain why a refolding defect might be observed that is not related to the role of Fes1, but rather the general health of the cells. Unfortunately, the actual contributions of Fes1 are difficult to assess because the high levels of chaperones in these cells could mask the contributions of this protein in optimal conditions. I wanted to test the functional contributions of Sse1 and Fes1 in conditions were the HSR remained repressed, to avoid any masking issues. All attempts to mutagenize, chemically alter, or build constructs to control expression of Hsf1 failed when in concert with the deletions strains. This suggests that inhibiting Hsf1 function in the absence of these NEFs is lethal, so I was not able to pursue this line of investigation.

There is another possibility as to why HSR activation occurs in the *fes1Δ* strain. If Fes1 acts as a negative regulator for Hsf1 along with Hsp70 and Hsp90, this would result in high levels of HSR activation in its absence. Further studies need to be performed to determine if Fes1 directly interacts with Hsf1, which will likely performed by Sara Peffer in my laboratory, who is currently working on chaperone-mediated regulation of Hsf1. Immunoprecipitation experiments performed in this study show that Fes1 interacts exclusively with Ssa in vivo (Fig.

4.6). In addition, I found that this is not likely due to competition with other proteins that interact with Ssb because in the absence of proteins in the RAC and NAC complexes as well as in the *sse1Δ* strain, Fes1 lacked interact with Ssb. This suggests that in vivo there is a mechanism in place that results in a specific interaction with Ssa. The most probable mechanism for this specificity is post-translational modification because in vitro Fes1 interacts equally with Ssa and Ssb, but the protein used for in vitro assays is purified from *E. coli,* which would not be modified the same as in yeast. Further studies will have to be done to test the difference in modification of these two proteins, which could include mass spectrometry or mutational analysis of predicted modification sites. Alternatively, there could be minor differences in the fold that allows it to be functional, but changes the interaction interface with Hsp70. This could be analyzed by using Fluorescence Resonance Energy Transer (FRET) of yeast purified Fes1.

One major caveat to the hypothesis that Fes1 directly regulates Hsf1 is how specificity would be imparted. Sse1 is the NEF most highly associated with Hsp70-mediated activity. In addition, Sse1 is also involved in Hsp90 regulation, and Hsp90 binds and represses Hsf1 as well. Sse1 presents as the most likely candidate for Hsf1 regulation, so why would Fes1 be more involved in this process? One possibility is that Sse1 is a very large co-chaperone much like Hsp90 and Hsp70, so the steric hindrance created by these two proteins could block Sse1 interaction leaving Fes1 as the only NEF that is small enough and can localize to the nucleus. This seems like a very simple explanation, but in some cases the simplest possibility is correct.

While the mechanism of Fes1 regulation of the HSR is currently unknown, I wanted to test if this was the reason we see suppression of the CPY*-GFP slow degradation phenotype in the *sse1Δfes1Δ* strain. To do this I heat shocked the cells in the *sse1Δ* strain to activate the HSR prior to the CPY*-GFP cycloheximide chase (Fig. 4.5). This improved the degradation kinetics suggesting that the upregulation of the HSR due to deletion of *FES1* is one possible mechanism by which the mutant CPY protein is cleared more efficiently in the *sse1Δfes1Δ* strain.

Understanding functional contribution of Snl1 to proteostasis – One of the most disconcerting findings in these studies was the lack of Snl1 process specificity. This NEF even more than the others should have a very specific activity because it is the only one that is localized to a distinct compartment, and this characteristic is unique to fungi to the best of my knowledge. This suggests that Snl1 localizes to optimize its function in yeast. Unfortunately the specific Hsp70-mediated processes that were tested in Chapter 4 did not identify any specific role of Snl1; although, one novel phenotype was revealed in this chapter. The synthetic slow growth phenotype observed at physiological temperatures in the *sse1Δsnl1Δ* strain suggests these two proteins either act redundantly in the same process or they act in two parallel processes (Fig. 4.1). This finding along with the ER localization, ribosomal interaction, and interaction with SSB in vivo led me to the hypothesis that Snl1 plays a role in modulating translation, but whether this role is direct has yet to be determined.

Previously Patrick Gibney, Ph.D. and I uncovered overexpression toxicity of Snl1 in an *sse1Δ* strain (Fig. 5.1, [193]). To further analyze this, I performed genetic analysis of Snl1 mutants produced and published by Jacob Verghese [124] in my laboratory to try to determine which aspects of Snl1 biology are important for the observed toxicity. This revealed that the primary characteristic of Snl1 related to overexpression toxicity is localization to the ER/nuclear membrane (Fig. 5.2-5.5). This could suggest that the primary role of Snl1 is associated with transport of proteins from the cytosol to the ER. Co-translational protein translocation is a tightly regulated process involving encoded signals and the signal recognition particle (SRP), and this is not known to involve Snl1. In addition, loss of proteins associated with this process results in upregulation of the stress response [197], and loss of Snl1 does not result in activation of the HSR (Fig 4.4). This does not completely eliminate the possibility of Snl1 playing a role in co-translational translocation, but more studies would have to be performed to understand the mechanism. Most likely if Snl1 is involved in this process, it is only active for specific substrates so a number of proteins would have to be tagged and folding or stability would have to be monitored in the presence and absence of Snl1.

Alternatively, post-translational translocation seems like a possible process that Snl1 might contribute to; although, it is not common in yeast, mating pheromone α-factor is a substrate requiring this pathway. Because α-factor is modified and processed in the ER, the only time the immature substrate (pre-pro-α factor) can be detected at high levels is when post-translational translocation is not properly functioning. Testing the accumulation of pre-pro-α factor revealed that Sse1, but not Snl1, was involved in translocation of this protein (Fig. 5.4).

In addition, I wanted to determine how the presence of Sse1 might prevent the Snl1 overexpression toxicity, so I tested an Sse1-Ssb binding mutant (*sse1*-N281A) in the presence and absence of Snl1 (Fig. 5.6-5.7). These results suggested that Sse1 interaction with Ssb was required to alleviate the toxicity exhibited by Snl1 overexpression, which supports a translation related role. It also appears that the presence of *sse1*-N281A in an Snl1 overexpression strain was more toxic than no Sse1 at all. It is unclear why this would occur. In addition, these studies revealed that Sse1 overexpression toxicity is mediated through the SSB interaction (Fig. 5.7-5.8). This is not surprising because nascent chains are the most sensitive to changing environments and stress, so if translation is somehow being altered it could easily lead to proteostasis issues. Because Sse1 interacts with both Ssa and Ssb it is likely that it is involved in the transition of nascent chains form co-translation folding to post-translational folding, which is more likely to be true if the substrate binding domain (SBD) of Sse1 of is active in the protein biogenesis process. To test this, Sse1 SBD mutants will need to be analyzed for efficiency of protein biogenesis.

The overexpression toxicity was mediated through Ssb, so I examined if it is maintained in cells lacking Ssb especially because Snl1/Hsp70 interaction was not required for this phenotype. The results showed partial alleviation of the Snl1-mediated overexpression toxicity in the absence of Ssb1/2. These data suggest that SSB compounds the problem associated with Snl1 overexpression toxicity in cells lacking Sse1. This makes sense if Sse1 is responsible for passing nascent chains from Ssb to Ssa because if Ssb proteins are out of the way, Ssa and another NEF can fold polypeptides being released from the ribosome; whereas

Ssb alone holds on to nascent chains for longer and folds with decreased efficiency without Sse1 [97]. This same study also shows that Ssa interaction with nascent chains increases in the absence of Ssb, which suggests that Ssa does come in and take over nascent chain folding when Ssb is not present.

It is still unclear what exactly is the physiological consequence when Snl1 is overexpressed in an *sse1Δ* strain. Obviously, this synthetic genetic situation is damaging as seen by the growth defect, so I wanted to test if the imbalance of these two NEFs was enough to activate the heat shock response more than the deletion of *SSE1* alone, and it was (Fig. 5.9). Although, it only appears to be a modest two-fold increase above the *sse1Δ* strain, overexpression of Snl1 increases stress associated with loss of Sse1.

Ultimately these data led me to two possible hypotheses: 1) Snl1 plays a general negative regulatory role in translation; or 2) It interacts with another unknown protein that regulates translation for vital proteins. Out of the two hypotheses, I think the first one is more plausible because this is an effect that would not be easily detected upon deletion of *SNL1*. If all other conditions in the cell are at optimum levels and translation rates are only slightly increased, this would not be an issue because the folding machinery and degradation machinery is intact. If this hypothesis is correct, Snl1 is primarily important for larger more unstructured proteins that need to be slowly translated so they can be folded to their native state without improper inter- or intra- molecular interactions occurring. This fits well with the data presented in 2012 by Judith Frydman's laboratory at Stanford University, that showed that the primary substrates associated with Ssb interaction were the ones with properties that slowed translation rates such as: protein length, secondary structure characteristics, hydrophobic elements, and aggregation propensity [115]. In theory, in the absence of Snl1 and Sse1, folding of large, complicated substrates is compromised and partially folded proteins are likely being locked in position with Ssb; therefore, even temporary pausing of translation due to slow release can lead to a loss of proteostasis. In addition, if Snl1 is overproduced, slow translation rates and general misfolding due to the absence of Sse1, could compound the proteostasis issue that already exists in the

sse1Δ strain. Thus proteins necessary to reset proteostasis in the *SSE1* null strain, such as Sse2, cannot be efficiently translated. This function of Snl1 would explain both observed phenotypes, but there is a caveat to this hypothesis; Snl1 localization and the low relative levels make its ability to affect global translation unlikely.

The alternative hypothesis is that Snl1 recruits another factor that affects translation of a subset of proteins. There is less evidence supporting this hypothesis, but it could explain how Snl1 seems to be impacting processes it is not known to be directly involved in such as nuclear pore biogenesis [179]. In addition, if Snl1 is not necessary for the recruitment of this unknown factor but improves efficiency of localization, it is unlikely there would be obvious phenotypes unless there are additional problems in the cell, such as upon the loss of Sse1. In a search for possible Snl1 interacting proteins that could fit this profile using The Biological General Repository for Interaction Datasets (BioGrid), I found Gis2. Gis2 is the only protein I was able to identify that also interacts with Sse1 and Ssb. In addition, this protein was found to control translation rates by interacting with hundreds of mRNA targets [198]. In addition, Gis2 was shown to localize to P-bodies and stress granules as well as interact with the translation initiation factor e1F4G [199], which suggests that this protein shuttles from granules where mRNA can remain latent until needed at the ribosome for translation. It would be interesting to see the genetic interactions between this protein and Snl1. More work needs to be done to determine the role of Snl1 in translation, but the evidence presented here provides support for this hypothesis.

The chaperone networks in process specificity – The primary cytosolic chaperones in yeast have a wide variety of substrates for which they bind to exposed hydrophobic regions. It is important to understand how these proteins are specifically required for so many different functions and how substrates are triaged in a very busy molecular environment. Chaperones also interact with each other to provide specificity; Hsp70 is part of the Hsp90 folding cycle and is thought to deliver substrate to Hsp90 [200]. In addition, Hsp70 and the yeast Hsp100,

Hsp104, interact and Hsp70 stimulates disaggregation activity of this protein [87]. In a yeast model for Huntington's disease, TRiC, the cytosolic chaperonin interacts with Hsp70 to prevent misfolding that leads to accumulation of amyloid deposits of polyglutamine proteins [201]. These are interactions of the primary chaperones, which I consider a top-level regulation, but other proteins that interact with the chaperones mentioned above also have to be considered.

One way that chaperones are involved in specific processes or bind to specific substrates is through the interaction with co-chaperones. The J-domain proteins, or Hsp40s, which activate the ATPase activity of Hsp70, are also well known for binding and delivering substrate to Hsp70. In yeast, there are 22 Hsp40s involved in a variety of processes and depending on which one is interacts with Hsp70 dictates substrate and pathway specificity. There are multiple levels at which this can happen. For example there are a number of general Hsp40s, such as Ydj1, that can interact with many substrate proteins to assist in folding. In this case, Ydj1 could also interact with an additional protein that interacts with a substrate. This paints a picture where several levels of interactions determine the process specific function at any given time. This means alone Hsp70 is one of the most promiscuous chaperones, but dependent on the complete network of interacting proteins, it can contribute to some of the most specific processes that exist in the cell.

One of the lesser-studied areas of the Hsp70 network is the NEFs. Many studies have been dedicated to Hsp110/Sse1 contributions to Hsp70 function, but the other families of NEFs have been somewhat overlooked. Hardly any work has been done on Hsp70-independent functions of these co-chaperones. It is important to understand how the NEFs act in the chaperone network because knowing their specific contributions to cellular processes is important for understanding the global picture of how proteostasis is maintained. Yeast is an important model system for studying these network interactions because homologs of nearly all of the chaperones and co-chaperones presented in this work exist in humans and models for many of the human neurodegenerative diseases have been generated in yeast [124, 202]]. Once the network interactions are understood, very specific complexes can be targeted to alter

processes associated with disease development or to reverse the negative effects of the disease.

Disease implications and relevance – There are currently no treatments that cure or reverse the effects of neurodegenerative diseases. Some of the most common neurodegenerative diseases, which have all been associated with specific proteins include: Alzheimer's disease (tau, amyloid-β), Huntington's disease (huntingtin, poly-Q), Amyotrophic Lateral Sclerosis (ALS; TDP-43, FUS), and Parkinson's disease (α-synuclein) [128]. Some of these proteins have been associated with multiple diseases and in some cases multiple proteins will aggregate leading to one disease state [203]. In addition, the prion protein, PrP, is a membrane protein that can also be cleaved and secreted for normal function, which is not completely understood [134]. PrP can assume alternative folds, on of which is known as Prp^{SC}, through an unknown post-translational process [204-206], and this conformation leads to the formation of aggregates that are found in extracellular deposits in diseases brains of people with Creutzfeldt-Jakob disease, fatal familial insomnia, Gerstmann-Straussler-Scheinker syndrome, and Kuru [207]. Much like the symptoms of the diseases themselves, the states of the proteins associated with each of these diseases all have a common theme. In each of these diseases a normal functioning protein can reach an alternative fold, which is thermodynamically favored and functions abnormally. This form of the protein can lead to aggregation, which can be either disordered or ordered such as in the formation of structured β-sheets. Ordered aggregation can develop into large amyloid structures and fibrils. Small pieces of these structures can break off to "seed" the production of more amyloid structures [208]. Ultimately, this process leads to cell damage and in humans, tissue damage and eventually death. Which state of each of these proteins is the most detrimental to cells is under constant debate and is still unclear; however, each state of the protein provides another process that can be targeted for treatment. Preventing initial misfolding and aggregation is dependent on the function of chaperones to target the alternatively folded proteins as they begin to aggregate in order to pull them apart

and refold to their proper structure. Once the aggregates are formed they can be stabilized so they do not form large disordered aggregates or amyloid structures, which can be mediated by chaperones such as the sHsps. Finally, once the amyloid structures are formed, the primary goal is to prevent seeding, which is mediated through disaggregase machines, such as Hsp104 in yeast [209-211]. Humans do not have Hsp104, but a complex of Hsp70, Hsp110, and one of the Hsp40s was shown to exhibit disaggregase activity [85, 86]. Disaggregase activity would have to be inhibited to stabilize the amyloid state of prion-like proteins to prevent seeding. The main question now is which state causes the most damage? Once that is identified as well as the chaperone networks involved in inhibiting that state, specific treatments to target chaperone protein interactions can be developed to prevent the progression of these debilitating diseases.

Future directions and new technologies targeting chaperones for disease treatment – The work presented here has uncovered some of the relative functions of cytosolic Hsp70 NEFs. I have also presented many questions that still need to be addressed. One major question is how Fes1 is contributing to the repression of Hsf1. This is important because the other chaperones involved in HSR regulation, Hsp70 and Hsp90, are general molecular chaperones, and alterations in their function will affect many different processes. If Fes1 could be targeted to selectively regulate the HSR, this could be beneficial in decreasing protein misfolding or aggregation. Cell biological analysis of wild-type Fes1 and mutants that cannot reside in the nucleus as well as biochemical analysis of Fes1 interactions need to be performed to provide enough data to develop a mechanism of how this regulation occurs.

Sse1 is the most studied of the NEFs, but the role of the SBD remains unclear. Sse1 is known to bind protein and act as a holdase for some substrates in vitro and maintain solubility of proteins in vivo, but is there a more specific function for substrate binding? Hsp110s have been shown to be important in preventing disease-causing proteins from accumulating in vivo, but it is difficult to separate these functions from that of Hsp70 [126, 163, 212]. In vitro Sse1 has been shown to specifically bind substrate with high affinity, and compared to Hsp70, it

tends to bind more aromatic residues suggesting that there are differential binding sites for Hsp70 and Hsp110 substrate interactions [94, 95]. Sse1 and the human Hsp110s were shown to stabilize firefly luciferase as a model and prevent aggregation of the unfolded protein directly in vitro [93, 194]. In addition a study in 2008 showed mutational analysis of Sse1 and suggested that Sse1 could assist Hsp70 by simultaneously associating with substrate [92]. What is left to determine is which Hsp70-mediated processes require Hsp110 substrate binding? Veronica Garcia in my laboratory is isolating Sse1 SBD mutants to characterize and test functional contributions to proteostasis.

Sse1 and Sse2 are paralogs that are 76% identical, but Sse2 is expressed at very low levels and is induced up to 20 fold in stress conditions [101]. In the studies presented in this thesis, I was unable to identify a distinct function for Sse2. A study done in 2010, uncovered data that suggested that Sse2 is more stable than Sse1 during heat stress [93]. They suggested that Sse2 may act as the primary NEF during heat stress because Sse1 begins to unfold in moderate heat whereas Sse2 unfolds at around 50°C, similar human Hsp110s, Hsp105α and Apg2, which suggests stability at all temperatures relevant to life [93]. Sse1 and Sse2 also differ in holdase activity, in this same study Sse1 held FFL in a soluble, folding ready conformation, but Sse2 bound FFL and it could not be recovered from the complex; this suggests that Sse2 is not a productive holdase. Understanding the function of Sse2 would require studying it under constant heat stress conditions to determine which Hsp70-mediated processes it contributes to. Also because the heat inducible versions of Hsp70 in the yeast cytosol are Ssa3/4, Sse2 should be studied in the context of these proteins rather than Ssa1/2 because Sse2 is more likely to work with these isoforms of Hsp70.

Snl1 is from a larger family of Bag-domain containing proteins known to be involved in a number of functions in humans including but not limited to influencing signal transduction pathways, protein folding, and protein degradation [181, 182, 184, 186, 213]. An interesting finding is that the Bag-family of proteins can act as both positive and negative regulators of Hsp70 activity [214]. Two studies have shown that Bag1 inhibits Hsp70-mediated reactivation

of FFL, but this appears to be dependent on the relative levels of Bag1 and Hsc70 [214, 215]. The C-terminus of Bag1 stimulates NEF activity, but the N-terminus modulates that activity [215]. There is not much conservation in the N-terminus of the Snl1 and Bag1 (22% for everything outside the Bag domain), and the yeast protein has a unique transmembrane domain that tethers it to the ER membrane, which is associated with the overexpression toxicity observed in an *sse1Δ* strain. This toxicity appears to be mediated through Ssb, but the role of Snl1 in proteostasis has not been determined. It is possible that it somehow acts as a negative regulator of translation, but more studies have to be done to test translation rates and other proteins that could be involved in this process.

The importance of this work is to get a better understanding of how chaperones and co-chaperones work together in networks dedicated to perform specific functions. Piecemeal-studies have been done providing bits of information for how each of the NEFs contributes to Hsp70-mediated functions, but many of these did not consider how the other NEFs compare. It is unlikely that all three families of NEFs would have been maintained throughout eukaryotic evolution if they did not have some specific purpose in the cell. It is important that I start to determine the function of a protein in the simplest way. Initially, starting with the protein itself using in vitro studies then determining if these functions are maintained in vivo, and ultimately examining how it works in the context of other proteins. Once the network has been established, the system can be manipulate in more specific ways to identify targets for treatment.

Based in this idea, small molecules are being created that target molecular chaperones. Hsp90 has classically been a target for the development of small molecule inhibitors due to its role in cancer [216]. Both inhibitors and activators of Hsp70 are now being developed, but it is important to understand the mechanism of these drugs [217]. For example a drug that directly binds Hsp70 NBD and inhibits ATPase activity is going to globally affect all functions related to Hsp70, but if only the Fes1 interaction with Hsp70 is inhibited, this would specifically upregulate the HSR according to the studies I have presented here. Now through the unpublished work of

Gestwicki and associates at University of California San Francisco, high throughput screens for small molecules that affect protein-protein interactions has led to the identification molecules that bind *E.coli* DnaK (Hsp70) and block the interface with DnaK and either DnaJ (J-domain protein) or GrpE (NEF). This sets a template for other protein interaction inhibitors. I am now screening a group of Hsp70 small molecules inhibitors that block the interaction between Hsp70 and the Bag-domain proteins. I am testing them in yeast to see if they also block Snl1 interaction with Ssa, so I can ultimately test the efficacy and specificity of these drugs. The purpose of this work, and by extension future investigations into the pharmacological manipulation of chaperone function, is to identify molecules that can very specifically alter protein interactions modulating activity of Hsp70 to improve or reverse the molecular effects of disease.

BIBLIOGRAPHY

1. Hartl, F.U., A. Bracher, and M. Hayer-Hartl, *Molecular chaperones in protein folding and proteostasis.* Nature, 2011. **475**(7356): p. 324-32.
2. Powers, E.T. and W.E. Balch, *Diversity in the origins of proteostasis networks--a driver for protein function in evolution.* Nat Rev Mol Cell Biol, 2013. **14**(4): p. 237-48.
3. Woolhead, C.A., P.J. McCormick, and A.E. Johnson, *Nascent membrane and secretory proteins differ in FRET-detected folding far inside the ribosome and in their exposure to ribosomal proteins.* Cell, 2004. **116**(5): p. 725-36.
4. Lu, J. and C. Deutsch, *Folding zones inside the ribosomal exit tunnel.* Nat Struct Mol Biol, 2005. **12**(12): p. 1123-9.
5. Beckmann, R., C.M. Spahn, N. Eswar, J. Helmers, P.A. Penczek, A. Sali, J. Frank, and G. Blobel, *Architecture of the protein-conducting channel associated with the translating 80S ribosome.* Cell, 2001. **107**(3): p. 361-72.
6. O'Brien, E.P., S.T. Hsu, J. Christodoulou, M. Vendruscolo, and C.M. Dobson, *Transient tertiary structure formation within the ribosome exit port.* J Am Chem Soc, 2010. **132**(47): p. 16928-37.
7. Ban, N., P. Nissen, J. Hansen, P.B. Moore, and T.A. Steitz, *The complete atomic structure of the large ribosomal subunit at 2.4 A resolution.* Science, 2000. **289**(5481): p. 905-20.
8. Kim, Y.E., M.S. Hipp, A. Bracher, M. Hayer-Hartl, and F.U. Hartl, *Molecular chaperone functions in protein folding and proteostasis.* Annu Rev Biochem, 2013. **82**: p. 323-55.
9. Dunker, A.K., I. Silman, V.N. Uversky, and J.L. Sussman, *Function and structure of inherently disordered proteins.* Curr Opin Struct Biol, 2008. **18**(6): p. 756-64.
10. Dobson, C.M. and R.J. Ellis, *Protein folding and misfolding inside and outside the cell.* EMBO J, 1998. **17**(18): p. 5251-4.

11. Bartlett, A.I. and S.E. Radford, *An expanding arsenal of experimental methods yields an explosion of insights into protein folding mechanisms.* Nat Struct Mol Biol, 2009. **16**(6): p. 582-8.

12. Hartl, F.U. and M. Hayer-Hartl, *Converging concepts of protein folding in vitro and in vivo.* Nat Struct Mol Biol, 2009. **16**(6): p. 574-81.

13. Gingold, H. and Y. Pilpel, *Determinants of translation efficiency and accuracy.* Mol Syst Biol, 2011. **7**: p. 481.

14. Abraham, A.K. and A. Pihl, *Effect of protein synthesis inhibitors on the fidelity of translation in eukaryotic systems.* Biochim Biophys Acta, 1983. **741**(2): p. 197-203.

15. Zaher, H.S. and R. Green, *Fidelity at the molecular level: lessons from protein synthesis.* Cell, 2009. **136**(4): p. 746-62.

16. Thompson, R.C. and A.M. Karim, *The accuracy of protein biosynthesis is limited by its speed: high fidelity selection by ribosomes of aminoacyl-tRNA ternary complexes containing GTP[gamma S].* Proc Natl Acad Sci U S A, 1982. **79**(16): p. 4922-6.

17. Fluitt, A., E. Pienaar, and H. Viljoen, *Ribosome kinetics and aa-tRNA competition determine rate and fidelity of peptide synthesis.* Comput Biol Chem, 2007. **31**(5-6): p. 335-46.

18. Albanese, V., A.Y. Yam, J. Baughman, C. Parnot, and J. Frydman, *Systems analyses reveal two chaperone networks with distinct functions in eukaryotic cells.* Cell, 2006. **124**(1): p. 75-88.

19. Albanese, V., S. Reissmann, and J. Frydman, *A ribosome-anchored chaperone network that facilitates eukaryotic ribosome biogenesis.* J Cell Biol, 2010. **189**(1): p. 69-81.

20. Morano, K.A., C.M. Grant, and W.S. Moye-Rowley, *The response to heat shock and oxidative stress in Saccharomyces cerevisiae.* Genetics, 2012. **190**(4): p. 1157-95.

21. Akerfelt, M., R.I. Morimoto, and L. Sistonen, *Heat shock factors: integrators of cell stress, development and lifespan.* Nat Rev Mol Cell Biol, 2010. **11**(8): p. 545-55.

22. Verghese, J., J. Abrams, Y. Wang, and K.A. Morano, *Biology of the heat shock response and protein chaperones: budding yeast (Saccharomyces cerevisiae) as a model system.* Microbiol Mol Biol Rev, 2012. **76**(2): p. 115-58.

23. Hubl, S.T., J.C. Owens, and H.C. Nelson, *Mutational analysis of the DNA-binding domain of yeast heat shock transcription factor.* Nat Struct Biol, 1994. **1**(9): p. 615-20.

24. Torres, F.A. and J.J. Bonner, *Genetic identification of the site of DNA contact in the yeast heat shock transcription factor.* Mol Cell Biol, 1995. **15**(9): p. 5063-70.

25. Flick, K.E., L. Gonzalez, Jr., C.J. Harrison, and H.C. Nelson, *Yeast heat shock transcription factor contains a flexible linker between the DNA-binding and trimerization domains. Implications for DNA binding by trimeric proteins.* J Biol Chem, 1994. **269**(17): p. 12475-81.

26. Voellmy, R., *On mechanisms that control heat shock transcription factor activity in metazoan cells.* Cell Stress Chaperones, 2004. **9**(2): p. 122-33.

27. Chen, Y., N.A. Barlev, O. Westergaard, and B.K. Jakobsen, *Identification of the C-terminal activator domain in yeast heat shock factor: independent control of transient and sustained transcriptional activity.* EMBO J, 1993. **12**(13): p. 5007-18.

28. Sorger, P.K., *Yeast heat shock factor contains separable transient and sustained response transcriptional activators.* Cell, 1990. **62**(4): p. 793-805.

29. Nieto-Sotelo, J., G. Wiederrecht, A. Okuda, and C.S. Parker, *The yeast heat shock transcription factor contains a transcriptional activation domain whose activity is repressed under nonshock conditions.* Cell, 1990. **62**(4): p. 807-17.

30. Sorger, P.K. and H.R. Pelham, *Purification and characterization of a heat-shock element binding protein from yeast.* EMBO J, 1987. **6**(10): p. 3035-41.

31. Jakobsen, B.K. and H.R. Pelham, *Constitutive binding of yeast heat shock factor to DNA in vivo.* Mol Cell Biol, 1988. **8**(11): p. 5040-2.

32. Hahn, J.S. and D.J. Thiele, *Activation of the Saccharomyces cerevisiae heat shock transcription factor under glucose starvation conditions by Snf1 protein kinase.* J Biol Chem, 2004. **279**(7): p. 5169-76.

33. Yao, J., K.M. Munson, W.W. Webb, and J.T. Lis, *Dynamics of heat shock factor association with native gene loci in living cells.* Nature, 2006. **442**(7106): p. 1050-3.

34. Zou, J., Y. Guo, T. Guettouche, D.F. Smith, and R. Voellmy, *Repression of heat shock transcription factor HSF1 activation by HSP90 (HSP90 complex) that forms a stress-sensitive complex with HSF1.* Cell, 1998. **94**(4): p. 471-80.

35. Taipale, M., D.F. Jarosz, and S. Lindquist, *HSP90 at the hub of protein homeostasis: emerging mechanistic insights.* Nat Rev Mol Cell Biol, 2010. **11**(7): p. 515-28.

36. Macario, A.J., L. Brocchieri, A.R. Shenoy, and E. Conway de Macario, *Evolution of a protein-folding machine: genomic and evolutionary analyses reveal three lineages of the archaeal hsp70(dnaK) gene.* J Mol Evol, 2006. **63**(1): p. 74-86.

37. Subjeck, J.R., J.J. Sciandra, and R.J. Johnson, *Heat shock proteins and thermotolerance; a comparison of induction kinetics.* Br J Radiol, 1982. **55**(656): p. 579-84.

38. Kampinga, H.H. and E.A. Craig, *The HSP70 chaperone machinery: J proteins as drivers of functional specificity.* Nat Rev Mol Cell Biol, 2010. **11**(8): p. 579-92.

39. Mayer, M.P., *Hsp70 chaperone dynamics and molecular mechanism.* Trends Biochem Sci, 2013. **38**(10): p. 507-14.

40. Swain, J.F., G. Dinler, R. Sivendran, D.L. Montgomery, M. Stotz, and L.M. Gierasch, *Hsp70 chaperone ligands control domain association via an allosteric mechanism mediated by the interdomain linker.* Mol Cell, 2007. **26**(1): p. 27-39.

41. Vogel, M., B. Bukau, and M.P. Mayer, *Allosteric regulation of Hsp70 chaperones by a proline switch.* Mol Cell, 2006. **21**(3): p. 359-67.

42. Kityk, R., J. Kopp, I. Sinning, and M.P. Mayer, *Structure and dynamics of the ATP-bound open conformation of Hsp70 chaperones.* Mol Cell, 2012. **48**(6): p. 863-74.

43. McCarty, J.S., A. Buchberger, J. Reinstein, and B. Bukau, *The role of ATP in the functional cycle of the DnaK chaperone system.* J Mol Biol, 1995. **249**(1): p. 126-37.

44. Karzai, A.W. and R. McMacken, *A bipartite signaling mechanism involved in DnaJ-mediated activation of the Escherichia coli DnaK protein.* J Biol Chem, 1996. **271**(19): p. 11236-46.

45. Misselwitz, B., O. Staeck, and T.A. Rapoport, *J proteins catalytically activate Hsp70 molecules to trap a wide range of peptide sequences.* Mol Cell, 1998. **2**(5): p. 593-603.

46. Laufen, T., M.P. Mayer, C. Beisel, D. Klostermeier, A. Mogk, J. Reinstein, and B. Bukau, *Mechanism of regulation of hsp70 chaperones by DnaJ cochaperones.* Proc Natl Acad Sci U S A, 1999. **96**(10): p. 5452-7.

47. Cyr, D.M., *Swapping nucleotides, tuning Hsp70.* Cell, 2008. **133**(6): p. 945-7.

48. Dragovic, Z., S.A. Broadley, Y. Shomura, A. Bracher, and F.U. Hartl, *Molecular chaperones of the Hsp110 family act as nucleotide exchange factors of Hsp70s.* Embo J, 2006. **25**(11): p. 2519-28.

49. Raviol, H., H. Sadlish, F. Rodriguez, M.P. Mayer, and B. Bukau, *Chaperone network in the yeast cytosol: Hsp110 is revealed as an Hsp70 nucleotide exchange factor.* EMBO J, 2006. **25**(11): p. 2510-8.

50. Shaner, L., R. Sousa, and K.A. Morano, *Characterization of hsp70 binding and nucleotide exchange by the yeast hsp110 chaperone sse1.* Biochemistry, 2006. **45**(50): p. 15075-84.

51. Sondermann, H., C. Scheufler, C. Schneider, J. Hohfeld, F.U. Hartl, and I. Moarefi, *Structure of a Bag/Hsc70 complex: convergent functional evolution of Hsp70 nucleotide exchange factors.* Science, 2001. **291**(5508): p. 1553-7.

52. Flaherty, K.M., C. DeLuca-Flaherty, and D.B. McKay, *Three-dimensional structure of the ATPase fragment of a 70K heat-shock cognate protein.* Nature, 1990. **346**(6285): p. 623-8.

53. Zhu, X., X. Zhao, W.F. Burkholder, A. Gragerov, C.M. Ogata, M.E. Gottesman, and W.A. Hendrickson, *Structural analysis of substrate binding by the molecular chaperone DnaK.* Science, 1996. **272**(5268): p. 1606-14.

54. Rudiger, S., A. Buchberger, and B. Bukau, *Interaction of Hsp70 chaperones with substrates.* Nat Struct Biol, 1997. **4**(5): p. 342-9.

55. Bertelsen, E.B., L. Chang, J.E. Gestwicki, and E.R. Zuiderweg, *Solution conformation of wild-type E. coli Hsp70 (DnaK) chaperone complexed with ADP and substrate.* Proc Natl Acad Sci U S A, 2009. **106**(21): p. 8471-6.

56. Nelson, R.J., T. Ziegelhoffer, C. Nicolet, M. Werner-Washburne, and E.A. Craig, *The translation machinery and 70 kd heat shock protein cooperate in protein synthesis.* Cell, 1992. **71**(1): p. 97-105.

57. Preissler, S. and E. Deuerling, *Ribosome-associated chaperones as key players in proteostasis.* Trends Biochem Sci, 2012. **37**(7): p. 274-83.

58. Otto, H., C. Conz, P. Maier, T. Wolfle, C.K. Suzuki, P. Jeno, P. Rucknagel, J. Stahl, and S. Rospert, *The chaperones MPP11 and Hsp70L1 form the mammalian ribosome-associated complex.* Proc Natl Acad Sci U S A, 2005. **102**(29): p. 10064-9.

59. Pfund, C., P. Huang, N. Lopez-Hoyo, and E.A. Craig, *Divergent functional properties of the ribosome-associated molecular chaperone Ssb compared with other Hsp70s.* Mol Biol Cell, 2001. **12**(12): p. 3773-82.

60. Brejning, J., N. Arneborg, and L. Jespersen, *Identification of genes and proteins induced during the lag and early exponential phase of lager brewing yeasts.* J Appl Microbiol, 2005. **98**(2): p. 261-71.

61. Gasch, A.P., P.T. Spellman, C.M. Kao, O. Carmel-Harel, M.B. Eisen, G. Storz, D. Botstein, and P.O. Brown, *Genomic expression programs in the response of yeast cells to environmental changes.* Mol Biol Cell, 2000. **11**(12): p. 4241-57.

62. Gautschi, M., H. Lilie, U. Funfschilling, A. Mun, S. Ross, T. Lithgow, P. Rucknagel, and S. Rospert, *RAC, a stable ribosome-associated complex in yeast formed by the DnaK-DnaJ homologs Ssz1p and zuotin.* Proc Natl Acad Sci U S A, 2001. **98**(7): p. 3762-7.

63. Pfund, C., N. Lopez-Hoyo, T. Ziegelhoffer, B.A. Schilke, P. Lopez-Buesa, W.A. Walter, M. Wiedmann, and E.A. Craig, *The molecular chaperone Ssb from Saccharomyces cerevisiae is a component of the ribosome-nascent chain complex.* EMBO J, 1998. **17**(14): p. 3981-9.

64. Huang, P., M. Gautschi, W. Walter, S. Rospert, and E.A. Craig, *The Hsp70 Ssz1 modulates the function of the ribosome-associated J-protein Zuo1.* Nat Struct Mol Biol, 2005. **12**(6): p. 497-504.

65. Leidig, C., G. Bange, J. Kopp, S. Amlacher, A. Aravind, S. Wickles, G. Witte, E. Hurt, R. Beckmann, and I. Sinning, *Structural characterization of a eukaryotic chaperone--the ribosome-associated complex.* Nat Struct Mol Biol, 2013. **20**(1): p. 23-8.

66. Koplin, A., S. Preissler, Y. Ilina, M. Koch, A. Scior, M. Erhardt, and E. Deuerling, *A dual function for chaperones SSB-RAC and the NAC nascent polypeptide-associated complex on ribosomes.* J Cell Biol, 2010. **189**(1): p. 57-68.

67. Wiedmann, B., H. Sakai, T.A. Davis, and M. Wiedmann, *A protein complex required for signal-sequence-specific sorting and translocation.* Nature, 1994. **370**(6489): p. 434-40.

68. Reimann, B., J. Bradsher, J. Franke, E. Hartmann, M. Wiedmann, S. Prehn, and B. Wiedmann, *Initial characterization of the nascent polypeptide-associated complex in yeast.* Yeast, 1999. **15**(5): p. 397-407.

69. del Alamo, M., D.J. Hogan, S. Pechmann, V. Albanese, P.O. Brown, and J. Frydman, *Defining the specificity of cotranslationally acting chaperones by systematic analysis of mRNAs associated with ribosome-nascent chain complexes.* PLoS Biol, 2011. **9**(7): p. e1001100.

70. Nelson, R.J., M.F. Heschl, and E.A. Craig, *Isolation and characterization of extragenic suppressors of mutations in the SSA hsp70 genes of Saccharomyces cerevisiae.* Genetics, 1992. **131**(2): p. 277-85.

71. Werner-Washburne, M., D.E. Stone, and E.A. Craig, *Complex interactions among members of an essential subfamily of hsp70 genes in Saccharomyces cerevisiae.* Molecular and cellular biology, 1987. **7**(7): p. 2568-77.

72. Sharma, D. and D.C. Masison, *Single methyl group determines prion propagation and protein degradation activities of yeast heat shock protein (Hsp)-70 chaperones Ssa1p and Ssa2p.* Proc Natl Acad Sci U S A, 2011. **108**(33): p. 13665-70.

73. Becker, J., W. Walter, W. Yan, and E.A. Craig, *Functional interaction of cytosolic hsp70 and a DnaJ-related protein, Ydj1p, in protein translocation in vivo.* Mol Cell Biol, 1996. **16**(8): p. 4378-86.

74. Kim, S., B. Schilke, E.A. Craig, and A.L. Horwich, *Folding in vivo of a newly translated yeast cytosolic enzyme is mediated by the SSA class of cytosolic yeast Hsp70 proteins.* Proc Natl Acad Sci U S A, 1998. **95**(22): p. 12860-5.

75. Unno, K., T. Kishido, M. Hosaka, and S. Okada, *Role of Hsp70 subfamily, Ssa, in protein folding in yeast cells, seen in luciferase-transformed ssa mutants.* Biological & pharmaceutical bulletin, 1997. **20**(12): p. 1240-4.

76. McClellan, A.J. and J.L. Brodsky, *Mutation of the ATP-binding pocket of SSA1 indicates that a functional interaction between Ssa1p and Ydj1p is required for post-translational translocation into the yeast endoplasmic reticulum.* Genetics, 2000. **156**(2): p. 501-12.

77. Shaner, L., H. Wegele, J. Buchner, and K.A. Morano, *The yeast Hsp110 Sse1 functionally interacts with the Hsp70 chaperones Ssa and Ssb.* J Biol Chem, 2005. **280**(50): p. 41262-9.

78. Satyanarayana, C., S. Schroder-Kohne, E.A. Craig, P.V. Schu, and M. Horst, *Cytosolic Hsp70s are involved in the transport of aminopeptidase 1 from the cytoplasm into the vacuole.* FEBS Lett, 2000. **470**(3): p. 232-8.

79. Shulga, N., P. Roberts, Z. Gu, L. Spitz, M.M. Tabb, M. Nomura, and D.S. Goldfarb, *In vivo nuclear transport kinetics in Saccharomyces cerevisiae: a role for heat shock protein 70 during targeting and translocation.* J Cell Biol, 1996. **135**(2): p. 329-39.

80. Doyle, S.M., O. Genest, and S. Wickner, *Protein rescue from aggregates by powerful molecular chaperone machines.* Nat Rev Mol Cell Biol, 2013. **14**(10): p. 617-29.

81. Bercovich, B., I. Stancovski, A. Mayer, N. Blumenfeld, A. Laszlo, A.L. Schwartz, and A. Ciechanover, *Ubiquitin-dependent degradation of certain protein substrates in vitro requires the molecular chaperone Hsc70.* J Biol Chem, 1997. **272**(14): p. 9002-10.

82. Meacham, G.C., C. Patterson, W. Zhang, J.M. Younger, and D.M. Cyr, *The Hsc70 co-chaperone CHIP targets immature CFTR for proteasomal degradation.* Nat Cell Biol, 2001. **3**(1): p. 100-5.

83. Youker, R.T., P. Walsh, T. Beilharz, T. Lithgow, and J.L. Brodsky, *Distinct roles for the Hsp40 and Hsp90 molecular chaperones during cystic fibrosis transmembrane conductance regulator degradation in yeast.* Molecular biology of the cell, 2004. **15**(11): p. 4787-97.

84. Muchowski, P.J. and J.L. Wacker, *Modulation of neurodegeneration by molecular chaperones.* Nat Rev Neurosci, 2005. **6**(1): p. 11-22.

85. Rampelt, H., J. Kirstein-Miles, N.B. Nillegoda, K. Chi, S.R. Scholz, R.I. Morimoto, and B. Bukau, *Metazoan Hsp70 machines use Hsp110 to power protein disaggregation.* EMBO J, 2012. **31**(21): p. 4221-35.

86. Shorter, J., *The mammalian disaggregase machinery: Hsp110 synergizes with Hsp70 and Hsp40 to catalyze protein disaggregation and reactivation in a cell-free system.* PLoS One, 2011. **6**(10): p. e26319.

87. Lee, J., J.H. Kim, A.B. Biter, B. Sielaff, S. Lee, and F.T. Tsai, *Heat shock protein (Hsp) 70 is an activator of the Hsp104 motor.* Proc Natl Acad Sci U S A, 2013. **110**(21): p. 8513-8.

88. Meyer, A.E., N.J. Hung, P. Yang, A.W. Johnson, and E.A. Craig, *The specialized cytosolic J-protein, Jjj1, functions in 60S ribosomal subunit biogenesis.* Proc Natl Acad Sci U S A, 2007. **104**(5): p. 1558-63.

89. Sahi, C. and E.A. Craig, *Network of general and specialty J protein chaperones of the yeast cytosol.* Proc Natl Acad Sci U S A, 2007. **104**(17): p. 7163-8.

90. Kabani, M., *Structural and functional diversity among eukaryotic Hsp70 nucleotide exchange factors.* Protein Pept Lett, 2009. **16**(6): p. 623-60.

91. Shaner, L. and K.A. Morano, *All in the family: atypical Hsp70 chaperones are conserved modulators of Hsp70 activity.* Cell Stress Chaperones, 2007. **12**(1): p. 1-8.

92. Polier, S., Z. Dragovic, F.U. Hartl, and A. Bracher, *Structural basis for the cooperation of Hsp70 and Hsp110 chaperones in protein folding.* Cell, 2008. **133**(6): p. 1068-79.

93. Polier, S., F.U. Hartl, and A. Bracher, *Interaction of the Hsp110 molecular chaperones from S. cerevisiae with substrate protein.* J Mol Biol, 2010. **401**(5): p. 696-707.

94. Goeckeler, J.L., A.P. Petruso, J. Aguirre, C.C. Clement, G. Chiosis, and J.L. Brodsky, *The yeast Hsp110, Sse1p, exhibits high-affinity peptide binding.* FEBS Lett, 2008. **582**(16): p. 2393-6.

95. Xu, X., E.B. Sarbeng, C. Vorvis, D.P. Kumar, L. Zhou, and Q. Liu, *Unique peptide substrate binding properties of 110-kDa heat-shock protein (Hsp110) determine its distinct chaperone activity.* J Biol Chem, 2012. **287**(8): p. 5661-72.

96. Schuermann, J.P., J. Jiang, J. Cuellar, O. Llorca, L. Wang, L.E. Gimenez, S. Jin, A.B. Taylor, B. Demeler, K.A. Morano, P.J. Hart, J.M. Valpuesta, E.M. Lafer, and R. Sousa, *Structure of the Hsp110:Hsc70 nucleotide exchange machine.* Mol Cell, 2008. **31**(2): p. 232-43.

97. Yam, A.Y., V. Albanese, H.T. Lin, and J. Frydman, *Hsp110 cooperates with different cytosolic HSP70 systems in a pathway for de novo folding.* J Biol Chem, 2005. **280**(50): p. 41252-61.

98. Shomura, Y., Z. Dragovic, H.C. Chang, N. Tzvetkov, J.C. Young, J.L. Brodsky, V. Guerriero, F.U. Hartl, and A. Bracher, *Regulation of Hsp70 function by HspBP1: structural analysis reveals an alternate mechanism for Hsp70 nucleotide exchange.* Mol Cell, 2005. **17**(3): p. 367-79.

99. Dragovic, Z., Y. Shomura, N. Tzvetkov, F.U. Hartl, and A. Bracher, *Fes1p acts as a nucleotide exchange factor for the ribosome-associated molecular chaperone Ssb1p.* Biol Chem, 2006. **387**(12): p. 1593-600.

100. Sondermann, H., A.K. Ho, L.L. Listenberger, K. Siegers, I. Moarefi, S.R. Wente, F.U. Hartl, and J.C. Young, *Prediction of novel Bag-1 homologs based on structure/function analysis identifies Snl1p as an Hsp70 co-chaperone in Saccharomyces cerevisiae.* J Biol Chem, 2002. **277**(36): p. 33220-7.

101. Ghaemmaghami, S., W.K. Huh, K. Bower, R.W. Howson, A. Belle, N. Dephoure, E.K. O'Shea, and J.S. Weissman, *Global analysis of protein expression in yeast.* Nature, 2003. **425**(6959): p. 737-41.

102. Mukai, H., T. Kuno, H. Tanaka, D. Hirata, T. Miyakawa, and C. Tanaka, *Isolation and characterization of SSE1 and SSE2, new members of the yeast HSP70 multigene family.* Gene, 1993. **132**(1): p. 57-66.

103. Trott, A., L. Shaner, and K.A. Morano, *The Molecular Chaperone Sse1 and the Growth Control Protein Kinase Sch9 Collaborate to Regulate Protein Kinase A Activity in Saccharomyces cerevisiae.* Genetics, 2005. **170**(3): p. 1009-21.

104. Kabani, M., C. McLellan, D.A. Raynes, V. Guerriero, and J.L. Brodsky, *HspBP1, a homologue of the yeast Fes1 and Sls1 proteins, is an Hsc70 nucleotide exchange factor.* FEBS Lett, 2002. **531**(2): p. 339-42.

105. Easton, D.P., Y. Kaneko, and J.R. Subjeck, *The hsp110 and Grp1 70 stress proteins: newly recognized relatives of the Hsp70s.* Cell Stress Chaperones, 2000. **5**(4): p. 276-90.

106. Steel, G.J., D.M. Fullerton, J.R. Tyson, and C.J. Stirling, *Coordinated activation of Hsp70 chaperones.* Science, 2004. **303**(5654): p. 98-101.

107. Lin, H.Y., P. Masso-Welch, Y.P. Di, J.W. Cai, J.W. Shen, and J.R. Subjeck, *The 170-kDa glucose-regulated stress protein is an endoplasmic reticulum protein that binds immunoglobulin.* Mol Biol Cell, 1993. **4**(11): p. 1109-19.

108. Meunier, L., Y.K. Usherwood, K.T. Chung, and L.M. Hendershot, *A subset of chaperones and folding enzymes form multiprotein complexes in endoplasmic reticulum to bind nascent proteins.* Mol Biol Cell, 2002. **13**(12): p. 4456-69.

109. Andreasson, C., J. Fiaux, H. Rampelt, M.P. Mayer, and B. Bukau, *Hsp110 is a nucleotide-activated exchange factor for Hsp70.* J Biol Chem, 2008. **283**(14): p. 8877-84.

110. de Keyzer, J., G.J. Steel, S.J. Hale, D. Humphries, and C.J. Stirling, *Nucleotide binding by Lhs1p is essential for its nucleotide exchange activity and for function in vivo.* J Biol Chem, 2009. **284**(46): p. 31564-71.

111. Howes, J., Y. Shimizu, M.J. Feige, and L.M. Hendershot, *C-terminal mutations destabilize SIL1/BAP and can cause Marinesco-Sjogren syndrome.* J Biol Chem, 2012. **287**(11): p. 8552-60.

112. Craven, R.A., M. Egerton, and C.J. Stirling, *A novel Hsp70 of the yeast ER lumen is required for the efficient translocation of a number of protein precursors.* EMBO J, 1996. **15**(11): p. 2640-50.

113. Tyson, J.R. and C.J. Stirling, *LHS1 and SIL1 provide a lumenal function that is essential for protein translocation into the endoplasmic reticulum.* EMBO J, 2000. **19**(23): p. 6440-52.

114. Saris, N., H. Holkeri, R.A. Craven, C.J. Stirling, and M. Makarow, *The Hsp70 homologue Lhs1p is involved in a novel function of the yeast endoplasmic reticulum, refolding and stabilization of heat-denatured protein aggregates.* J Cell Biol, 1997. **137**(4): p. 813-24.

115. Willmund, F., M. del Alamo, S. Pechmann, T. Chen, V. Albanese, E.B. Dammer, J. Peng, and J. Frydman, *The cotranslational function of ribosome-associated Hsp70 in eukaryotic protein homeostasis.* Cell, 2013. **152**(1-2): p. 196-209.

116. Liu, X.D., K.A. Morano, and D.J. Thiele, *The yeast Hsp110 family member, Sse1, is an Hsp90 cochaperone.* J Biol Chem, 1999. **274**(38): p. 26654-60.

117. Fan, Q., K.W. Park, Z. Du, K.A. Morano, and L. Li, *The role of Sse1 in the de novo formation and variant determination of the [PSI+] prion.* Genetics, 2007. **177**(3): p. 1583-93.

118. Kryndushkin, D. and R.B. Wickner, *Nucleotide exchange factors for Hsp70s are required for [URE3] prion propagation in Saccharomyces cerevisiae.* Mol Biol Cell, 2007. **18**(6): p. 2149-54.

119. McClellan, A.J., M.D. Scott, and J. Frydman, *Folding and quality control of the VHL tumor suppressor proceed through distinct chaperone pathways.* Cell, 2005. **121**(5): p. 739-48.

120. Heck, J.W., S.K. Cheung, and R.Y. Hampton, *Cytoplasmic protein quality control degradation mediated by parallel actions of the E3 ubiquitin ligases Ubr1 and San1.* Proc Natl Acad Sci U S A, 2010. **107**(3): p. 1106-11.

121. Mandal, A.K., P.A. Gibney, N.B. Nillegoda, M.A. Theodoraki, A.J. Caplan, and K.A. Morano, *Hsp110 chaperones control client fate determination in the hsp70-Hsp90 chaperone system.* Mol Biol Cell, 2010. **21**(9): p. 1439-48.

122. Prasad, R., S. Kawaguchi, and D.T. Ng, *A nucleus-based quality control mechanism for cytosolic proteins.* Mol Biol Cell, 2010. **21**(13): p. 2117-27.

123. Gowda, N.K., G. Kandasamy, M.S. Froehlich, R.J. Dohmen, and C. Andreasson, *Hsp70 nucleotide exchange factor Fes1 is essential for ubiquitin-dependent degradation of misfolded cytosolic proteins.* Proc Natl Acad Sci U S A, 2013. **110**(15): p. 5975-80.

124. Verghese, J. and K.A. Morano, *A lysine-rich region within fungal BAG domain-containing proteins mediates a novel association with ribosomes.* Eukaryotic cell, 2012. **11**(8): p. 1003-11.

125. Senderek, J., M. Krieger, C. Stendel, C. Bergmann, M. Moser, N. Breitbach-Faller, S. Rudnik-Schoneborn, A. Blaschek, N.I. Wolf, I. Harting, K. North, J. Smith, F. Muntoni, M. Brockington, S. Quijano-Roy, F. Renault, R. Herrmann, L.M. Hendershot, J.M. Schroder, H. Lochmuller, H. Topaloglu, T. Voit, J. Weis, F. Ebinger, and K. Zerres, *Mutations in SIL1 cause Marinesco-Sjogren syndrome, a cerebellar ataxia with cataract and myopathy.* Nat Genet, 2005. **37**(12): p. 1312-4.

126. Eroglu, B., D. Moskophidis, and N.F. Mivechi, *Loss of Hsp110 leads to age-dependent tau hyperphosphorylation and early accumulation of insoluble amyloid beta.* Mol Cell Biol, 2010. **30**(19): p. 4626-43.

127. Zhang, S., R. Binari, R. Zhou, and N. Perrimon, *A genomewide RNA interference screen for modifiers of aggregates formation by mutant Huntingtin in Drosophila.* Genetics, 2010. **184**(4): p. 1165-79.

128. Soto, C., *Unfolding the role of protein misfolding in neurodegenerative diseases.* Nature reviews. Neuroscience, 2003. **4**(1): p. 49-60.

129. Simmons, L.K., P.C. May, K.J. Tomaselli, R.E. Rydel, K.S. Fuson, E.F. Brigham, S. Wright, I. Lieberburg, G.W. Becker, D.N. Brems, and et al., *Secondary structure of amyloid beta peptide correlates with neurotoxic activity in vitro.* Mol Pharmacol, 1994. **45**(3): p. 373-9.

130. Ebrahimi-Fakhari, D., I. Cantuti-Castelvetri, Z. Fan, E. Rockenstein, E. Masliah, B.T. Hyman, P.J. McLean, and V.K. Unni, *Distinct roles in vivo for the ubiquitin-proteasome system and the autophagy-lysosomal pathway in the degradation of alpha-synuclein.* J Neurosci, 2011. **31**(41): p. 14508-20.

131. Ebrahimi-Fakhari, D., L. Wahlster, and P.J. McLean, *Molecular chaperones in Parkinson's disease--present and future.* J Parkinsons Dis, 2011. **1**(4): p. 299-320.

132. Kalia, S.K., T. Sankar, and A.M. Lozano, *Deep brain stimulation for Parkinson's disease and other movement disorders.* Curr Opin Neurol, 2013. **26**(4): p. 374-80.

133. Ha, A.D. and V.S. Fung, *Huntington's disease.* Curr Opin Neurol, 2012. **25**(4): p. 491-8.

134. Andre, R. and S.J. Tabrizi, *Misfolded PrP and a novel mechanism of proteasome inhibition.* Prion, 2012. **6**(1): p. 32-6.

135. Aulic, S., M.L. Bolognesi, and G. Legname, *Small-Molecule Theranostic Probes: A Promising Future in Neurodegenerative Diseases.* Int J Cell Biol, 2013. **2013**: p. 150952.

136. Cuanalo-Contreras, K., A. Mukherjee, and C. Soto, *Role of Protein Misfolding and Proteostasis Deficiency in Protein Misfolding Diseases and Aging.* Int J Cell Biol, 2013. **2013**: p. 638083.

137. Calamini, B. and R.I. Morimoto, *Protein homeostasis as a therapeutic target for diseases of protein conformation.* Curr Top Med Chem, 2012. **12**(22): p. 2623-40.

138. Cohen, E., *Aging, protein aggregation, chaperones, and neurodegenerative disorders: mechanisms of coupling and therapeutic opportunities.* Rambam Maimonides Med J, 2012. **3**(4): p. e0021.

139. Chang, L., E.B. Bertelsen, S. Wisen, E.M. Larsen, E.R. Zuiderweg, and J.E. Gestwicki, *High-throughput screen for small molecules that modulate the ATPase activity of the molecular chaperone DnaK.* Anal Biochem, 2008. **372**(2): p. 167-76.

140. Jinwal, U.K., Y. Miyata, J. Koren, 3rd, J.R. Jones, J.H. Trotter, L. Chang, J. O'Leary, D. Morgan, D.C. Lee, C.L. Shults, A. Rousaki, E.J. Weeber, E.R. Zuiderweg, J.E. Gestwicki, and C.A. Dickey, *Chemical manipulation of hsp70 ATPase activity regulates tau stability.* J Neurosci, 2009. **29**(39): p. 12079-88.

141. Rafii, M.S. and P.S. Aisen, *Recent developments in Alzheimer's disease therapeutics.* BMC Med, 2009. **7**: p. 7.

142. Wisen, S., E.B. Bertelsen, A.D. Thompson, S. Patury, P. Ung, L. Chang, C.G. Evans, G.M. Walter, P. Wipf, H.A. Carlson, J.L. Brodsky, E.R. Zuiderweg, and J.E. Gestwicki,

Binding of a small molecule at a protein-protein interface regulates the chaperone activity of hsp70-hsp40. ACS Chem Biol, 2010. **5**(6): p. 611-22.

143. Mumberg, D., R. Muller, and M. Funk, *Yeast vectors for the controlled expression of heterologous proteins in different genetic backgrounds.* Gene, 1995. **156**(1): p. 119-22.

144. Gietz, R.D. and R.A. Woods, *Transformation of yeast by lithium acetate/single-stranded carrier DNA/polyethylene glycol method.* Methods Enzymol, 2002. **350**: p. 87-96.

145. Morano, K.A. and D.J. Thiele, *Heat shock factor function and regulation in response to cellular stress, growth and differentiation signals.* Gene Expression, 1999. **7**: p. 271-282.

146. Abrams, J.L. and K.A. Morano, *Coupled assays for monitoring protein refolding in Saccharomyces cerevisiae.* J Vis Exp, 2013(77): p. e50432.

147. Nicolet, C.M. and E.A. Craig, *Isolation and characterization of STI1, a stress-inducible gene from Saccharomyces cerevisiae.* Mol Cell Biol, 1989. **9**(9): p. 3638-46.

148. Tkach, J.M. and J.R. Glover, *Nucleocytoplasmic trafficking of the molecular chaperone Hsp104 in unstressed and heat-shocked cells.* Traffic, 2008. **9**(1): p. 39-56.

149. Morimoto, R.I., *The heat shock response: systems biology of proteotoxic stress in aging and disease.* Cold Spring Harb Symp Quant Biol, 2011. **76**: p. 91-9.

150. Grantcharova, V., E.J. Alm, D. Baker, and A.L. Horwich, *Mechanisms of protein folding.* Curr Opin Struct Biol, 2001. **11**(1): p. 70-82.

151. Parsell, D.A., A.S. Kowal, M.A. Singer, and S. Lindquist, *Protein disaggregation mediated by heat-shock protein Hsp104.* Nature, 1994. **372**(6505): p. 475-8.

152. Glover, J.R. and S. Lindquist, *Hsp104, Hsp70, and Hsp40: a novel chaperone system that rescues previously aggregated proteins.* Cell, 1998. **94**(1): p. 73-82.

153. Lum, R., M. Niggemann, and J.R. Glover, *Peptide and protein binding in the axial channel of Hsp104. Insights into the mechanism of protein unfolding.* J Biol Chem, 2008. **283**(44): p. 30139-50.

154. Cashikar, A.G., M. Duennwald, and S.L. Lindquist, *A chaperone pathway in protein disaggregation. Hsp26 alters the nature of protein aggregates to facilitate reactivation by Hsp104.* J Biol Chem, 2005. **280**(25): p. 23869-75.

155. Nakatsu, T., S. Ichiyama, J. Hiratake, A. Saldanha, N. Kobashi, K. Sakata, and H. Kato, *Structural basis for the spectral difference in luciferase bioluminescence.* Nature, 2006. **440**(7082): p. 372-6.

156. Marques, S.M. and J.C. Esteves da Silva, *Firefly bioluminescence: a mechanistic approach of luciferase catalyzed reactions.* IUBMB Life, 2009. **61**(1): p. 6-17.

157. Conti, E., L.F. Lloyd, J. Akins, N.P. Franks, and P. Brick, *Crystallization and preliminary diffraction studies of firefly luciferase from Photinus pyralis.* Acta Crystallogr D Biol Crystallogr, 1996. **52**(Pt 4): p. 876-8.

158. Seliger, H.H., J.B. Buck, W.G. Fastie, and W.D. McElroy, *The Spectral Distribution of Firefly Light.* J Gen Physiol, 1964. **48**: p. 95-104.

159. Nathan, D.F., M.H. Vos, and S. Lindquist, *In vivo functions of the Saccharomyces cerevisiae Hsp90 chaperone.* Proc Natl Acad Sci U S A, 1997. **94**(24): p. 12949-56.

160. Penna, T.C., M. Ishii, A.P. Junior, and O. Cholewa, *Thermal stability of recombinant green fluorescent protein (GFPuv) at various pH values.* Appl Biochem Biotechnol, 2004. **113-116**: p. 469-83.

161. Abrams, J.L., J. Verghese, P.A. Gibney, and K.A. Morano, *Hierarchical Functional Specificity of Cytosolic Heat Shock Protein 70 (Hsp70) Nucleotide Exchange Factors in Yeast.* J Biol Chem, 2014.

162. Xiao, J., L.S. Kim, and T.R. Graham, *Dissection of Swa2p/auxilin domain requirements for cochaperoning Hsp70 clathrin-uncoating activity in vivo.* Mol Biol Cell, 2006. **17**(7): p. 3281-90.

163. Saxena, A., Y.K. Banasavadi-Siddegowda, Y. Fan, S. Bhattacharya, G. Roy, D.R. Giovannucci, R.A. Frizzell, and X. Wang, *Human heat shock protein 105/110 kDa (Hsp105/110) regulates biogenesis and quality control of misfolded cystic fibrosis*

transmembrane conductance regulator at multiple levels. J Biol Chem, 2012. **287**(23): p. 19158-70.

164. Shi, Y., D.D. Mosser, and R.I. Morimoto, *Molecular chaperones as HSF1-specific transcriptional repressors.* Genes Dev, 1998. **12**(5): p. 654-66.

165. Duina, A.A., H.M. Kalton, and R.F. Gaber, *Requirement for Hsp90 and a CyP-40-type cyclophilin in negative regulation of the heat shock response.* J Biol Chem, 1998. **273**(30): p. 18974-8.

166. Wang, Y., P.A. Gibney, J.D. West, and K.A. Morano, *The yeast Hsp70 Ssa1 is a sensor for activation of the heat shock response by thiol-reactive compounds.* Molecular biology of the cell, 2012. **23**(17): p. 3290-8.

167. Liu, X.D., P.C. Liu, N. Santoro, and D.J. Thiele, *Conservation of a stress response: human heat shock transcription factors functionally substitute for yeast HSF.* Embo J, 1997. **16**(21): p. 6466-77.

168. Trotter, E.W., L. Berenfeld, S.A. Krause, G.A. Petsko, and J.V. Gray, *Protein misfolding and temperature up-shift cause G1 arrest via a common mechanism dependent on heat shock factor in Saccharomycescerevisiae.* Proc Natl Acad Sci U S A, 2001. **98**(13): p. 7313-8.

169. Trotter, E.W., C.M. Kao, L. Berenfeld, D. Botstein, G.A. Petsko, and J.V. Gray, *Misfolded proteins are competent to mediate a subset of the responses to heat shock in Saccharomyces cerevisiae.* J Biol Chem, 2002. **277**(47): p. 44817-25.

170. McClellan, A.J., S. Tam, D. Kaganovich, and J. Frydman, *Protein quality control: chaperones culling corrupt conformations.* Nat Cell Biol, 2005. **7**(8): p. 736-41.

171. Park, S.H., N. Bolender, F. Eisele, Z. Kostova, J. Takeuchi, P. Coffino, and D.H. Wolf, *The cytoplasmic Hsp70 chaperone machinery subjects misfolded and endoplasmic reticulum import-incompetent proteins to degradation via the ubiquitin-proteasome system.* Mol Biol Cell, 2007. **18**(1): p. 153-65.

172. James, P., C. Pfund, and E.A. Craig, *Functional specificity among Hsp70 molecular chaperones.* Science, 1997. **275**(5298): p. 387-9.

173. Hundley, H., H. Eisenman, W. Walter, T. Evans, Y. Hotokezaka, M. Wiedmann, and E. Craig, *The in vivo function of the ribosome-associated Hsp70, Ssz1, does not require its putative peptide-binding domain.* Proc Natl Acad Sci U S A, 2002. **99**(7): p. 4203-8.

174. Gautschi, M., A. Mun, S. Ross, and S. Rospert, *A functional chaperone triad on the yeast ribosome.* Proc Natl Acad Sci U S A, 2002. **99**(7): p. 4209-14.

175. Martineau, C.N., J.M. Beckerich, and M. Kabani, *Flo11p-independent control of "mat" formation by hsp70 molecular chaperones and nucleotide exchange factors in yeast.* Genetics, 2007. **177**(3): p. 1679-89.

176. Craig, E.A. and C.A. Gross, *Is hsp70 the cellular thermometer?* Trends Biochem Sci, 1991. **16**(4): p. 135-40.

177. Morano, K.A., P.C. Liu, and D.J. Thiele, *Protein chaperones and the heat shock response in Saccharomyces cerevisiae.* Curr Opin Microbiol, 1998. **1**(2): p. 197-203.

178. Sadlish, H., H. Rampelt, J. Shorter, R.D. Wegrzyn, C. Andreasson, S. Lindquist, and B. Bukau, *Hsp110 chaperones regulate prion formation and propagation in S. cerevisiae by two discrete activities.* PLoS One, 2008. **3**(3): p. e1763.

179. Ho, A.K., G.A. Raczniak, E.B. Ives, and S.R. Wente, *The integral membrane protein snl1p is genetically linked to yeast nuclear pore complex function.* Mol Biol Cell, 1998. **9**(2): p. 355-73.

180. Harrison, C.J., M. Hayer-Hartl, M. Di Liberto, F. Hartl, and J. Kuriyan, *Crystal structure of the nucleotide exchange factor GrpE bound to the ATPase domain of the molecular chaperone DnaK.* Science, 1997. **276**(5311): p. 431-5.

181. Luders, J., J. Demand, and J. Hohfeld, *The ubiquitin-related BAG-1 provides a link between the molecular chaperones Hsc70/Hsp70 and the proteasome.* J Biol Chem, 2000. **275**(7): p. 4613-7.

182. Takayama, S., Z. Xie, and J.C. Reed, *An evolutionarily conserved family of Hsp70/Hsc70 molecular chaperone regulators.* J Biol Chem, 1999. **274**(2): p. 781-6.

183. Wang, H.G., S. Takayama, U.R. Rapp, and J.C. Reed, *Bcl-2 interacting protein, BAG-1, binds to and activates the kinase Raf-1.* Proc Natl Acad Sci U S A, 1996. **93**(14): p. 7063-8.

184. Kriegenburg, F., V. Jakopec, E.G. Poulsen, S.V. Nielsen, A. Roguev, N. Krogan, C. Gordon, U. Fleig, and R. Hartmann-Petersen, *A chaperone-assisted degradation pathway targets kinetochore proteins to ensure genome stability.* PLoS Genet, 2014. **10**(1): p. e1004140.

185. Frebel, K. and S. Wiese, *Signalling molecules essential for neuronal survival and differentiation.* Biochem Soc Trans, 2006. **34**(Pt 6): p. 1287-90.

186. Matsumura, Y., L.L. David, and W.R. Skach, *Role of Hsc70 binding cycle in CFTR folding and endoplasmic reticulum-associated degradation.* Mol Biol Cell, 2011. **22**(16): p. 2797-809.

187. Takayama, S., D.N. Bimston, S. Matsuzawa, B.C. Freeman, C. Aime-Sempe, Z. Xie, R.I. Morimoto, and J.C. Reed, *BAG-1 modulates the chaperone activity of Hsp70/Hsc70.* EMBO J, 1997. **16**(16): p. 4887-96.

188. Homma, T., H. Iwahashi, and Y. Komatsu, *Yeast gene expression during growth at low temperature.* Cryobiology, 2003. **46**(3): p. 230-7.

189. Murata, Y., T. Homma, E. Kitagawa, Y. Momose, M.S. Sato, M. Odani, H. Shimizu, M. Hasegawa-Mizusawa, R. Matsumoto, S. Mizukami, K. Fujita, M. Parveen, Y. Komatsu, and H. Iwahashi, *Genome-wide expression analysis of yeast response during exposure to 4 degrees C.* Extremophiles, 2006. **10**(2): p. 117-28.

190. Shaner, L., A. Trott, J.L. Goeckeler, J.L. Brodsky, and K.A. Morano, *The function of the yeast molecular chaperone Sse1 is mechanistically distinct from the closely related hsp70 family.* J Biol Chem, 2004. **279**(21): p. 21992-2001.

191. Escusa-Toret, S., W.I. Vonk, and J. Frydman, *Spatial sequestration of misfolded proteins by a dynamic chaperone pathway enhances cellular fitness during stress.* Nat Cell Biol, 2013. **15**(10): p. 1231-43.

192. Kumar, N., D. Gaur, D.C. Masison, and D. Sharma, *The BAG Homology Domain of Snl1 Cures Yeast Prion [URE3] Through Regulation of Hsp70 Chaperones.* G3 (Bethesda), 2014.

193. Gibney, P.A., *The Eukaryotic Cellular Stress Response: Biochemical and Genetic Analysis in Saccharomyces cerevisiae*. 2009, The Graduate School of Biomedical Sciences at Houston: Houston, TX. p. 228.

194. Mattoo, R.U., S.K. Sharma, S. Priya, A. Finka, and P. Goloubinoff, *Hsp110 is a bona fide chaperone using ATP to unfold stable misfolded polypeptides and reciprocally collaborate with Hsp70 to solubilize protein aggregates.* J Biol Chem, 2013. **288**(29): p. 21399-411.

195. Kominek, J., J. Marszalek, C. Neuveglise, E.A. Craig, and B.L. Williams, *The complex evolutionary dynamics of Hsp70s: a genomic and functional perspective.* Genome Biol Evol, 2013. **5**(12): p. 2460-77.

196. Levy, E.J., J. McCarty, B. Bukau, and W.J. Chirico, *Conserved ATPase and luciferase refolding activities between bacteria and yeast Hsp70 chaperones and modulators.* FEBS Lett, 1995. **368**(3): p. 435-40.

197. Rossi, D., F.C. Galvao, H.M. Bellato, P.E. Boldrin, B.J. Andrews, S.R. Valentini, and C.F. Zanelli, *eIF5A has a function in the cotranslational translocation of proteins into the ER.* Amino Acids, 2014. **46**(3): p. 645-53.

198. Scherrer, T., C. Femmer, R. Schiess, R. Aebersold, and A.P. Gerber, *Defining potentially conserved RNA regulons of homologous zinc-finger RNA-binding proteins.* Genome Biol, 2011. **12**(1): p. R3.

199. Rojas, M., G.W. Farr, C.F. Fernandez, L. Lauden, J.C. McCormack, and S.L. Wolin, *Yeast Gis2 and its human ortholog CNBP are novel components of stress-induced RNP granules.* PLoS One, 2012. **7**(12): p. e52824.

200. Prodromou, C., G. Siligardi, R. O'Brien, D.N. Woolfson, L. Regan, B. Panaretou, J.E. Ladbury, P.W. Piper, and L.H. Pearl, *Regulation of Hsp90 ATPase activity by tetratricopeptide repeat (TPR)- domain co-chaperones.* Embo J, 1999. **18**(3): p. 754-62.

201. Behrends, C., C.A. Langer, R. Boteva, U.M. Bottcher, M.J. Stemp, G. Schaffar, B.V. Rao, A. Giese, H. Kretzschmar, K. Siegers, and F.U. Hartl, *Chaperonin TRiC promotes the assembly of polyQ expansion proteins into nontoxic oligomers.* Mol Cell, 2006. **23**(6): p. 887-97.

202. Tenreiro, S., M.C. Munder, S. Alberti, and T.F. Outeiro, *Harnessing the power of yeast to unravel the molecular basis of neurodegeneration.* J Neurochem, 2013. **127**(4): p. 438-52.

203. Ebrahimi-Fakhari, D., L.J. Saidi, and L. Wahlster, *Molecular chaperones and protein folding as therapeutic targets in Parkinson's disease and other synucleinopathies.* Acta Neuropathol Commun, 2013. **1**(1): p. 79.

204. Prusiner, S.B., *Prions.* Proc Natl Acad Sci U S A, 1998. **95**(23): p. 13363-83.

205. Ma, J. and S. Lindquist, *Conversion of PrP to a self-perpetuating PrPSc-like conformation in the cytosol.* Science, 2002. **298**(5599): p. 1785-8.

206. Pan, K.M., M. Baldwin, J. Nguyen, M. Gasset, A. Serban, D. Groth, I. Mehlhorn, Z. Huang, R.J. Fletterick, F.E. Cohen, and et al., *Conversion of alpha-helices into beta-sheets features in the formation of the scrapie prion proteins.* Proc Natl Acad Sci U S A, 1993. **90**(23): p. 10962-6.

207. Prusiner, S.B., *Novel proteinaceous infectious particles cause scrapie.* Science, 1982. **216**(4542): p. 136-44.

208. Jucker, M. and L.C. Walker, *Self-propagation of pathogenic protein aggregates in neurodegenerative diseases.* Nature, 2013. **501**(7465): p. 45-51.

209. Winkler, J., J. Tyedmers, B. Bukau, and A. Mogk, *Chaperone networks in protein disaggregation and prion propagation.* J Struct Biol, 2012. **179**(2): p. 152-60.

210. Ohta, S., S. Kawai-Noma, A. Kitamura, C.G. Pack, M. Kinjo, and H. Taguchi, *The interaction of Hsp104 with yeast prion Sup35 as analyzed by fluorescence cross-correlation spectroscopy.* Biochem Biophys Res Commun, 2013. **442**(1-2): p. 28-32.

211. Taguchi, H. and S. Kawai-Noma, *Amyloid oligomers: diffuse oligomer-based transmission of yeast prions.* FEBS J, 2010. **277**(6): p. 1359-68.

212. Kuo, Y., S. Ren, U. Lao, B.A. Edgar, and T. Wang, *Suppression of polyglutamine protein toxicity by co-expression of a heat-shock protein 40 and a heat-shock protein 110.* Cell Death Dis, 2013. **4**: p. e833.

213. Knapp, R.T., M.J. Wong, L.K. Kollmannsberger, N.C. Gassen, A. Kretzschmar, J. Zschocke, K. Hafner, J.C. Young, and T. Rein, *Hsp70 cochaperones HspBP1 and BAG-1M differentially regulate steroid hormone receptor function.* PLoS One, 2014. **9**(1): p. e85415.

214. Nollen, E.A., J.F. Brunsting, J. Song, H.H. Kampinga, and R.I. Morimoto, *Bag1 functions in vivo as a negative regulator of Hsp70 chaperone activity.* Mol Cell Biol, 2000. **20**(3): p. 1083-8.

215. Gassler, C.S., T. Wiederkehr, D. Brehmer, B. Bukau, and M.P. Mayer, *Bag-1M accelerates nucleotide release for human Hsc70 and Hsp70 and can act concentration-dependent as positive and negative cofactor.* J Biol Chem, 2001. **276**(35): p. 32538-44.

216. Theodoraki, M.A. and A.J. Caplan, *Quality control and fate determination of Hsp90 client proteins.* Biochim Biophys Acta, 2012. **1823**(3): p. 683-8.

217. Wisen, S. and J.E. Gestwicki, *Identification of small molecules that modify the protein folding activity of heat shock protein 70.* Anal Biochem, 2008. **374**(2): p. 371-7.

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