Substrate binding by the yeast Hsp110 nucleotide exchange factor and molecular chaperone Sse1 is not obligate for its biological activities

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ABSTRACT **The highly conserved heat shock protein 70 (Hsp70) is a ubiquitous molecular chaperone essential for maintaining cellular protein homeostasis. The related protein Hsp110 (Sse1/Sse2 in *Saccharomyces cerevisiae*) functions as a nucleotide exchange factor (NEF) to regulate the protein folding activity of Hsp70. Hsp110/Sse1 also can prevent protein aggregation in vitro via its substrate-binding domain (SBD), but the cellular roles of this "holdase" activity are poorly defined. We generated and characterized an Sse1 mutant that separates, for the first time, its nucleotide exchange and substrate-binding functions. $Sse1_{sbd}$ retains nucleotide-binding and nucleotide exchange activities while exhibiting severe deficiencies in chaperone holdase activity for unfolded polypeptides. In contrast, we observed no effect of the SBD mutation in reconstituted disaggregation or refolding reactions in vitro. In vivo, $Sse1_{sbd}$ successfully heterodimerized with the yeast cytosolic Hsp70s Ssa and Ssb and promoted normal growth, with the exception of sensitivity to prolonged heat but not other proteotoxic stress. Moreover, $Sse1_{sbd}$ was fully competent to support Hsp90-dependent signaling through heterologously expressed glucocorticoid receptor and degradation of a permanently misfolded protein, two previously defined roles for Sse1. We conclude that despite conservation among eukaryotic homologues, chaperone holdase activity is not an obligate function in the Hsp110 family.**

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INTRODUCTION

Proteins must fold into a proper three-dimensional configuration, or native state, to execute their intended functions. Proteomic stressors such as exposure to harmful chemicals, oxidative stress, and aging can inhibit protein folding, disrupting protein homeostasis and resulting in cell death and human disease (Hartl *et al.*, 2011). Misfolded proteins or amyloid aggregates contribute to the development or progression of neurodegenerative disorders: Alzheimer, Huntington, and Parkinson diseases are all fundamentally diseases of protein misfolding (Soto, 2003; Broadley and Hartl, 2009). Cell survival during and after stress conditions is promoted by molecular chaperones that optimize protein folding by stabilizing folding intermediates until native conformations have been obtained. The highly conserved Hsp70 chaperone is integral to protein biogenesis, quality control, and degradation of terminally misfolded proteins (Kampinga and Craig, 2010). The Hsp70 protein-folding cycle is ATP dependent and is regulated by cochaperones such as Hsp40s and nucleotide exchange factors (NEFs) that stimulate ATP hydrolysis and exchange, respectively (Mayer and Bukau, 2005; Mayer, 2013). The budding yeast *Saccharomyces cerevisiae* expresses three classes of cytosolic NEFs, all with human orthologues: the Hsp110-type proteins Sse1/Sse2, the HSPBP1-type protein Fes1, and the BAG-1–type protein Snl1 (Bracher and Verghese, 2015). *SSE1* deletion results in slow growth and temperature sensitivity, whereas

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Abbreviations used: HSP, heat shock protein; NBD, nucleotide-binding domain; NEF, nucleotide exchange factor; SBD, substrate-binding domain.

a combined deletion of *SSE1* and *SSE2* is lethal despite the presence of Fes1 and Snl1, suggesting a potentially unique role for the Hsp110 proteins (Trott *et al.*, 2005; Raviol *et al.*, 2006). The Hsp110 proteins are highly homologous to Hsp70, composed of an amino-terminal nucleotide-binding domain (NBD) and a substrate-binding domain (SBD), which is further subdivided into a β-sandwich domain and an α-helical "lid" domain (Liu and Hendrickson, 2007; Polier *et al.*, 2008; Schuermann *et al.*, 2008). Distinct from Hsp70, Sse1/2 bind ATP, which stabilizes the NBD, but catalytic activity (ATP hydrolysis) is not required to functionally complement the null mutant in vivo or accelerate Hsp70 nucleotide exchange in vitro (Shaner *et al.*, 2005, 2006; Dragovic *et al.*, 2006; Raviol *et al.*, 2006; Andreasson *et al.*, 2008a; Nillegoda and Bukau, 2015).

Whereas the NEF function of Hsp110/Sse is well established, possible biological roles for substrate binding by the SBD remain speculative. Crystal structures of the Hsp70-Sse1 complex depict the Hsp70 SBD in close proximity to the Sse1 β-domain, suggesting possible cooperative substrate binding (Polier *et al.*, 2008; Schuermann *et al.*, 2008). The Hsp110 SBD is structurally similar, but not identical, to that of Hsp70, and it is suggested that it binds peptides much like Hsp70 through interactions with both β-sheets and the connecting loops within the β-domain (Oh *et al.*, 1997, 1999; Polier *et al.*, 2010). Hsp110s are highly efficient at blocking aggregation of misfolded substrates in vitro (defined as "holdase" activity) and Sse1 possesses a unique peptide binding preference for regions enriched in aromatic amino acids relative to the yeast Hsp70, Ssa1 (Goeckeler *et al.*, 2008; Xu *et al.*, 2012). Although contributions to substrate selection and targeting to Hsp70 by Hsp40 cochaperones are established, it is unclear whether the holdase activity of Sse1 or other Hsp110 chaperones contributes to Hsp70-dependent functions in vivo (Johnson and Craig, 2001). Deletion mutagenesis to remove the Sse1 SBD is complicated by the fact that carboxyl-terminal deletions render the protein unstable that the α-helical domain is required for heterodimerization with Hsp70 (Shaner *et al.*, 2005; Polier *et al.*, 2008; Schuermann *et al.*, 2008). Site-specific mutagenesis targeting residues in the Sse1 SBD modeled on the peptide-binding site of the bacterial Hsp70, DnaK, was likewise unsuccessful in significantly impairing holdase activity (Polier *et al.*, 2008). Yeast cells lacking Sse1 are defective in folding of newly synthesized polypeptides and degradation of some misfolded proteins (Dragovic *et al.*, 2006; Gowda *et al.*, 2013; Abrams *et al.*, 2014). However, overexpression of either Fes1 or a soluble, truncated mutant form of the normally endoplasmic reticulum-associated NEF Snl1, both of which lack demonstrated holdase activities, partially suppresses these phenotypes (Raviol *et al.*, 2006; Mandal *et al.*, 2010). In contrast, other NEFs cannot substitute for Hsp110 in protein disaggregation reactions, suggesting that Hsp110 possesses specific properties that could be linked to its unique SBD (Rampelt *et al.*, 2012; Gao *et al.*, 2015; Nillegoda and Bukau, 2015; Nillegoda *et al.*, 2015).

We generated an Sse1 variant that separates, for the first time, the nucleotide exchange and substrate-binding functions of this chaperone. Multiple targeted single-residue substitutions in the β-sandwich region of the SBD were introduced to generate a novel mutant ($Sse1_{sbd}$) that exhibits greatly reduced aggregation-preventing activity while retaining nucleotide-binding and Hsp70 nucleotide exchange potency. Strikingly, $Sse1_{sbd}$ was competent to restore growth to cells lacking *SSE1* and/or *SSE2*, promote disaggregase activity in a reconstituted in vitro system, and support Hsp70-dependent signal transduction and protein degradation while exhibiting minor defects in stress resistance and protein quality control. The data presented here suggest that the substrate-binding function of Sse1, despite being conserved among the eukaryotic Hsp110 proteins, plays a minor role in maintaining protein homeostasis in the yeast system.

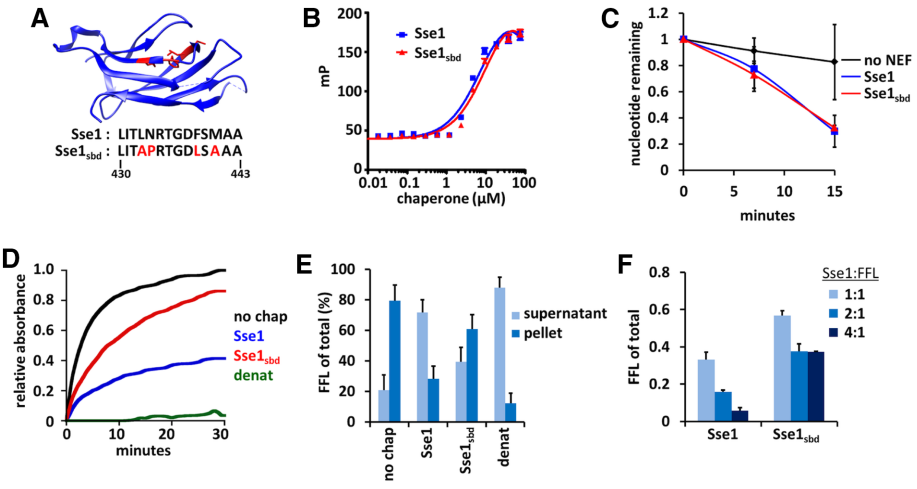

FIGURE 1: A novel SBD mutant exhibits impaired chaperone holdase activity but retains Hsp70 nucleotide exchange capacity. (A) Crystal structure of the Sse1 β-domain, with amino acids selected for mutations highlighted in red (Xu *et al.*, 2012). (B) Fluorescence anisotropy was performed with increasing concentrations of chaperone (Sse1 or $Sse1_{sbd}$) binding fluorescently labeled ATP-FAM. (C) Nucleotide exchange activity assays using HSPA8 (Hsp70) prebound to α-^{32}P-ATP in the presence or absence of Sse1. (D) Holdase experiments were conducted using chemically denatured FFL (200 nM) diluted into refolding buffer without chaperone (no chap), with Sse1 (400 nM), or with $Sse1_{sbd}$ (400 nM). FFL diluted into denaturing buffer instead of folding buffer was used as an aggregation control (denat). (E) Differential centrifugation analysis of FFL aggregation in the absence of chaperone or with Sse1 or $Sse1_{sbd}$ after a 30-min holdase assay. Samples were visualized by SDS–PAGE, followed by Coomassie stain, and scanning densitometry quantitation was performed to determine FFL aggregation under each condition. (F) Endpoint analysis of holdase experiments performed as in D, using denatured FFL with varying ratios of chaperone to substrate, quantified as fraction of total aggregation.

RESULTS

A novel SBD mutation specifically impairs Sse1 holdase activity

We generated a novel Sse1 SBD mutant based on previous structural studies (Polier *et al.*, 2008; Xu *et al.*, 2012) that indicated the region mutated could be within a putative peptide-binding site (Figure 1A). This putative substrate binding–defective mutant ($Sse1_{sbd}$) includes four specific amino acid substitutions (L433A, N434P, F439L, and M441A) within the $L_{3,4}$ region of the β-sandwich domain in Sse1. We first verified that the introduced mutations exclusively targeted substrate binding while maintaining proper nucleotide binding in the NBD. Using recombinant proteins purified from *Escherichia coli* (Supplemental Figure S1A), we measured ATP binding with fluorescently labeled nucleotide through fluorescence anisotropy. Compared with the wild-type protein, $Sse1_{sbd}$ bound FAM-ATP with approximately the same affinity (K_d = 12.1 ± 1.9 μM for $Sse1_{sbd}$ vs. 8.6 ± 1.4 μM for wild-type Sse1;

Figure 1B). It was also essential that the mutant protein could still function as a nucleotide exchange factor (NEF) for Hsp70. We measured the exchange of α-^{32}P-ATP loaded onto human Hsc70 (HSPA8) in the absence of NEF or in the presence of Sse1 or $Sse1_{sbd}$ and found no discernible difference in the accelerated exchange rates (Figure 1C). Together these results demonstrate that $Sse1_{sbd}$ retains critical nucleotide-binding and NEF features of the Hsp110 chaperone.

To assess whether substrate binding was impaired as predicted, we measured the ability of Sse1 and $Sse1_{sbd}$ to prevent the aggregation of chemically denatured firefly luciferase (FFL) using an established assay system (Garcia *et al.*, 2016). Whereas wild-type Sse1 effectively reduced FFL aggregation relative to that observed in the absence of chaperone, the $Sse1_{sbd}$ protein was significantly impaired in aggregate prevention (Figure 1D). To verify that the spectrophotometric assays reflected substrate aggregation into insoluble material, we analyzed endpoint samples by differential centrifugation followed by SDS–PAGE and densitometry quantitation (Figure 1E). Sse1 maintained 72% of FFL in a soluble state after 30 min, whereas only 39% of FFL is soluble in the presence of $Sse1_{sbd}$ as the chaperone. Similar results were obtained with citrate synthase as the unfolded substrate (Supplemental Figure S1, B–E). Increasing the ratio of Sse1 to FFL allowed for better aggregate prevention, whereas increasing the ratio of $Sse1_{sbd}$ only mildly improved protection of the denatured substrate (Figure 1F). These data indicate that the novel $Sse1_{sbd}$ mutant is defective in its ability to passively chaperone unfolded proteins, whereas NEF function and nucleotide binding remain intact. However, we note that substrate binding, although significantly reduced, is not completely abolished.

Reduction in Sse1 substrate-binding capacity does not reduce disaggregation or refolding by the Hsp70/Hsp40/Hsp110 machine

Hsp110 boosts the aggregate solubilization activity of the Hsp70-based disaggregase machine in a manner that depends on its nucleotide exchange activity (Shorter, 2011; Rampelt *et al.*, 2012; Gao *et al.*, 2015; Nillegoda *et al.*, 2015). We therefore tested whether the substrate-binding function of Sse1 is also required in this capacity. As a first step, we tested whether substrate binding by Sse1 was important for refolding of thermally denatured monomeric FFL. We heat denatured FFL in the presence of HSPA8 (hHsc70), DnaJB1 (hHsp40), and yeast Hsp26 (yHsp26) for 10 min at 42°C (Rampelt *et al.*, 2012). The samples were shifted to 30°C, and a nucleotide regeneration system was added. Refolding of FFL was measured in the presence of no NEF, Sse1, $Sse1_{sbd}$, or HSPH2 (hHsp110) as a control. It was previously established that yeast and human Hsp110s are functionally interchangeable (Rampelt *et al.*, 2012). Sse1 and $Sse1_{sbd}$ were observed to aid Hsp70/Hsp40 equally in refolding FFL (Figure 2A). Next we tested whether the substrate-binding function of Sse1 might be necessary for the more difficult task of solubilizing aggregated FFL. FFL aggregates were formed by heat denaturation (15 min, 45°C) in the presence of Hsp26, and the aggregates were mixed with a cocktail of chaperones and cochaperones containing hHsc70, hHsp40, and no NEF, Sse1, $Sse1_{sbd}$, or hHsp110. Again, substrate binding–deficient mutant $Sse1_{sbd}$ functioned with Hsp70/Hsp40 as effectively as the wild-type Sse1 or the hHsp110 control (Figure 2B). Because $Sse1_{sbd}$ is significantly reduced in substrate binding, we infer from these experiments that full Sse1 holdase activity is not obligatory for effective refolding or disaggregase activity of at least the model substrate FFL.

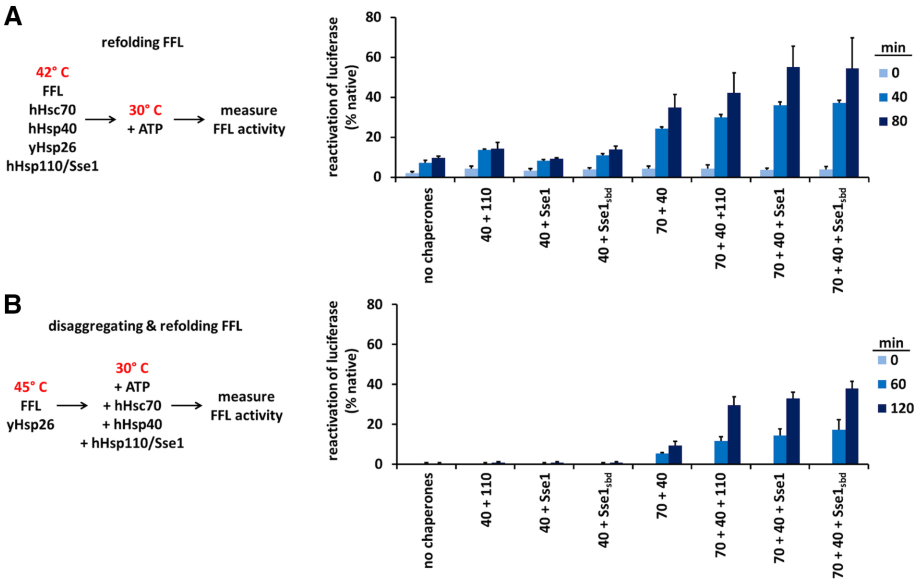

FIGURE 2: Intact Sse1 SBD function is not obligate for the Hsp70/Hsp40/Hsp110 machine to disaggregate or refold substrates. (A) FFL refolding over time in the presence of different chaperone mixtures as indicated in the schematic and described in detail in *Materials and Methods*. (B) FFL disaggregation and refolding over time in the presence of the same chaperone mixtures shown in A, as indicated in the schematic and described in detail in *Materials and Methods*. Human proteins HSPA8, DnaJB1, and HSPH2 are labeled as Hsp70, Hsp40, and Hsp110, respectively, in the experimental schematic and as 70, 40, and 110 in the plots. Yield is calculated as the percentage reactivation of luciferase relative to activity before denaturation.

Sse1 substrate binding domain function is required during heat stress

Sse1 is a critical component of the protein quality control machineries. Indeed, *sse1*Δ cells demonstrate significant growth deficiencies, including temperature sensitivity, and *sse1*Δ*sse2*Δ cells are inviable (Trott *et al.*, 2005). Among known cytosolic NEFs, Sse1 and Sse2 are unique for possessing an ability to bind nonnative substrates, raising the possibility that this activity is important for protein quality control processes in vivo. To test this hypothesis, we began by determining the expression of $Sse1_{sbd}$ to ensure that the introduced mutations did not affect its stability in vivo. At 30 and 37°C, plasmid-borne Sse1, $Sse1_{sbd}$, and a previously described NEF-defective mutant carrying the G233D mutation (here designated $Sse1_{nbd}$) were expressed at similar levels, but both slightly higher than endogenous Sse1 (Figure 3, A and B, and Supplemental Figure S2, A and B). We also wanted to ensure that $Sse1_{sbd}$ retained interaction with the yeast cytosolic Hsp70s (Ssa and Ssb) to function as a NEF in vivo. All Sse1 proteins were expressed with a FLAG tag fused to the N-terminus, and coimmunoprecipitations were performed (Shaner *et al.*, 2005). $Sse1_{sbd}$ was found to associate with the cytosolic Hsp70s, Ssa, and Ssb at both

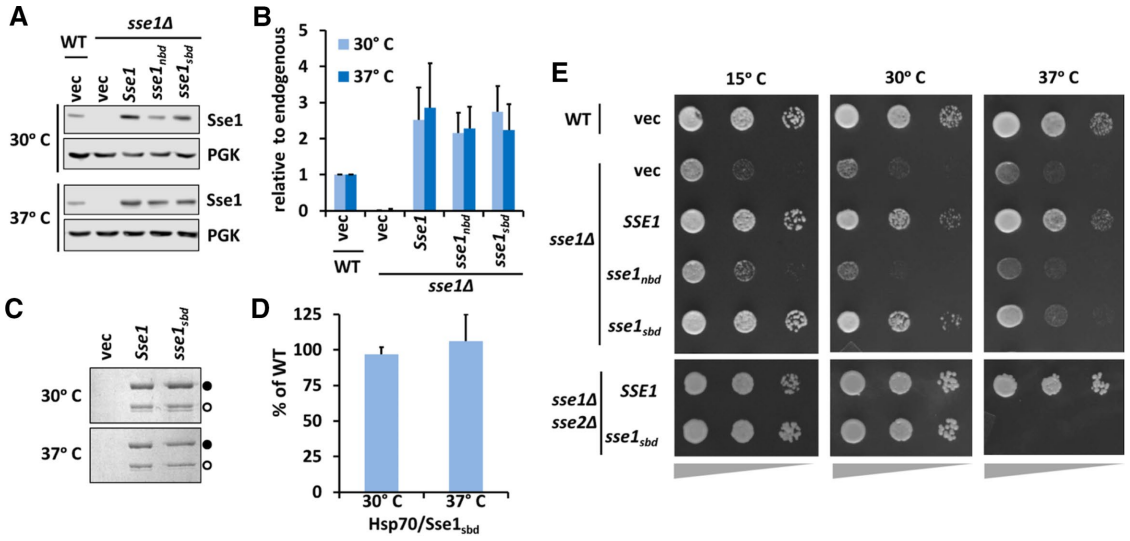

FIGURE 3: $Sse1_{sbd}$ stably interacts with endogenous yeast Hsp70 proteins and supports growth at normal but not heat shock temperatures. (A) Protein lysates from cells expressing the indicated *SSE1* alleles and cultured at 30 or 37°C were analyzed by immunoblot to determine expression levels and stability. (B) Quantitative analysis of the immunoblots in A. (C) Coimmunoprecipitation experiments using FLAG-tagged Sse1 variants were performed to assess interactions with endogenous Ssa and Ssb proteins. Samples were analyzed via SDS–PAGE and Coomassie staining. Sse1; closed circle, Ssa/Ssb; open circle. (D) Quantitation of C. (E) Serial dilution plating of *sse1*Δ or *sse1*Δ*sse2*Δ cells complemented with the indicated plasmid-expressed Sse1 alleles and cultured at the indicated temperatures. Wedges below images represent relative cell density.

temperatures in a manner indistinguishable from wild-type Sse1 (Figure 3, C and D), whereas $Sse1_{nbd}$ failed to do so (Supplemental Figure S2, C and D).

Given that $Sse1_{sbd}$ displayed normal stability and retained Hsp70 interaction at both standard and heat shock temperatures, we next assessed the contribution of substrate binding to Sse1 functions in yeast. As previously mentioned, the $sse1_{nbd}$ allele contains the mutation G233D, which renders it unable to bind nucleotide, interact with Hsp70, or act as a NEF (Shaner *et al.*, 2004, 2005; Dragovic *et al.*, 2006). We compared the growth of *sse1*Δ cells expressing *SSE1*, $sse1_{nbd}$, or $sse1_{sbd}$ under cold stress or heat stress. Whereas $sse1_{sbd}$ fully complements *sse1*Δ cells grown in optimal conditions and under cold stress, the mutant allele could not confer normal growth under heat stress (Figure 3E). This behavior contrasted with the inability of the $sse1_{nbd}$ allele to complement under any condition, suggesting that thermal stress may impose distinct requirements for Sse1 functions that include NEF and substrate holdase activities. To further probe this question and ask whether the presence of the closely related Sse2 protein masked growth defects of $sse1_{sbd}$ under non–heat shock conditions, we transformed *sse1*Δ*sse2*Δ cells with $sse1_{sbd}$- or *SSE1*-expressing plasmids using a plasmid shuffle technique (Trott *et al.*, 2005). We again observed indistinguishable growth between the two alleles at 30°C, whereas $sse1_{sbd}$ was unable to maintain viability at 37°C (Figure 3E and Supplemental Figure S3A). Consistent with the phenotypes seen under thermal stress, cells grown in the presence of formamide, which acts as a general protein denaturant, exhibited phenotypes consistent with heat stress (Supplemental Figure S3B). Cells expressing the $sse1_{nbd}$, or $sse1_{sbd}$ were hypersensitive to formamide, and this phenotype was augmented with combined heat stress. Of interest, exposing cells to ethanol stress at 30°C did not affect cells expressing $sse1_{sbd}$, as they grew at least as well as cells expressing wild-type *SSE1* (Supplemental Figure S3C; Trotter *et al.*, 2002). These results suggest that despite being unnecessary for substrate refolding and disaggregation in vitro or resistance to other forms of proteotoxic stress, the Sse1 SBD and its holdase activity are important for cell physiology and survival under prolonged thermal stress. Previous work has established that defects in thermal tolerance in *sse1*Δ cells are linked to deficiencies in cell wall integrity rather than overall protein homeostasis and correspondingly remediated by growth in an osmotic support medium (Shaner *et al.*, 2008). In keeping with this interpretation, addition of 1 M sorbitol suppressed the temperature sensitivity of *sse1*Δ and $sse1_{sbd}$ cells without altering the slow-growth phenotype of the $sse1_{nbd}$ mutant (Supplemental Figure S3D).

Reduction in Sse1 holdase activity results in mild proteotoxicity

We envisioned two possible explanations to account for the results obtained from the growth analyses with $sse1_{sbd}$. One is that Sse1 substrate binding is important only during heat stress due to physiological insults that occur exclusively under those conditions. The second possibility is that the Sse1 SBD is functioning at all times to maintain proteostasis and is dispensable under normal growth conditions but is required to endure the increased burden on protein quality control systems during heat stress. To determine whether a nonfunctional Sse1 SBD has any effect on the proteome while cells are grown under optimal conditions, we assessed the activation of the heat shock response (HSR) as a proxy for disruption of proteostasis using an established HSE-lacZ reporter. To prevent possible variability from plasmid expression in these and subsequent experiments, we chose to directly integrate the *SSE1* mutants into the yeast chromosome at the endogenous locus (Supplemental Table S1). It was previously shown that *sse1*Δ cells exhibit a twofold to fourfold elevated HSR, consistent with chronic proteostatic imbalance (Liu *et al.*, 1999). We confirmed that cells expressing the NEF-defective allele $sse1_{nbd}$ also demonstrated an activated HSR (Figure 4A). Of interest, cells expressing $sse1_{sbd}$ exhibited modest activation of the HSR (~1.8-fold) supporting the idea that the Sse1 SBD may

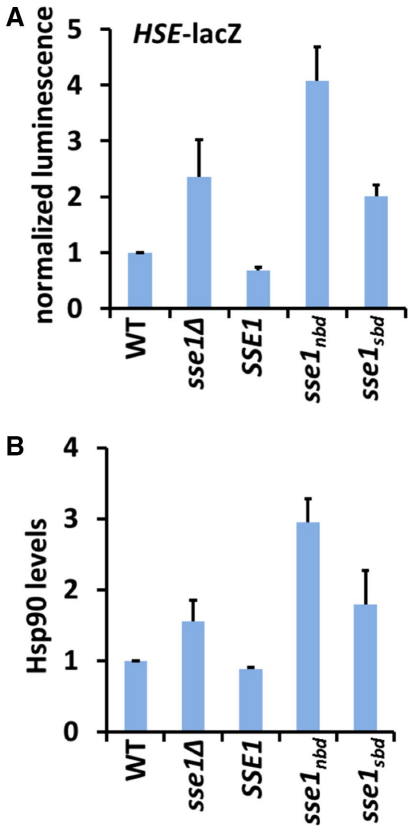

FIGURE 4: Reduced Sse1 holdase activity results in mild proteotoxicity. (A) β-Galactosidase activity assays from cells expressing the indicated *SSE1* alleles integrated at the endogenous locus and expressing the HSF reporter *pSSA3HSE-lacZ*. (B) Protein lysates from cells grown to mid log phase were analyzed for Hsp90 protein levels by SDS–PAGE and immunoblot, quantified, and normalized to PGK load control. Experiments were repeated in triplicate, and error bars represent SD.

play some role in proteome maintenance during nonstress conditions. Supplementation of the growth medium with 1 M sorbitol under nonstress conditions before determination of HSR status did not appreciably alter the general pattern observed in Figure 4A but decreased overall activation of the HSR in all strains tested by 20–30% (Supplemental Figure S3E). As a complementary approach, we assessed Hsp90 protein levels because expression of the two Hsp90-encoding genes *HSC82* and *HSP82* is under the exclusive transcriptional control of Hsf1 (Erkine *et al.*, 1995; Solis *et al.*, 2016). Using immunoblot analysis, we determined that the *sse1*Δ, *sse1*$_{nbd}$, *sse1*$_{sbd}$ cells exhibited a modest 1.5- to 3-fold increase in steady-state Hsp90 levels, in accordance with the HSR reporter activation results (Figure 4B). These data suggest that the holdase activity of Sse1 nominally contributes to proper functioning of the chaperone network under normal physiological conditions. In addition, we infer that the ability of sorbitol to rescue the 37°C growth defects of *sse1* mutant strains without changing HSR activation status reflects a deficiency in cell wall integrity distinct from maintenance of protein quality control.

Reduction in Sse1 SBD function does not negatively affect Hsp90-based signal transduction or clearance of terminally misfolded proteins

In addition to general contributions to proteostasis, Sse1 supports signal transduction and functions of Hsp90 (Liu *et al.*, 1999; Mandal *et al.*, 2010). To assess whether this biological role required Sse1 to functionally interact with unfolded substrates, we used the maturation and activation of the mammalian glucocorticoid receptor (GR) in yeast cells as a benchmark of Hsp90 activity. β-Galactosidase activity was measured in cells coexpressing a glucocorticoid response element (GRE)–lacZ reporter and the different *SSE1* alleles after activation of the GR via the synthetic hormone deoxycorticosterone (DOC; Figure 5A). Wild-type cells exhibited a robust response to DOC treatment, indicative of GR activation. Similarly, cells expressing *sse1*$_{sbd}$ were also able to activate the GR, whereas activation was abolished in *sse1*Δ and *sse1*$_{nbd}$ cells.

Another established cellular role for Sse1 is its participation in the triage decision for Hsp70-mediated protein folding versus degradation, in which Sse1 is required for targeting terminally misfolded proteins to the proteasome for degradation (Mandal *et al.*, 2010). Specifically, Sse1 stimulates ubiquitination and degradation of the model misfolded protein CPY‡–green fluorescent protein (GFP), an engineered variant of the vacuolar protease carboxypeptidase Y that lacks the endoplasmic reticulum (ER) signal sequence and is permanently misfolded (Heck *et al.*, 2010; Abrams *et al.*, 2014). We used CPY‡-GFP to assess whether Sse1 substrate binding was important for targeting terminally misfolded proteins for degradation. After treating cells with cycloheximide, we tracked the clearance of CPY‡-GFP in cells expressing *SSE1*, *sse1*$_{ndb}$, or *sse1*$_{sbd}$ by immunoblot. We found that *sse1*$_{nbd}$-expressing cells matched *sse1*Δ cells in their inability to clear the terminally misfolded protein after 2 h of cycloheximide chase (Figure 5B). In contrast, *sse1*$_{sbd}$-expressing cells fully cleared CPY‡-GFP, indicating that the Sse1 SBD function is not required for targeting terminally misfolded proteins for degradation. In addition to immunoblot analysis, we assessed the amount of CPY‡-GFP aggregates forming in cells expressing the different Sse1 alleles and tracked their clearance over time using fluorescence microscopy. CPY‡-GFP aggregate clearance correlated precisely with protein clearance (Figure 5, C and D). To test whether Sse2 could be masking a substrate-binding role for Sse1 in protein degradation, we constructed *SSE1sse2*Δ and *sse1*$_{sbd}$*sse2*Δ strains and tracked the ability of these cells to clear the CPY‡-GFP aggregates. Cells expressing the substrate binding–deficient mutant cleared the aggregates at the same rate as *SSE1sse2*Δ cells (Figure 5, E and F). Together these data strongly support the contention that Sse1 substrate binding is not required to support Hsp90 signaling activities or promote the degradation of terminally misfolded cytosolic proteins.

DISCUSSION

Among the three classes of cytosolic NEFs, Hsp110/Sse is the sole family demonstrated to possess holdase activity for unfolded proteins, yet no in vivo role has been exclusively attributed to this domain. To address this quandary, we generated a novel Sse1 allele that disrupts the ability of the chaperone to prevent aggregation, presumably via substrate binding and sequestration, while maintaining interaction with Hsp70 and NEF activity. Data from our laboratory and others strongly suggest that the yeast cytosolic Hsp110s, Sse1 and Sse2, play critical cellular roles in maintaining protein homeostasis during physiological and stress conditions. This interpretation is bolstered by the fact that *sse1*Δ*sse2*Δ cells are inviable and, whereas overexpression of the other yeast NEFs can only partially complement growth phenotypes at 30°C, the complete absence of Hsp110 proteins can only be fully remedied by overexpression of either Sse1 or Sse2 (Mandal *et al.*, 2010). In all cases studied, elimination of Hsp110/Sse NEF activity phenocopies the gene deletion, suggesting that, indeed, the NEF function is a primary, if not

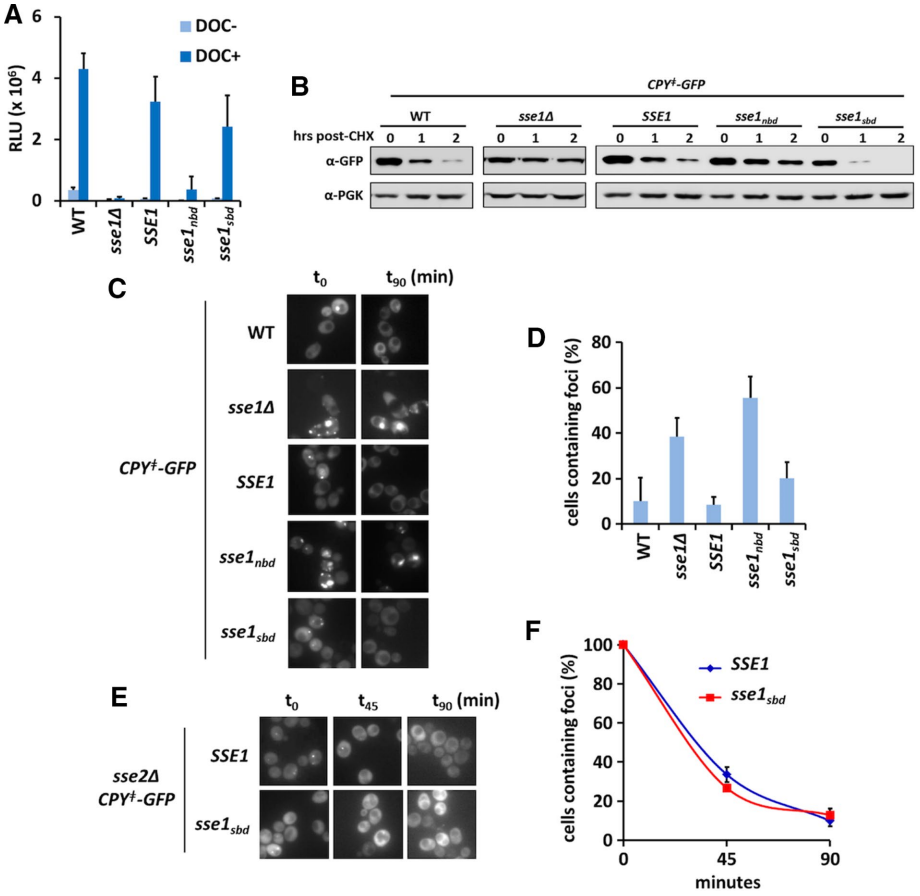

FIGURE 5: Reduced SBD function does not impair Sse1-dependent biological processes. (A) Activation of the rat glucocorticoid receptor was measured via a *lacZ* reporter in the absence and presence of 10 μM DOC. (B) Degradation of CPY*GFP after cycloheximide treatment analyzed using anti-GFP antibody. (C) Representative micrographs of the various *SSE1* strains at 0 and 90 min after cycloheximide treatment to track CPY*GFP aggregate clearance in the cell population. (D) Quantitation of the experiments in C; percentage calculated as aggregate-containing cells relative to time zero. (E) Representative micrographs of *SSE1sse2*Δ or *sse1*$_{sbd}$*sse2*Δ strains, tracking CPY*GFP aggregate clearance at the indicated time points. (F) Quantitation of the experiments in D; percentage calculated as aggregate-containing cells relative to time zero. All experiments were performed using cells expressing the indicated *SSE1* alleles integrated at the endogenous locus.

dominant, role for this class of chaperone. To determine which known Sse1 roles might additionally be affected by loss of Hsp110/Sse holdase activity, we tested refolding and disaggregation in vitro, responses to different proteotoxic stresses, signaling through Hsp90, and targeting of terminally misfolded cytosolic proteins for degradation. Strikingly, we did not observe a demonstrable role for Sse1 SBD function in the reconstituted luciferase refolding or disaggregation reactions, leading us to conclude that the holdase activity is dispensable for these activities. Similarly, Hsp90-dependent signaling and protein degradation was fully supported by the Sse1$_{sbd}$ mutant. This is in apparent contrast to a recent study that identified distinct sequences in two secretory pathway proteins, immunoglobulin γ_1 heavy chain and NS-1 κ light chain, which are preferentially bound by the ER homologue of Hsp110, Grp170, and, when eliminated, disrupt processing of these substrates (Behnke *et al.*, 2016). Although these findings suggest a biological role for Grp170 substrate binding in human cells, these same regions within the substrates are recognized by the ER Hsp40 cochaperones ERdj4 and ERdj5, precluding a clear interpretation that holdase activity by Grp170 is specifically required. However, the finding that Grp170 and ERdj4/5 modulation of substrate processing can occur independently of the ER Hsp70 BiP also suggests that the passive holdase activity of these chaperones and, by extension, Sse1/Hsp110 affects proteome maintenance. This observation might explain the modest activation of the HSR we detect with the Sse1$_{sbd}$ mutant. It would be of interest to determine whether introduction of mutations in the Grp170 SBD analogous to those we demonstrated reduce substrate binding by Sse1 phenocopy elimination of the proposed target sequences in the secretory protein substrates. In addition, models have been proposed in which Hsp110 chaperones are competent to promote the folding of unfolded substrates when assisted by Hsp40 cochaperones in an ATP-dependent folding cycle, an activity that would presumably rely on the SBD (Mattoo *et al.*, 2013). However, the ability of catalytically inactive *SSE1* mutant alleles to fully support known Sse1-dependent activities challenges the biological relevance of this observation (Shaner *et al.*, 2004; Raviol *et al.*, 2006).

It is possible that the Hsp110/Sse SBD plays a (minor and perhaps redundant) role in protein folding events that is magnified under certain stress conditions. For example, we found that Sse1$_{sbd}$ was unable to serve as the sole Hsp110 allele under extended growth at 37°C or in the presence of formamide, the latter a phenotype that we and others have demonstrated to be functionally analogous to thermal stress (Hampsey, 1997; Trott and Morano, 2004). We cannot exclude, however, that these phenotypes are ultimately more tightly linked with cell wall integrity than protein homeostasis, an idea reinforced by the clear suppression of *sse1* mutant phenotypes with sorbitol, an osmotic stabilizing agent (Shaner *et al.*, 2008; Supplemental Figure S3D). Moreover, Sse1$_{sbd}$ was insensitive to proteotoxicity caused by chronic exposure to 8% ethanol, further challenging the notion that Hsp110/Sse holdase activity is a major contributor to protein homeostasis.

It is relevant to consider that the Sse1$_{sbd}$ mutant is not completely defective in substrate binding, retaining between 20 and 50% of its aggregation prevention potential in a substrate-specific manner. It is possible that a complete abrogation of substrate interaction is necessary to reveal more dramatic phenotypes in the different Sse1 functions tested. However, we attempted to generate a more severe holdase-defective mutant through additional targeted amino acid substitutions based on the work of Xu *et al.* (2012) but without success. Of importance, we observed nearly identical outcomes in multiple in vitro and in vivo assays that are highly dependent on Sse1 and sensitive to perturbations in its status. Tellingly, the recently described role for Hsp110/Sse as a critical component of the eukaryotic disaggregase machine provided a prime opportunity to answer the open question of whether substrate holding by this family of proteins contributed to the remarkable ability of the

Hsp110•Hsp70•Hsp40 complex to extract and refold aggregated proteins. Our findings support the growing contention that Hsp110 NEF activity, not holdase activity, is the key accelerator of disaggregation in this context (Nillegoda and Bukau, 2015). Recent findings reveal that the yeast Hsp104-based disaggregation activity is coordinated in a manner that requires the interaction of Sse1 and Sse2 with Hsp70 (Kaimal *et al.*, 2017). Efficient recruitment of Hsp104 to aggregates was found to require Sse1/2, and in turn recruitment of Sse1/2 was dependent on Ssa1/Hsp70, demonstrated by using the Hsp70 binding–defective *SSE1* allele *sse1-2,3* (Polier *et al.*, 2008). However, Sse1/2 was also shown to be required for efficient Ssa1/Hsp70 association with aggregates, suggesting that Hsp110 may be limiting for generating sufficient cellular levels of Ssa1/Hsp70-ATP (Kaimal *et al.*, 2017). Because the *sse1-2,3* allele presumably retains substrate-binding function, it may be inferred that the Sse1 SBD is insufficient for recruitment to luciferase aggregates, consistent with our finding that disaggregation and clearance of CPY*-GFP aggregates proceeded unimpeded in cells expressing Sse1$_{sbd}$.

Because the Sse1$_{sbd}$ mutant is not completely without substrate-binding capacity, we cannot yet formally exclude a role for substrate binding by Hsp110 chaperones in proteostasis. The passive holdase activity of Hsp110/Sse has been shown to promote the refolding of luciferase in yeast cytosol, likely by stabilizing the unfolded polypeptide and preventing its aggregation. This activity may also be compared with subtle interactions under certain conditions with the Sup35 prion in yeast, which appear to be independent of Sse1 NEF function (Brodsky *et al.*, 1999; Sadlish et al., 2008). In both of the latter scenarios, the Sse1 holdase function is likely operating independently of Hsp70. It may be of interest to further probe potential contributions of Hsp110/Sse1 holdase activity in aggregate prevention for specific aggregation-prone substrates. For example, Hsp105α (HSPH1) in human cells is known to modulate cystic fibrosis transmembrane conductance regulator folding and processing (Saxena *et al.*, 2012). Hsp110 suppresses the aggregation and associated toxicity of the mutant proteins that lead to amyotrophic lateral sclerosis and Alzheimer disease, respectively, when expressed in *Caenorhabditis elegans* and mice (Wang *et al.*, 2009; Eroglu *et al.*, 2010). Hsp110 has also been found to be an important modulator of neuronal degeneration caused by the expression of toxic polyglutamine proteins that model Huntington disease in the fly (Zhang *et al.*, 2010; Kuo *et al.*, 2013). Strikingly, Hsp110 can also ameliorate toxicity caused by the G85R variant of SOD1, a contributor to amyotrophic lateral sclerosis, significantly extending survival of SOD1G85R-YFP transgenic mice when overexpressed in motor neurons (Song *et al.*, 2013; Nagy *et al.*, 2016). The specific mechanisms by which Hsp110 prevents aggregation and disease progression in these model systems are unknown. Given the increasing significance of Hsp110 chaperones in modulation of proteotoxic aggregation, it will be important to define more precisely the features that contribute to such activities as a precursor to therapeutically manipulating the chaperone network to combat progression of protein-misfolding disorders.

MATERIALS AND METHODS

Strains, plasmids, and yeast culture

All yeast strains are derived from either BY4741 or W303 parent background (Supplemental Table S1). Yeast were propagated on standard rich (YPD; 1% yeast extract, 2% peptone, 2% glucose) or synthetic minimal complete media lacking amino acids for marker selection (Sunrise Science, San Diego, CA) at 30°C unless otherwise specified. Complementation of the lethal sse1Δsse2Δ mutant strain was conducted using a standard yeast plasmid shuffle technique with a *URA3*-based, *SSE1*-expressing plasmid and counterselection using 5-fluroorotic acid. Mutant *sse1$_{sbd}$* was constructed via site-directed mutagenesis by PCR using the plasmid p413TEF-FLAG-SSE1 as a template. This *SSE1* allele and the *sse1$_{nbd}$* mutant (*sse1-G233D*; previously described in Shaner *et al.*, 2004) were subcloned into the 413TEF vector using *Spe*I/*Xho*I restriction sites (Mumberg *et al.*, 1995). For growth complementation and immunoprecipitation experiments, *SSE1* alleles were expressed from the p413TEF plasmid. A FLAG epitope tag (DYKDDDDK) was added to the 5′ end of the *SSE1* genes immediately after the start codon by using primers that included the FLAG-encoding, yeast-optimized sequence (GACTACAAGGACGACGATGACAAAATG). Strains expressing the various *SSE1* alleles from the endogenous locus (YPL106C) were constructed by gene replacement. *SSE1* amplicons were generated from plasmids containing a *CYC1* terminator sequence using primers (5′-ATAACTCTGTCCTTGCCGT-3′) and (5′-TACTCTGTCAGAAACGGCCTGTACCGGCCGCAAATTAAAGCC-3′) to PCR amplify from nucleotide +35 relative to the ATG in *SSE1* (forward primer) to the end of the *CYC1* terminator with an overhang with homology to the *LEU2* terminator (reverse primer). The *LEU2* cassette was PCR amplified from plasmid DNA using a forward primer matching the *CYC1* terminator (5′-GCTTTAATTTGCGGCCGGTACAGGCCGTTTCTGACAGAGTAAAATTCTTG) and a reverse primer with an overhang matching the endogenous *SSE1* terminator (5′-AATCTTTTTTTAACTATACAGAGAAGATATTAGTATTTCACACCGCATATCG-3′). The two PCR amplicons were cotransformed into the BY4741 parent strain, and successful Leu^{+} double recombinants were selected. Individual clones were obtained and sequenced to verify correct integration, presence of desired mutations, and absence of additional nucleotide substitutions.

Hsf1 activity was measured with strains harboring plasmid pSSA3HSE-*lacZ* as described (Abrams *et al.*, 2014). For experiments testing CPY*-GFP degradation, strains were constructed using pRH2081 (generous gift from Randy Hampton, University of California, San Diego), a plasmid that carries *TDH3*-driven CPY*-GFP (Heck *et al.*, 2010). The integrative plasmid was linearized using restriction endonuclease Van91I and transformed into indicated strains with Ura^{+} selection. For growth analysis, cells were grown to mid logarithmic phase and normalized to an OD_{600} of 1.0. Tenfold dilutions were plated on minimal medium and incubated as described in figure legends. Spot plates were imaged after 2–3 d of growth.

Protein purification

Purified firefly luciferase (Sigma L-9506) and citrate synthase (Sigma C-2360) were obtained from Sigma-Aldrich (St. Louis, MO). Sse1 was purified from *E. coli* BL21 (DE3) by metal affinity chromatography followed by size exclusion chromatography as described in Garcia et al. (2016). HSPA8 (hHsp70), DNAJB1 (hHsp40), and HSPH2 (hHsp110) were expressed in *E. coli* BL21 (DE3) with an N-terminal His6-Smt3 tag and purified by affinity chromatography using a Ni-IDA matrix and further subjected to size exclusion chromatography. The His6-Smt3 tag was cleaved with Ulp1 as published earlier (Andreasson *et al.*, 2008b). Purified firefly luciferase (Sigma L-9506) and citrate synthase (Sigma C-2360) were obtained from Sigma-Aldrich.

Nucleotide-binding assay

Fluorescently labeled nucleotide, N^{6}-(6-amino)hexyl-ATP-5-FAM (ATP-FAM; Jena Bioscience, Jena, Germany), was incubated at a concentration of 20 nM with increasing amounts of Sse1 chaperone in buffer (25 mM Tris-HCl, pH 7.5, 100 mM NaCl, 5 mM $MgCl_2$, 50 mM KCl, 5% glycerol) for 30 min at room temperature as described (Rauch and Gestwicki, 2014). Fluorescence polarization was

measured (excitation λ = 485 nm, emission λ = 535 nm) using a SpectraMax M5 plate reader. Equilibrium binding constants were calculated using a saturation binding one-site equation via GraphPad Prism version 6 (GraphPad Software, La Jolla, CA).

Nucleotide exchange assay

The HSPA8 (Hsc70) protein was a generous gift from Betty Craig (University of Wisconsin, Madison, WI). HSPA8 (70 µg) was loaded with 100 µCi of α-^{32}P-ATP in a total volume of 120 µl of complex buffer (25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid [HEPES]–KOH, pH 7.5, 100 mM KCl, 11 mM MgOAc, and 25 µM ATP) for 30 min at 4°C, and HSPA8–^{32}P-ATP complex was obtained by centrifugation through a Microspin G-25 column (GE Healthcare, Chicago, IL). Labeled HSPA8 (7.8 µg) was incubated in the presence or absence of 5 µg of Sse1 or Sse1$_{sbd}$ at 30°C. At 0, 7, and 15 min, the HSPA8-Sse1 reactions were again passed over G-25 columns to separate from released nucleotide. Radiolabeled nucleotide that remained bound to HSPA8 was determined using a TRI-CARB 2900TR Liquid Scintillation Analyzer and normalized to counts obtained at time zero.

Protein aggregation assay

Substrate aggregation was measured in a Synergy MX Microplate Reader (BioTek, Winooski, VT) as described, with the following modifications (Garcia *et al.*, 2016). Stock concentrations of firefly luciferase or citrate synthase were incubated in denaturing buffer (6 M guanidinium chloride, 5 mM dithiothreitol [DTT]) for 1 h at room temperature. In a 96-well, half-area, ultraviolet-transmissible plate (675801; Greiner Bio-One, Monroe, NC) refolding buffer alone (25 mM Tris-HCl, pH 7.5, 100 mM NaCl), varying concentrations of chaperone in refolding buffer, or denaturing buffer was preequilibrated at 25°C for 5 min, and baseline light scattering was determined. After equilibration, chemically denatured substrate was added to each sample at a final concentration of 200 nM into the refolding buffer to a final volume of 180 µl. The samples were mixed vigorously for 5 s, and aggregation was measured at 320 nm at 30-s intervals for 30 min. Changes in absorbance were calculated after subtracting baseline absorbance at time zero. To assess fractionation of protein into soluble and insoluble aggregates, samples (175 µl) were taken from the endpoint of the substrate aggregation experiments and subjected to centrifugation at 16,000 × *g* for 4 min. We recovered 170 µl as the supernatant or soluble fraction. The lower 5-µl fraction was considered the pellet or insoluble fraction, and volume was normalized to 170 µl with the addition of refolding buffer. We separated 30 µl of each fraction by 12% SDS–PAGE and stained them with Coomassie blue. Band densities were calculated using Image Studio Software (Li-Cor Biosciences, Lincoln, NE).

FFL refolding and disaggregation

Solubilization of aggregated luciferase was performed as described (Rampelt *et al.*, 2012; Nillegoda *et al.*, 2015). In brief, thermal aggregation was performed by incubating 0.02 µM of native luciferase with 0.1 µM of yHsp26 at 45°C for 15 min in refolding buffer (40 mM HEPES-KOH, pH 7.5, 50 mM KCl, 5 mM $MgCl_2$, 2 mM DTT, 2 mM ATP, 10 µM bovine serum albumin) without the ATP-regenerating system in a water bath. The disaggregation reaction was started by adding 3 mM phosphoenolpyruvate and 20 ng/µl pyruvate kinase (ATP-regenerating system) with the indicated chaperone combinations at the concentrations 2 µM HSPA8 (hHsp70), 1 µM DNAJB1 (hHsp40), 0.2 µM HSPH2 (hHsp110), 0.2 µM Sse1 (yHsp110), and 0.2 µM Sse1$_{sbd}$ (yHsp110$_{sbd}$), and shifting the reaction to 30°C. For the refolding-only assays, 0.02 µM luciferase was incubated with the indicated chaperone cocktails (2 µM HSPA8 [hHsp70], 1 µM DNAJB1 [hHsp40], HSPH2 0.2 µM [hHsp110], 0.2 µM Sse1 [yHsp110], 0.2 µM Sse1$_{sbd}$ [yHsp110$_{sbd}$], and 0.1 µM yHsp26) and heat denatured at 42°C for 10 min in refolding buffer to generate thermally denatured luciferase (Nillegoda *et al.*, 2015). Luciferase refolding was initiated by adding an ATP-regenerating system and shifting the reaction to 30°C. Luciferase reactivation was monitored at the indicated time points with a Lumat LB 9507 luminometer (Berthold Technologies) by transferring 1 µl of sample to 100 µl of assay buffer (25 mM glycylglycine, pH 7.4, 5 mM ATP, pH 7, 100 mM KCl, and 15 mM $MgCl_2$) mixed with 100 µl of 0.25 mM luciferin.

Protein levels in vivo

Cultures were grown overnight and secondary cultures started and allowed to grow to an OD_{600} of 0.8, at which point cells were shifted to 37°C or maintained at 30°C for 6 h. Cells were collected and processed for protein lysates. Sse1 protein levels were detected by immunoblot using anti-Sse1 antiserum (generous gift from Jeff Brodsky, University of Pittsburgh, Pittsburgh, PA), and anti–phosphoglycerate kinase (PGK; Invitrogen, Carlsbad, CA) was used as a loading control. Band quantitation was performed using Image Studio Software, and Sse1 levels were normalized to the levels of PGK.

Immunoprecipitation and immunoblotting

Sse1 proteins were expressed with an N-terminal FLAG tag. Protein extracts were prepared from 30 ml of cultures grown at 30 or 37°C for 6 h. Protein lysates were incubated with 40 µl of M2 resin (Sigma-Aldrich) in TEGN (20 mM Tris-HCl, pH 7.9, 0.5 mM EDTA, 10% glycerol, 50 mM NaCl) at 4°C for 2 h. After washing with 4 ml of buffer, the resin was incubated with 40 µl of FLAG peptide for 25 min at room temperature to elute the FLAG-Sse1 complexes. Immunoprecipitated proteins were analyzed by SDS–PAGE and Coomassie stain. Band analysis was performed using Image Studio Software, and the coimmunoprecipitation efficiency of Hsp70 was calculated relative to the amount of Sse1 immunoprecipitated. Hsp90 levels were assessed by immunoblot using anti-Hsp90 (a generous gift from Avrom Caplan, CUNY, New York, NY) with anti-Sse1 and anti-PGK as internal controls. Band analysis was performed using Image Studio Software, and Hsp90 levels were normalized to PGK levels.

Glucocorticoid receptor activation

The various Sse1 strains were transformed with plasmids pCH-Flag-RatGR and pYRP-G2, expressing the glucocorticoid receptor protein and a GR-*lacZ* transcriptional reporter, respectively (Liu *et al.*, 1999). Cells grown to mid logarithmic phase were treated with dimethyl sulfoxide (DMSO) only (–DOC) or 10 µM DOC in DMSO (+DOC) for 1.5 h. β-Galactosidase activity was measured by adding 50 µl of cell suspension at OD_{600} of 0.4 and 50 µl of Beta-Glo reagent (Promega, Madison, WI) and incubating for 30 min at 30°C, followed by luminescence detection using a Synergy MX Microplate Reader.

CPY‡-GFP degradation assay

To track the degradation of the CPY‡-GFP protein in vivo, cells were grown to mid logarithmic phase and treated with 100 µg/ml cycloheximide, and 10 ml of culture was collected at 0, 1, and 2 h. Denatured protein extracts were prepared using a glass bead lysis method with SUME buffer (1% SDS, 8M urea, 10 mM 3-(*N*-morpholino)propanesulfonic acid, pH 6.8, 10 mM EDTA). The CPY‡-GFP protein was detected by immunoblot using anti-GFP (Roche, Basel, Switzerland), and anti-PGK was used an internal control. In parallel experiments, CPY‡-GFP–expressing cells were collected immediately after treatment at 0, 45, and 90 min and visualized using an

Olympus IX81-ZDC inverted microscope as described in Abrams and Morano (2013).

Statistical analysis

All experiments were performed at least in triplicate and quantitation shown as the mean, with error bars indicating SD as calculated in Microsoft Excel.

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