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The Hsp110 protein chaperone Sse1 is required for yeast cell wall integrity and morphogenesis

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Abstract

Molecular chaperones direct refolding and triage decisions and support signal transduction responses to cytotoxic stress. The eukaryotic chaperone Hsp110 is represented by the *SSE1/2* genes in *Saccharomyces cerevisiae*, which act as nucleotide exchange factors (NEFs) for cognate cytosolic Hsp70 chaperones. In this report, we present evidence that Sse1 is required for signaling through the cell integrity pathway via partnership with Hsp90 and the terminal MAP kinase Slt2. We found that *sse1*Δ and *sti1*Δ mutant cells share the typical cell integrity mutant phenotypes of osmoremediated temperature-sensitive growth and sensitivity to cell wall-damaging agents. Sse1 binds to Slt2 in vivo and similar to Hsp90 mutants, Slt2 stability and phosphorylation is not compromised in *sse1*Δ cells, whereas activation of the downstream transcription factor Rlm1 is abolished. In addition to Rlm1, Slt2 activates the Swi4/Swi6 heterodimer SBF in response to cell wall damage. *SSE1* displayed dramatic synthetic phenotypes when disrupted in combination with mutations in SBF and the related Mbp1/Swi6 heterodimer MBF, characterized by severe growth and morphological defects. These defects were reversed by restoration of Hsp70 NEF activity, providing a mechanistic model wherein Sse1 functionally partners with Hsp90 as an Hsp70 NEF to promote client protein maturation and interaction with downstream effectors.

Keywords

Yeast; Heat shock; Morphogenesis; Cell integrity; Chaperone; Hsp110; Hsp70; Hsp90

Introduction

All cells respond to heat shock and other cytotoxic stresses by production of heat shock proteins (HSPs), many of which function as molecular chaperones. Chaperones typically bind stretches of hydrophobic amino acids not normally exposed to the aqueous cellular milieu, thereby preventing their aggregation and assisting in refolding. Molecular chaperones such as those of the Hsp70 family are required for proper folding of proteins as they emerge from the translating ribosome, for translocation across biological membranes, assembly of multimeric complexes, and are also involved in protein degradation decisions

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(Mayer and Bukau 2005; Brodsky and Chiosis 2006). In contrast, the Hsp90 chaperone system is distinct from “general” chaperones such as Hsp70 that are involved in bulk protein folding in that its client proteins are typically kinases or transcription factors that rely on Hsp90 for proper signaling activity (Mayer and Bukau 1999; Pearl and Prodromou 2001). Similar to other chaperones, Hsp90 requires the action of several other cochaperones for proper function. The Hsp110 class of eukaryotic chaperones is represented in budding yeast by the essential Sse1 and Sse2 proteins (Mukai et al. 1993). Hsp110/Sse1 chaperones are divergent members of the Hsp70 superfamily, and recent work has established an unique role for this group as nucleotide exchange factors (NEFs) in a stable, heterodimeric complex with yeast and mammalian cytosolic Hsp70 chaperones (Shaner et al. 2004, 2005, 2006; Yam et al. 2005; Dragovic et al. 2006; Raviol et al. 2006; Shaner and Morano 2007). Sse1 was previously shown to be required for maturation and activity of Hsp90 substrates in yeast, consistent with a requirement for regulated transfer of client proteins from Hsp70 to Hsp90 in the early stages of the chaperone cycle (Liu et al. 1999; Goeckeler et al. 2002; Lee et al. 2004). However, the extent to which Sse1 participates in Hsp70-dependent and Hsp90-dependent activities is unknown.

The cell integrity signaling pathway is induced by cell wall perturbations caused by heat and chemical agents including Congo red and caffeine [reviewed in (Levin 2005)]. Cell wall damage is transmitted by protein kinase C to a MAP kinase module terminating in the MAPK Slt2 (Torres et al. 1991; Levin and Bartlett-Heubusch 1992; Paravicini et al. 1992). Slt2 (also known as Mpk1) phosphorylates and activates two transcription factors, Rlm1 and the Swi4/6 cell-cycle box binding factor (SBF), the latter a heterodimer composed of Swi4 and Swi6 proteins (Watanabe et al. 1995; Dodou and Treisman 1997; Madden et al. 1997; Jung et al. 2002). Rlm1 and SBF activate expression of distinct sets of genes with predicted roles in cell wall synthesis and repair (Lagorce et al. 2003; Garcia et al. 2004). In addition to cell wall stress, the pathway is activated during the G_1 to S phase transition of the cell cycle due to remodeling of the cell wall at the bud site (Igual et al. 1996; Zarzov et al. 1996). In addition to Swi4, Swi6 partners with the Mbp1 protein to form the transcription factor MBF (*Mlu*I cell-cycle box binding factor), also required for G_1/S-specific gene expression (Moll et al. 1992; Koch et al. 1993). Mutations of components in the cell integrity pathway share common phenotypes including sensitivity to cell wall-damaging drugs and temperature-sensitive growth at 37°C, both of which can be remediated by osmotic support in the growth medium (Levin 2005). Hsp90 was identified as an Slt2 binding partner in a two-hybrid screen, and multiple lines of evidence demonstrate that the chaperone is a critical component of cell wall integrity signaling: (1) Hsp90 preferentially binds dually phosphorylated (activated) Slt2, (2) mutants defective in Hsp90 function in vivo display osmotically remediated temperature-sensitive growth, and (3) Hsp90 mutants are defective in heat shock-activated expression of Rlm1-dependent target genes (Millson et al. 2005; Piper et al. 2006; Truman et al. 2006, 2007). Unlike many other protein kinase clients of Hsp90, stability and phosphorylation of Slt2 is not impaired in the absence of chaperone function, suggesting a specific chaperone requirement for interaction with the downstream effectors Rlm1 and SBF (Truman et al. 2007). With the exception of Cdc37 and Ydj1, the role of other Hsp90 cochaperones in cell integrity signaling has not been investigated (Hawle et al. 2007; Wright et al. 2007).

In this study, we present evidence that Sse1 is required for signaling through the cell integrity pathway via partnership with Slt2. Cells lacking Sse1 or the Hsp70/Hsp90 organizing protein (HOP) homolog Sti1 share the prototypical cell integrity phenotype of osmoremediated temperature-sensitive growth and sensitivity to cell wall-damaging agents. Sse1 associates with Slt2 in vivo but is not required for its stability or phosphorylation by Mkk1/2. Expression of a synthetic Rlm1 target gene reporter was abolished in cells lacking *SSE1*, suggesting that the cochaperone is essential for Slt2 kinase maturation. In addition to a role in activating Rlm1, elimination of *SSE1* resulted in dramatic synthetic phenotypes in combination with mutations in SBF and MBF, characterized by severe growth and morphological defects. These defects were reversed by restoration of Hsp70 NEF activity, providing a mechanistic model wherein Sse1 functionally partners with Hsp70, and by extension Hsp90, as an NEF to promote Slt2 maturation and interaction with downstream effectors required for cell integrity and morphogenesis.

Materials and methods

Strains and plasmids

Standard yeast propagation and transformation procedures were employed. Yeast strains used in this study are described in Table 1. Congo Red was included in solid media at a final concentration of 35 μg/mL and caffeine at a final concentration of 10 mM. Chaperone mutations were engineered into a variety of backgrounds, including W303, S288C (BY4741), and DS10, and are used throughout. While we observed similar responses in all backgrounds, the magnitude of effects, most notably growth phenotypes, varied considerably. Backgrounds are identified in the figure legends for each experiment. The plasmid-based *Sse1*Δ::*LEU2* and *Sse1*Δ::*URA3* disruption cassettes, and pRS413*SSE1* have been described previously (Mukai et al. 1993; Shaner et al. 2004). The collection of *SSE1* point mutants, corresponding wild type *SSE1*, and the *SSE2* ORFS were all subcloned from previously constructed p414TEF (*TRP1*) vectors into p415TEF to recreate the series in a *LEU2*-selectable plasmid (Shaner et al. 2004). A plasmid-based *mbp1*Δ::*HIS3* disruption cassette was created by PCR amplification of a 300-bp fragment upstream and a 400-bp fragment downstream of the *MBP1* open reading frame from BY4741 genomic DNA followed by cloning into a plasmid containing the *HIS3* gene. The *swi4*Δ::*LEU2* disruption cassettes were constructed in a similar manner, amplifying 300 bp of upstream and 250 bp of downstream genomic sequence. All disruptions were confirmed by PCR and phenotypic analysis. p416GPD-FLAG-*SLT2* was constructed by PCR amplification of the *SLT2* open reading frame from BY4741 yeast genomic DNA using primers containing the coding sequence for the FLAG epitope followed by cloning into the p416GPD vector. The *PST1-lacZ* transcriptional fusion was generated by cloning a region −697 to −107 relative to the translational start site of *PST1*. The *SLT2-13xmyc* strain (BY2207) and isogenic wild type were generous gifts of B. Andrews (University of Toronto). The ssa1-45 allele and the isogenic wild type strain JN516 were kind gifts of E. Craig (University of Wisconsin) (Becker et al. 1996). The *FES1* ORF was subcloned via *Spe*I and *Xho*I sites from p413GPD*FES1* into plasmid p423GPD to create p423GPD*FES1* (Shaner et al. 2006). Sequences of all primers are available upon request.

Immunoprecipitation and immunoblot analysis

FLAG immunoprecipitations, SDS-PAGE and immunoblot analysis were performed as described (Shaner et al. 2004; Shaner et al. 2005). Anti-Sse1 polyclonal antibody was used at 1:2,000 dilution (kindly provided by J. Brodsky, University of Pittsburgh). M2 monoclonal antibody (anti-FLAG, Sigma, St Louis, MO) was used at 1:1,000 dilution. Anti-*myc* monoclonal antibody (Invitrogen, Carlsbad, CA) was used at 1:1,000 dilution. Anti-phospho-p44/42 MAPK monoclonal antibody (recognizes dually phosphorylated Slt2) was used at 1:1,000 dilution (Cell Signaling Technology, Danvers, MA).

Microscopy

All microscopy was performed using differential interference contrast (DIC, “Nomarski”) optics with an Olympus BX60 microscope at both 400 × and 1,000 × magnifications using DIC-compatible objectives. Images were captured using a Photometrics CCD camera and QED Imaging software (Media Cybernetics, Bethesda, MD). Cells were grown to logarithmic phase in selective medium where appropriate. Three classes of morphology were defined for the purposes of this study: normal, elongated, and amorphous. Cells of the typical size and round shape of a budding haploid yeast cell were considered normal. Elongated cells were defined as having a long axis at least twice as long as the short axis. Amorphous cells were defined as having multiple elongated buds or aberrant projections per mother cell. In these cases, all unseparated buds or aberrant projections were counted as part of the parent cell. Between 100 and 200 individual cells taken from multiple microscopic fields of view were counted for each strain.

Enzymatic assays

Liquid β-galactosidase assays were performed on yeast strains grown to mid-log phase (OD_{600} ~ 0.6) at 30°C, followed by determination of β-galactosidase activity as described (Trott et al. 2005). The alkaline phosphatase cell leakage assay was performed as described with minor modifications (Paravicini et al. 1992). Cells were suspended to a density of approximately 10^6 cells/mL in 180 μL of YPD in a sterile 96-well plate, and a multiprong replicator (Sigma) was used to spot each strain onto YPD plates. The plates were incubated at 30°C for 1 day, and then shifted to 39°C for two additional days. Next, 10 mL of the alkaline phosphatase assay solution [1% agar, 0.05 M glycine (pH = 9.5), 10 mM 5-bromo-4-chloro-3-indolyl phosphate disodium salt (BCIP)] was overlaid onto each plate, allowed to solidify, and incubated at room temperature. Plates were photographed at multiple time intervals. In repeated experiments, initial coloration was observed within 20 min, while intact spots remained white for over 80 min.

Results

The Hsp90/Hsp70 cochaperones Sse1 and Sti1 are required for yeast cell integrity

Hypomorphic alleles of Hsp90 (*HSC82* or *HSP82* genes) or the heat shock transcription factor *HSF1* exhibit temperature-sensitive growth at 37°C that can be remediated by the addition of the osmotic stabilizers sorbitol or sodium chloride (Piper et al. 2006; Truman et al. 2007). Although deletion of both *SSE1* and *SSE2* is lethal, *sse1*Δ strains are likewise

temperature-sensitive at 37°C(Mukai et al. 1993; Trott et al. 2005). Given that Sse1 is required for Hsp90 chaperone activity in *S. cerevisiae*, we sought to determine if the temperature-sensitive growth phenotype reflected a role in cell integrity signaling. Wild type and *sse1*Δ cells were serially diluted and plated on YPD medium with or without 1 M sorbitol or 0.4 M NaCl and incubated at 30, 37, or 39°C (Fig. 1a). Strikingly, *sse1*Δ temperature sensitivity was completely suppressed in the presence of 0.4 M NaCl or 1 M sorbitol, consistent with phenotypes exhibited by strains compromised in cell integrity signaling. Likewise, an isogenic strain lacking the Hsp90 cochaperone *STI1* displayed temperature-sensitive growth remediated by osmotic stabilization (Nicolet and Craig 1989). This observation suggests that loss of these Hsp70 and Hsp90 protein chaperone cofactors renders cells temperature-sensitive not because of a global protein folding defect, but rather due to specific attenuation of cell integrity pathway function.

Because the temperature-sensitive growth phenotypes of *HSF1*, *SSE1*, *STI1*, and some *HSP82/HSC82* mutants are suppressed by osmotic stabilization, we next asked whether loss of function of the cytosolic Ssa family of Hsp70 chaperones could be suppressed by osmotic stabilization. The *ssa1-45* allele of *SSA1* is inactivated at high temperature, and in the absence of the partially redundant *SSA2, SSA3 and SSA4* genes results in inviability at 37°C (Becker et al. 1996). Growth of this mutant at the restrictive temperature, or a strain lacking both *SSE1* and *SSE2*, was not restored in the presence of salt or sorbitol, demonstrating that osmotic stabilization does not indiscriminately rescue loss of global chaperone function, but rather compensates for a specific defect in cell integrity signaling (Fig. 1b and data not shown).

Cell integrity pathway mutants are commonly sensitive to compounds that damage the cell wall. Therefore, we sought to determine if this was the case for *sse1*Δ cells growing on media containing Congo red or caffeine, two compounds known to interfere with cell wall assembly and stability. As shown in Fig. 1c, growth of *sse1*Δ cells was strongly inhibited in the presence of both compounds when compared to wild type. Sse1 and its paralog Sse2 function as nucleotide exchange factors for the cytosolic families of Hsp70, Ssa, and Ssb (Dragovic et al. 2006; Raviol et al. 2006; Shaner et al. 2006). Likewise, metazoan Hsp110 performs a similar role for its cognate Hsp70 (Dragovic et al. 2006). Mutations in *SSE1* that perturb association with Hsp70 abrogate NEF function and phenocopy the *sse1*Δ null mutation (Shaner et al. 2004, 2005). We therefore asked whether the observed cell integrity defects were consistent with loss of Hsp70 NEF activity using a previously generated series of *SSE1* alleles. The *sse1-K69Q*, allele, defective in ATP hydrolysis, and the *SSE2* gene have been shown to functionally complement all known *Sse1*Δ phenotypes at 30°C (Shaner et al. 2004). In contrast, the G233D mutation abolishes Sse1–Hsp70 interaction and fails to complement (Shaner et al. 2004). These results were recapitulated with respect to tolerance of cell wall-inhibiting compounds, as shown in Fig. 1c. These results demonstrate that Sse1 is likely acting in the cell integrity pathway as an Hsp70 NEF, possibly in concert with the Hsp90 chaperone complex.

Sse1 is required for Slt2 MAP kinase activity

Hsp90 is required for activity of Slt2, the terminal MAP kinase in the cell integrity kinase cascade (Piper et al. 2006; Truman et al. 2007). Multiple findings support the idea that the relationship between Hsp90 and Slt2 is unique. Hsp90 and the associated cochaperone Cdc37 are required for the stability of a majority of protein kinases, and loss of chaperone activity results in lowered client protein abundance (Farrell and Morgan 2000; Lee et al. 2004; Hawle et al. 2007; Mandal et al. 2007). However, Slt2 stability is unaffected in numerous mutants with reduced Hsp90 activity (Piper et al. 2006; Truman et al. 2007). Moreover, dual phosphorylation of Slt2 (Thr^{190}/Tyr^{192}) by the MAPK kinases Mkk1 and Mkk2 is likewise unaffected (Piper et al. 2006; Hawle et al. 2007; Truman et al. 2007). Lastly, Hsp82 is preferentially recruited to dually phosphorylated Slt2, consistent with a role postactivation (Piper et al. 2006). Because Sse1 has been shown to participate in Hsp90 signal transduction activities, we sought to determine if Sse1 physically interacts with Slt2. To this end, we performed coimmunoprecipitation analysis using a functional FLAG-epitope tagged Slt2 (Fig. 2a). Indeed, when FLAG-Slt2 was immunoprecipitated from yeast protein extracts, Sse1 was found to copurify with the kinase and was not enriched from control extracts.

To further probe for signaling defects through the cell integrity MAP kinase cascade in *sse1*Δ cells during heat shock, we examined stability and phosphorylation of Slt2. A functional *myc*-tagged genomic allele of *SLT2* was utilized in this experiment, along with a commercially available antibody that specifically recognizes Slt2 dually phosphorylated at Thr^{190}/Tyr^{192}. Wild type and *sse1*Δ cells bearing the *SLT2-13xMyc* allele were subject to a 1-h heat shock, and protein extracts were made from aliquots taken at the indicated time points and subjected to SDS-PAGE and immunoblot. Slt2 protein levels remained unchanged in response to the heat shock and loss of *SSE1*, as shown in Fig. 2b. In addition, Slt2 was phosphorylated in response to heat shock in *sse1*Δ cells to levels indistinguishable from wild type. This experiment indicates that signaling through the cell integrity pathway is intact through phosphorylation of the terminal MAP kinase, Slt2, and that any phenotypic consequences of *SSE1* mutation must result from the inability of Slt2 to phosphorylate its downstream targets.

Slt2 is known to phosphorylate and activate at least two transcription factors, Rlm1 and the Swi4/Swi6 heterodimer SBF (Watanabe et al. 1995; Dodou and Treisman 1997; Madden et al. 1997; Jung et al. 2002)). To determine if Rlm1 function is impaired in *sse1*Δ cells, we constructed a transcriptional reporter using promoter sequences amplified from the known Rlm1 target gene *PST1* and β-galactosidase (*lacZ*). *PST1* induction in response to heat shock requires Slt2, and thus directly reports on cell integrity pathway signaling (Garcia et al. 2004). Although Slt2 phosphorylation attenuates rapidly, Rlm1-dependent transcription as assayed by reporter fusion increases over time up to 15 h (Jung et al. 2002). In wild type cells, expression of *PST1-lacZ* was low at 30°C but induced nearly eightfold by a 1.5-h heat shock at 37°C. Consistent with previous reports, both basal and heat shock-induced expression of *PST1-lacZ* was abolished in *rlm1*Δ cells (Garcia et al. 2004). Interestingly, an identical result was observed in *sse1*Δ cells, demonstrating that Sse1 is also required for stress signaling between Slt2 and Rlm1. Cells lacking SBF (*swi4*Δ) displayed nearly wild

type levels of induction, consistent with a previous report that *PST1* expression is independent of this transcription factor (Garcia et al. 2004). In contrast to the dramatic reduction in Rlm1-dependent gene expression observed in *sse1*Δ cells, no defects in heat shock expression of the Slt2/SBF target gene *PCL1* (Baetz et al. 2001) were detected (data not shown), suggesting differential involvement of Sse1 in cell integrity-regulated transcription.

Synthetic genetic interaction between *SSE1* and SBF

To further investigate the relationship between Sse1 and SBF, we constructed a series of mutant strains with deletions in *SSE1*, *SWI4*, and *SWI6* (see Table 1). In the BY4741 strain background, the *swi4*Δ and *swi6*Δ single deletion strains exhibited wild-type growth at 30°C and 37°C, while the *sse1*Δ mutant displayed a slow growth defect without additional temperature sensitivity (Fig. 3a). A strain containing mutant alleles of both *SWI4* and *SSE1* grew at 30°C at a rate similar to the *sse1*Δ single mutant strain, but was temperature-sensitive at 37°C. A more dramatic phenotype was observed in the *swi6*Δ *sse1*Δ strain, which was nearly inviable at both temperatures. Intriguingly, we were successful in obtaining a *swi4*Δ *swi6*Δ strain, previously thought to be inviable, in the BY4741 background that also displayed a severe growth defect (Koch et al. 1993). To ask whether these growth phenotypes correlated with defects in cell integrity, we assessed leakage of endogenous alkaline phosphatase. This assay provides a colorimetric readout of cell lysis using the chromogenic substrate BCIP (Paravicini et al. 1992). All the single mutants, as well as the *swi4*Δ *sse1*Δ double mutant strain, exhibited little to no alkaline phosphatase activity after 2 days of growth at 37°C. In contrast, but in keeping with the shared severe growth phenotypes, both the *swi6*Δ *sse1*Δ and the *swi4*Δ *swi6*Δ strains displayed substantial leakage, demonstrating that cell integrity was severely compromised (Fig. 3a, “BCIP”).

When these strains were examined by differential interference contrast microscopy, both the *swi6*Δ *sse1*Δ (LSY8) and the *swi4*Δ *swi6*Δ strains (LSY7) exhibited striking morphological defects typified by extremely large, amorphous cells suggestive of defects in cell cycle regulation and septum formation (Fig. 3b). The *swi6*Δ and *swi4*Δ *sse1*Δ strains displayed only moderately perturbed morphologies that included elongated daughter cells. The distribution of morphological classes (normal, elongated, and amorphous) was quantified for a minimum of 100 cells per strain and is shown in Fig. 3c. By this analysis, nearly 95% of *swi6*Δ *sse1*Δ cells examined were amorphous (black bars) or exhibited an elongated phenotype (gray bars). Similarly, approximately 77% of *swi4*Δ *swi6*Δ cells exhibited abnormal morphology. Loss of *SSE1* therefore appears to phenocopy loss of *SWI4* with respect to synthetic genetic interaction with the *swi6*Δ mutation.

Loss of Hsp70 NEF activity is responsible for morphological defects

Because sensitivity to cell wall-inhibiting compounds in *sse1*Δ cells could be suppressed by *SSE2*, but not by the *sse1-G233D* allele incapable of acting as an Hsp70 NEF, we asked whether the NEF activity of Sse1 was likewise required for proper morphogenesis in cells lacking *SWI6*. Plasmids carrying wild type SSE1, SSE2, or mutant alleles were introduced into *sse1*Δ *swi6*Δ cells and the morphological distribution of each resulting transformant is calculated, as shown in Fig. 4. *sse1*Δ *swi6*Δ cells harboring either an empty vector or the

sse1-G233D allele exhibited significant morphological defects, with slight variance in the percentages of normal versus abnormal daughter cells. In contrast, reintroduction of wild type *SSE1* or *SSE2*, or the functional *SSE1-K69Q* allele, restored normal cell growth and morphology.

To further validate that loss of Hsp70 NEF activity is a significant cause of the growth and morphology defects in *sse1*Δ *swi6*Δ cells, we asked whether overexpression of another cytosolic NEF, Fes1, would suppress the observed phenotypes (Kabani et al. 2002). As shown in Fig. 4, over-expressed Fes1 reversed the morphology defects to a level nearly indistinguishable from overexpressed Sse1. These data indicate that either NEF may partner with Hsp70, and by extension, Hsp90, to facilitate cell integrity signaling and proper proliferation and morphogenesis.

SSE1 and *RLM1* are required for growth in the absence of SBF and MBF

Results from the preceding experiments suggest that Sse1, as an Hsp70 NEF, is required for proper morphogenesis under normal growth conditions as well as gene expression governed by the cell integrity pathway, likely through Slt2. We next asked whether these genetic interactions extended to the other major Slt2-controlled factor, Rlm1. In contrast to the growth and morphological defects observed with deletions of *SSE1* and SBF components, deletion of *RLM1* caused no detectable growth impairment in combination with *SSE1* at 30 or 37°C (Fig. 5 and data not shown). Surprisingly, *rlm1*Δ *swi4*Δ cells also exhibited no growth defects, indicating that Slt2-activated cell integrity signaling requiring these transcription factors (but perhaps not Slt2-dependent, Rlm1/SBF-independent gene expression, see “Discussion”) is largely dispensable in the BY4741 genetic background.

Because deletion of *SSE1* had dramatic consequences in the context of cells lacking *SWI6*, we also generated multiple deletion strains to test for genetic interactions with the heterodimeric transcription factor MBF, composed of the *SWI6* and *MBP1* gene products. When gene disruption combinations affecting all three transcription factors (Rlm1, SBF, and MBF) were constructed, several interesting results were obtained. First, we were able to successfully obtain a *swi4*Δ *mbp1*Δ strain, previously reported to be inviable (Fig. 5) (Koch et al. 1993). This result suggests that cells from the BY4741 strain background are able to regulate expression of genes important for the G_1 to S phase transition in an unknown manner. Importantly, when all three transcription factors were disrupted (*rlm1*Δ *swi4*Δ *mbp1*Δ), severe growth impairment was observed at 30°C. A nearly identical phenotype was obtained when *SSE1*, *MBP1*, and *SWI4* were deleted in combination (Fig. 5), demonstrating that loss of the Sse1 protein chaperone is functionally equivalent to loss of the cell integrity-specific transcription factor Rlm1 in combination with inactivation of the Swi6-containing cell cycle-regulated transcription factors (SBF, MBF). This result is consistent with the hypothesis that Hsp90/Hsp70 and the essential cochaperone Sse1 are required for proper activation of Rlm1 by Slt2.

Discussion

In this report, we have demonstrated that the Hsp70 and Hsp90 cochaperone Sse1 is required for yeast cell integrity signaling through the Slt2 MAP kinase. Moreover, Sse1 genetically

interacts with the SBF and MBF cell cycle-regulated transcription factors to support growth and proliferation. A number of recent reports have linked conclusively protein chaperone function to the cell integrity pathway, with Slt2 as the likely nexus of interaction (Millson et al. 2005; Piper et al. 2006; Truman et al. 2006). Hsp90 physically associates with the kinase, and Hsp90 mutants display phenotypes consistent with impaired cell integrity signaling. Moreover, identical results are observed with the HSF(1-583) truncation mutant of Hsf1, previously shown to be deficient in Hsp90 production (Morano et al. 1999; Truman et al. 2007). Pharmacological inhibition of Hsp90 by radicicol likewise mirrors these mutant phenotypes (Truman et al. 2006, 2007). Our results documenting cell integrity pathway phenotypes with *SSE1* and *STI1* mutants are completely consistent with these findings. In addition, with the recent assignment of Sse1 as an Hsp70 nucleotide exchange factor, we have been able to extend our investigation to show that Hsp70 NEF activity is required for both cell integrity signaling and morphogenesis (Dragovic et al. 2006; Raviol et al. 2006; Shaner et al. 2006; Shaner and Morano 2007).

Unlike most other Hsp90 clients that require the chaperone for stability in vivo, the role of Hsp90 and its associated cochaperones in Slt2 function appears to be more subtle. Slt2 stability and activation via phosphorylation of Thr^{190}/Tyr^{192} in response to heat shock was unaffected in *sse1*Δ cells and in cells defective in Hsp90 function. In contrast, Slt2 kinase activity as measured using the model substrate myelin basic protein (MBP) was abolished in the presence of radicicol (Truman et al. 2007). These interactions are likely to be conserved in higher eukaryotes, as the human MAP kinase ERK5 can functionally substitute for Slt2 and is also Hsp90-dependent (Truman et al. 2006). We have not observed major defects in Slt2 kinase activity in *sse1*Δ cells using a similar assay, nor is Slt2-dependent; heat shock-induced phosphorylation of Swi6 diminished in vivo (data not shown). However, Rlm1-dependent transcriptional activation in response to heat shock is strongly reduced both in *Sse1*Δ cells and after treatment with radicicol (Truman et al. 2007). Together, these data suggest that while Hsp90 and the dedicated kinase chaperone Cdc37 are directly responsible for supporting cell integrity signaling through Slt2, Sse1, Sti1, and other cochaperones may play a more ancillary role that is amplified in vivo during heat shock conditions. A model summarizing these interactions is shown in Fig. 6. The cell integrity pathway is initially activated by a chemical or physical assault to the cell wall leading to signaling through protein kinase C and the MAP kinase pathway, ultimately resulting in the dual Thr/Tyr phosphorylation and activation of Slt2. Hsp90 and its cochaperones are required for Slt2 to phosphorylate and activate SBF and Rlm1 to induce a battery of cell wall integrity genes, with a greater bias toward Rlm1 function. In addition, Hsp110 appears to influence cellular morphogenesis in a pathway tightly linked to SBF and MBF, although the data presented in this report do not conclusively link Slt2 to this phenomenon. It is possible that the Hsp90 chaperone complex may differentially promote productive interaction between kinase and substrates, potentially as a scaffold. Because the strongest transcriptional defects in *sse1*Δ cells were observed with the Rlm1-dependent reporter, more precise investigation of these later stages in the pathway await development of an in vivo assay for Rlm1 phosphorylation by Slt2 (Jung et al. 2002).

We also observed strong genetic interactions between *SSE1* and genes encoding the two MBF subunits, *SWI6* and *MBP1*. Cells lacking *SSE1* and *SWI6* displayed severe growth

inhibition and morphological defects at 30°C, whereas *sse1*Δ *swi4*Δ mutants exhibited only minor temperature sensitivity. Cells lacking *MBP1* and *SWI4* displayed no apparent growth defects, whereas the additional deletion of *SSE1* rendered these cells nearly inviable, as did disruption of *RLM1*. A possible explanation for these observations is that deletion of *SSE1* (and by extension inhibition of Hsp90 function) results in diminished activity of Slt2, which would then manifest as “hypomorphism” in Rlm1 and SBF, both Slt2-dependent factors. Further deletion of *MBP1* may reduce cell cycle-regulated transcription below a critical threshold. Interestingly, Slt2 itself possesses transcriptional activation potential that requires physical interaction with SBF at target promoters but not kinase activity (Baetz et al. 2001; Kim et al. 2007, 2008). In this scenario, Slt2 acts as a DNA-binding factor to recruit Swi4, and ultimately Swi6, to activate gene expression. Our inability to detect defects in Slt2 kinase activity in *sse1*Δ mutants but clear cell wall integrity signaling phenotypes leads us to speculate that Sse1, in partnership with Hsp70, may be involved in this novel transcriptional activation complex.

Because Swi6 is a component of both SBF and MBF, *swi6*Δ mutants are simultaneously defective in transcription dependent on both factors, whereas loss of *SWI4* or *MBP1* is expected to eliminate SBF or MBF, respectively. Therefore, *sse1*Δ *swi6*Δ mutants are predicted to be genetically equivalent to *sse1*Δ *swi4*Δ *mbp1*Δ mutants, consistent with the growth phenotypes obtained in these studies. In addition, the identical growth defects observed in *rlm1*Δ *swi4*Δ *mbp1*Δ and *sse1*Δ *swi4*Δ *mbp1*Δ cells demonstrates that in the absence of SBF and MBF function, *SSE1* and *RLM1* are genetically equivalent, consistent with the similar reduction of *PST1-lacZ* induction in these strains (Fig. 2). Additional consideration must be given to the finding that HSF(1-583) mutants are defective in at least one synthetic transcriptional target of MBF, and this defect is remediated by overexpression of Hsp90 (Truman et al. 2007). While this result may be explained by the fact that Slt2 phosphorylates Swi6, the observed remediation also occurs in an *slt2*Δ background, suggesting that Hsp90 may interact with MBP in an Slt2-independent manner. It is also noteworthy to point out that many of our observations would not have been possible in other strain backgrounds. For example, we were able to engineer several mutant strains in the BY4741 strain that were previously reported to be inviable (*swi4*Δ *swi6*Δ, *mbp1*Δ *swi4*Δ), allowing us to uncover novel phenotypes (Koch et al. 1993). At this time, it is not clear if these differences reflect general strain “robustness” or differential reliance on SBF, MBF, or the novel Slt2-SBF transcription factors.

An important conclusion that can be drawn from our findings is that many temperature-sensitive growth phenotypes of Hsp90 and cochaperone mutants [HSF(1-583), *sse1*Δ, *sti1*Δ, *ydj1*Δ] are not simply due to indiscriminate destabilization or aggregation of cellular proteins (Truman et al. 2007; Wright et al. 2007). Instead, these phenotypes are due to improper signaling through Slt2 resulting in weakened cell walls leading to cell lysis. This view is supported by the fact that osmotic stabilization of the growth medium could not replace Hsp70 (*ssa1-45*) or Hsp110 (*sse1*Δ *sse2*Δ) functions to allow viability. This is almost certain because these chaperones have other essential functions in the cell, such as protein biogenesis, that cannot be rescued by this treatment. In summary, our results demonstrate that in addition to well-documented duties in protein refolding and repair, protein chaperones, especially Hsp110, Hsp90, Hsp70, and their associated cochaperones, play

important roles in supporting stress-responsive signal transduction critical for high-temperature growth.

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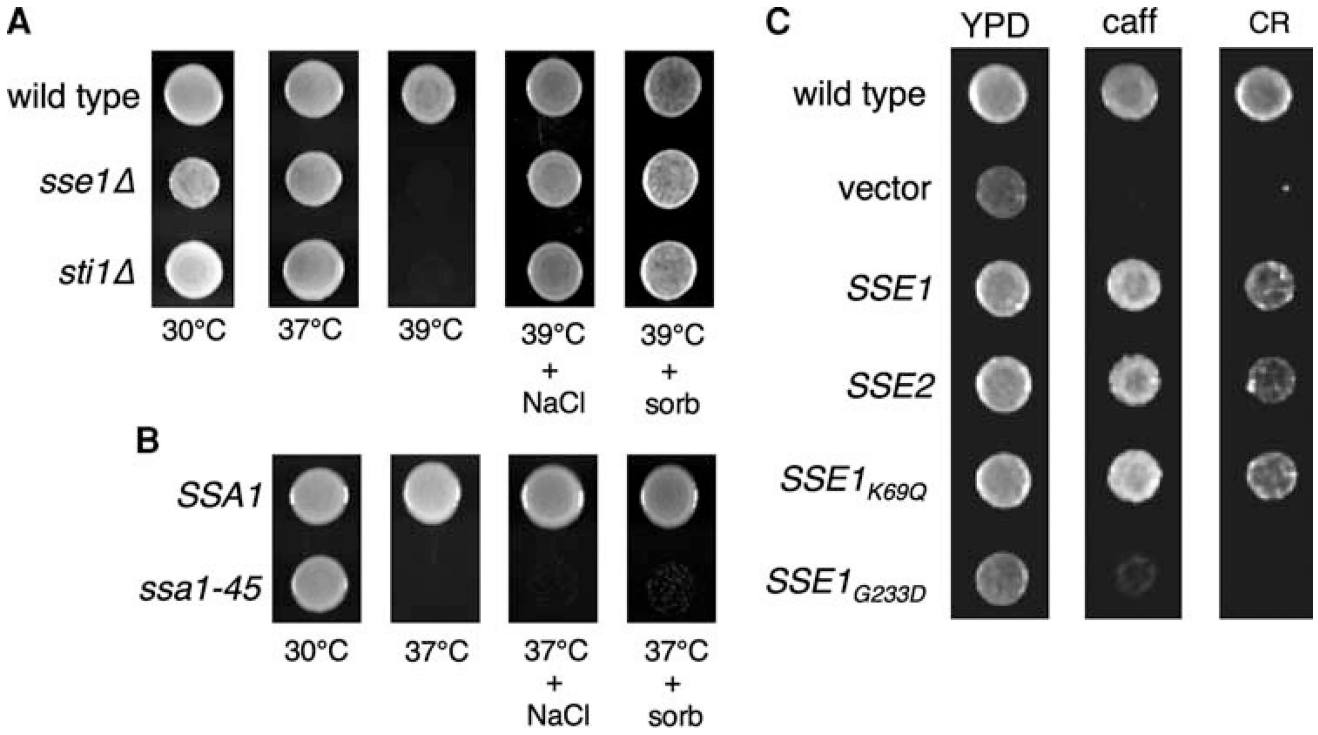

Fig. 1.

SSE1 and *STI1* mutants exhibit defects in cell integrity. **a** Serial dilutions of wild type (DS10) and the indicated isogenic knockouts, *sse1*Δ (DTY249) and *sti1*Δ (CN11), were spotted onto YPD, YPD containing 1 M sorbitol, or YPD containing 0.4 M NaCl plates and incubated at 30, 37, or 39°C for 2 days. A single equivalent dilution representing approximately 10^3 cells is shown for each condition. **b** A strain containing the *ssa1-45* allele as the sole copy of Ssa Hsp70 and its isogenic *SSA1* wild type parent were serially diluted and grown at 30 or 37°C on YPD plates containing 1 M sorbitol or 0.4 M NaCl as described in (**a**). **c** Serial dilutions of wild type (BY4741) and the isogenic *sse1*Δ disruption (LSY4) containing the indicated alleles were spotted onto YPD, YPD plus caffeine, or YPD plus Congo Red and incubated at 30°C for 2 days. A single dilution is shown as in (**a**)

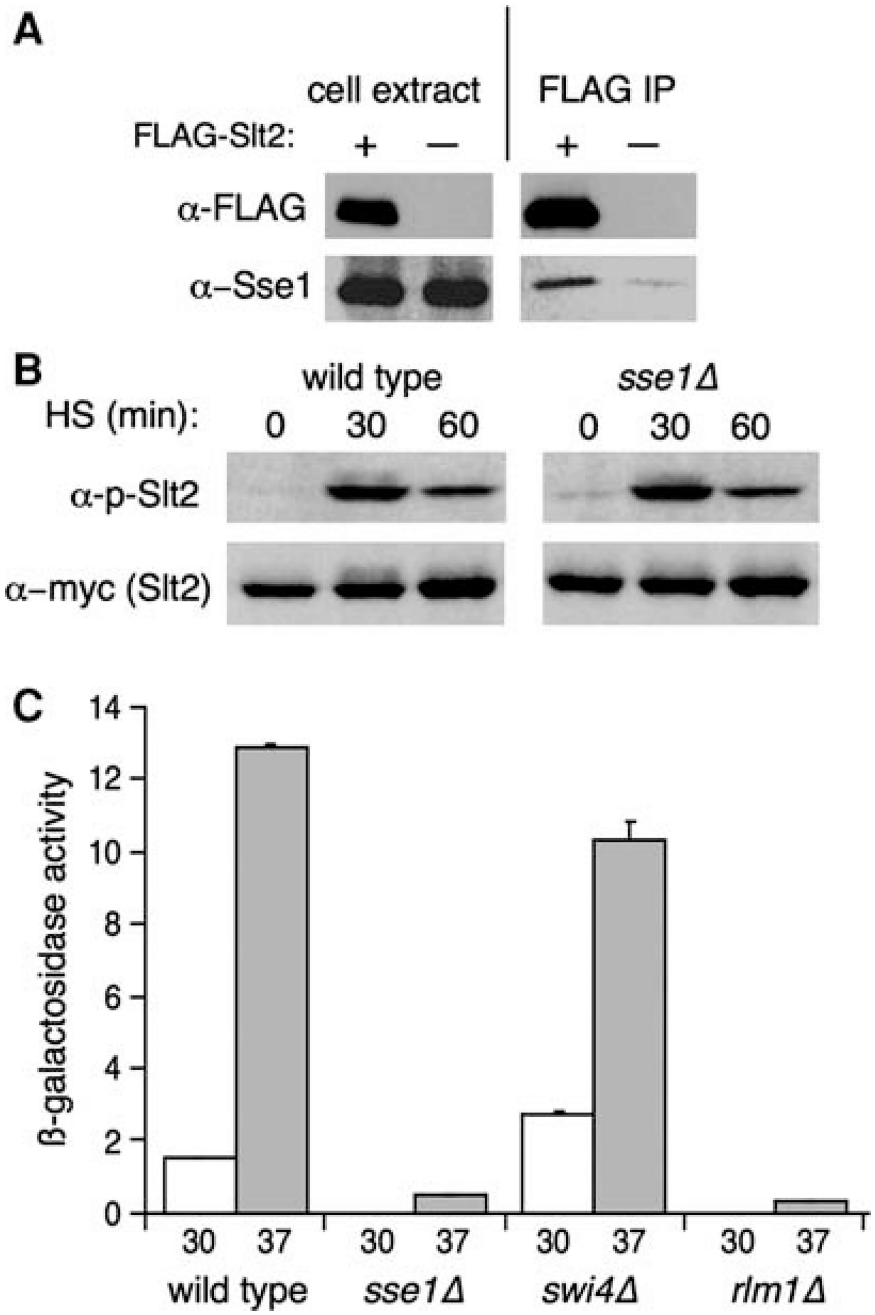

Fig. 2.

Sse1 physically interacts with Slt2 and is required for its activation. **a** FLAG immunoprecipitations were performed on protein extracts derived from W303 cells harboring p416GPD-FLAG-*SLT2* or p416GPD as described in “Materials and methods.” Immunoblot analysis using anti-Sse1 and anti-FLAG antibodies was carried out on the extracts and immunoprecipitates. The *extract lanes* represent 5% of the total amount of extract used for the immunoprecipitation. **b** A strain carrying an integrated 13×-myc-tagged *SLT2* allele (wild type, BY2207) and the isogenic *SSE1* deletion (*sse1*Δ, LSY19) were grown to mid-log phase at 25°C followed by shifting of the cultures to 37°C and removal of

aliquots at 0, 30, and 60 min postshift. Protein extracts were prepared followed by anti-myc and anti-phospho-Slt2 immunoblot analysis. **c** Wild type (DS10), *sse1*Δ (KMY69), *swi4*Δ (LSY1), and *rlm1*Δ (LSY2) cells were transformed with the *PST1-lacZ* reporter plasmid to measure transcriptional activation by Rlm1 and β-galactosidase activity assayed before and after a 1.5 h heat shock at 37°C

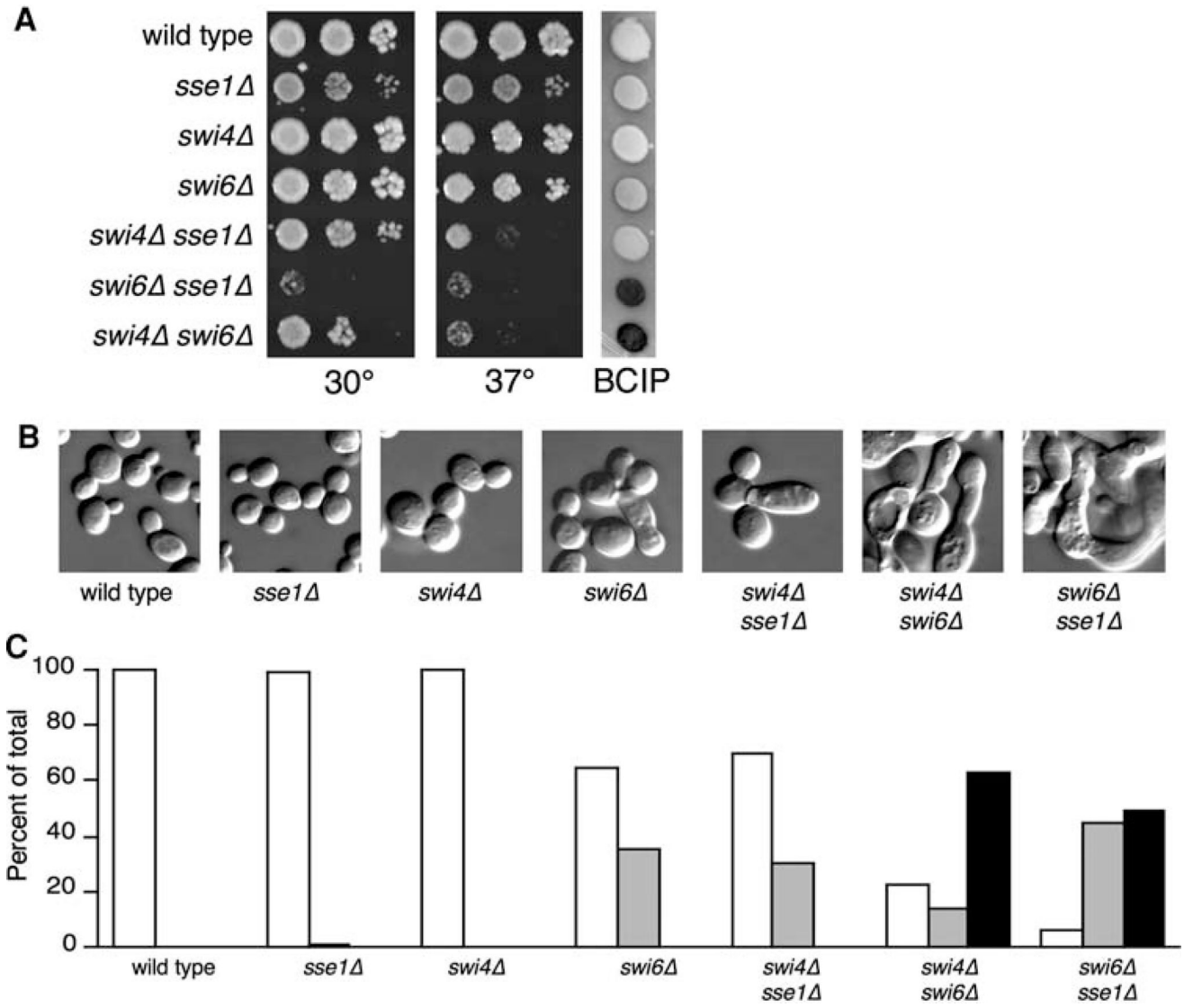

Fig. 3.

Synthetic growth and morphology defects between *SSE1* and SBF components. **a** Wild type (BY4741), and the indicated isogenic derivative strains (see Table 1) were serially diluted, plated on YPD and incubated at 30 and 37°C for 2 days. A parallel dilution series was subject to the alkaline phosphatase cell leakage assay after 2 days of growth at 37°C as described in “Materials and methods,” and a representative dilution spot is shown for all strains (BCIP). **b** The same strains were analyzed for morphology using Normarski microscopy. Representative micrographs are shown. **c** Quantitation of the morphological types observed in (**b**). Populations of at least 100 cells were categorized as normal (*white bars*), elongated (*gray bars*), or amorphous (*black bars*) as described in “Materials and methods”

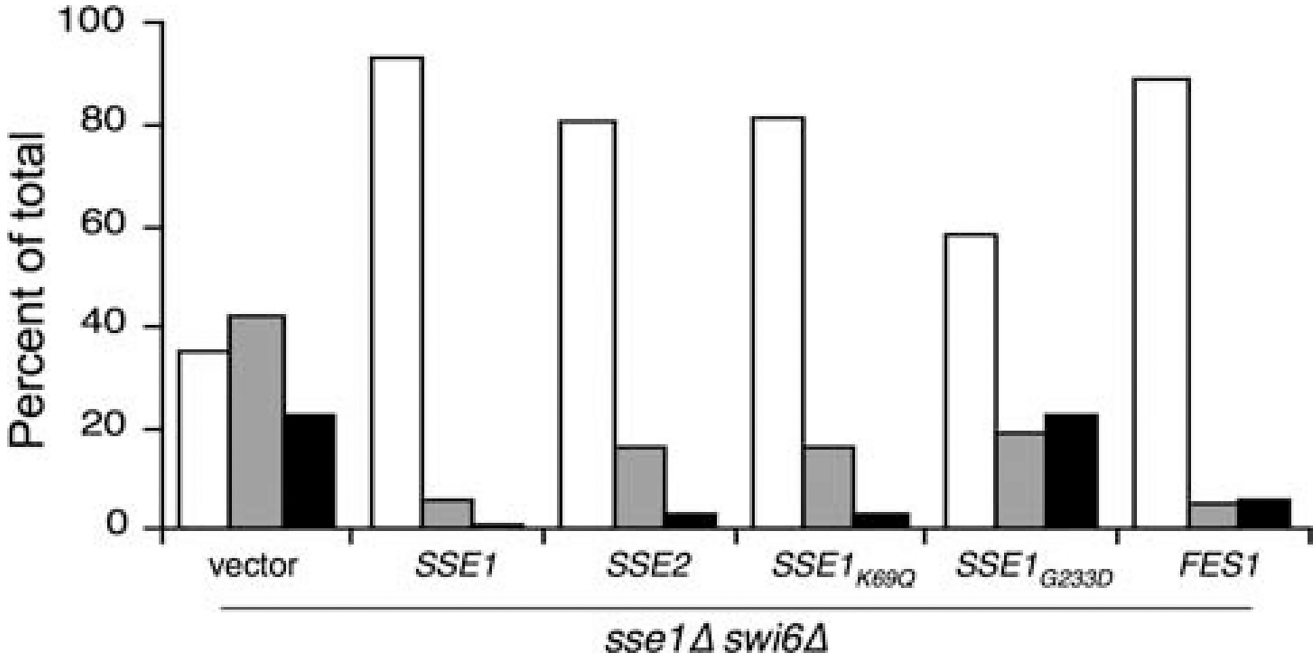

Fig. 4.

Hsp70 nucleotide exchange factor activity is required for normal growth and morphology in *swi6*Δ mutants. *sse1*Δ *swi6*Δ cells were transformed with the indicated plasmids bearing *SSE1* alleles, *SSE2* or *FES1* (see text for explanation), and morphological defects assessed as in Fig. 3. Populations of at least 200 cells for each strain were categorized as normal (*white bars*), elongated (*gray bars*), or amorphous (*black bars*) as described in "Materials and methods"

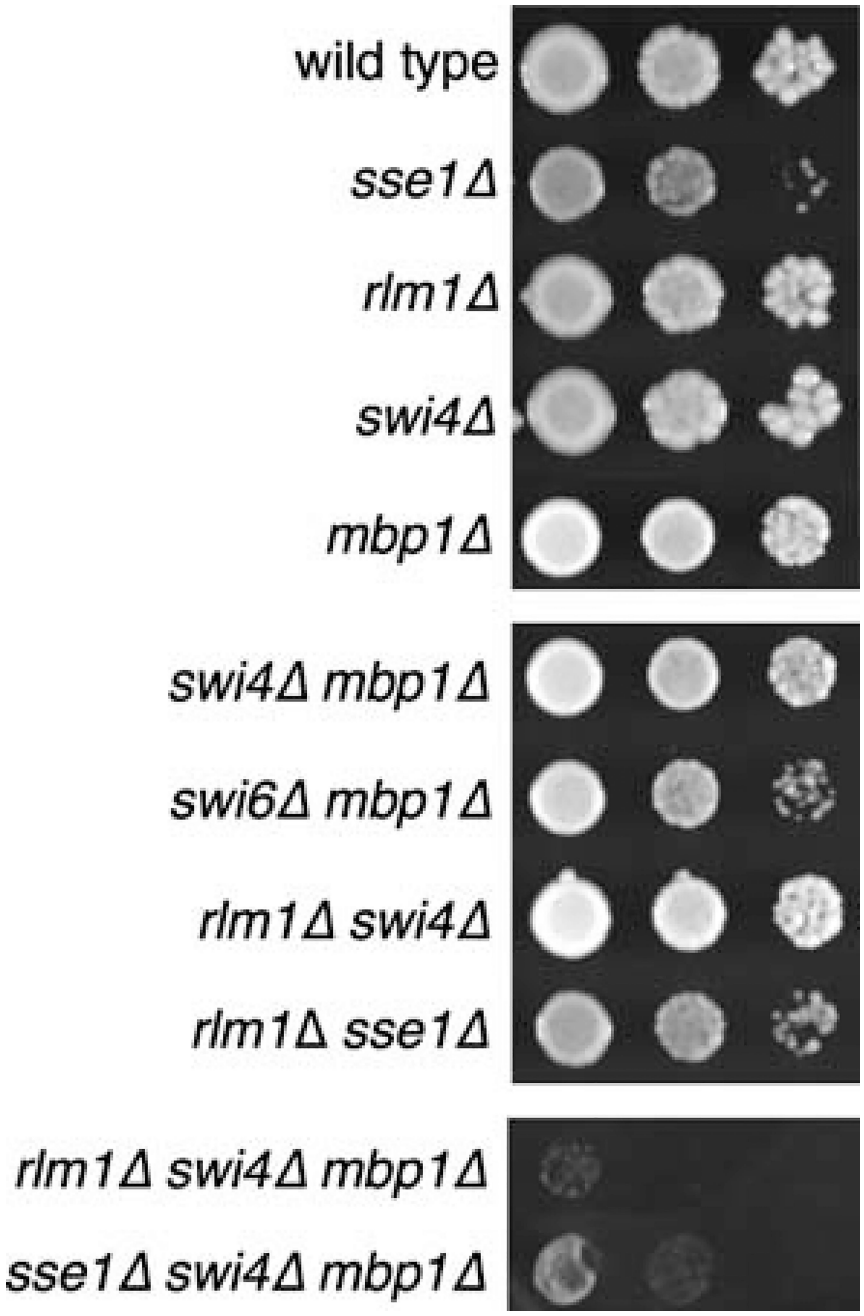

Fig. 5.

SSE1 genetically interacts with *RLM1* and MBF in addition to SBF. Wild type (BY4741) and the indicated isogenic derivative strains (see Table 1) were serially diluted, spotted onto YPD plates and incubated at 30°C for 2 days

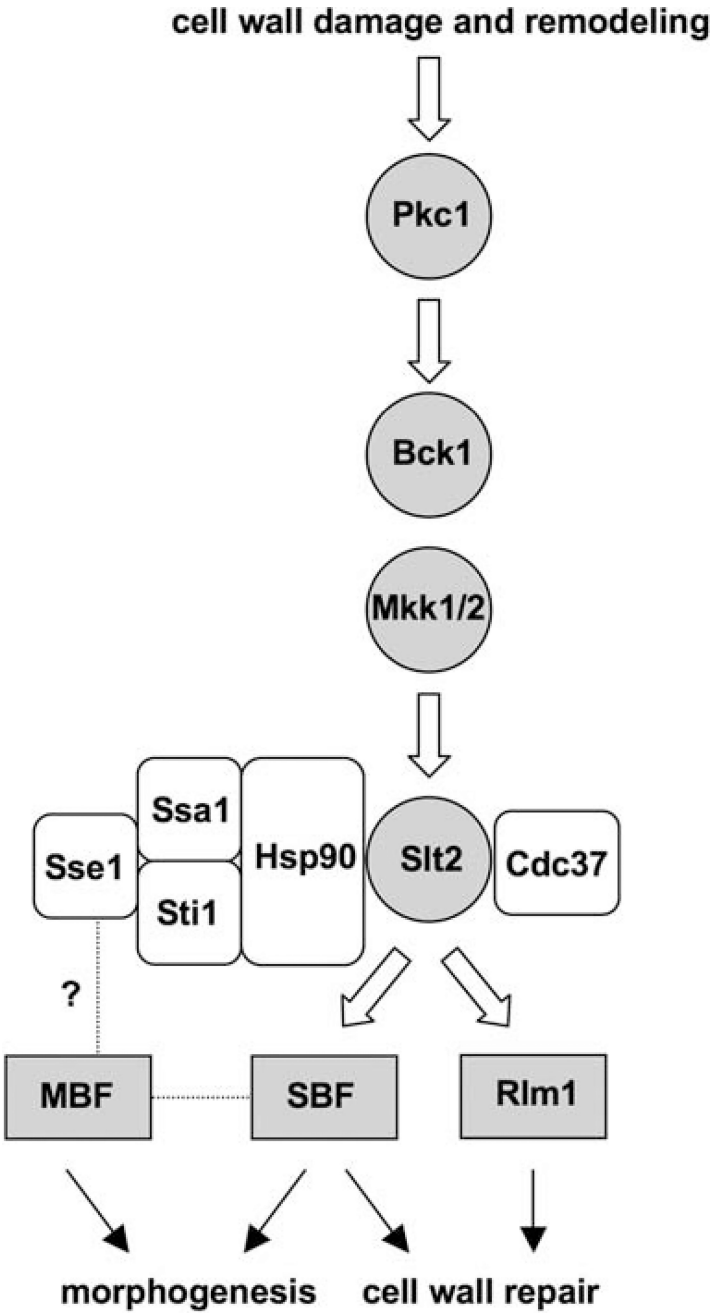

Fig. 6.

A multichaperone complex supports cell integrity signaling. A multichaperone complex that includes at least Hsp90, Hsp70 (Ssa), Hsp110 (Sse), and Stil interacts with the terminal Slt2 MAP kinase and is required for proper signaling to the effector transcription factors SBF and Rlm1. SBF and Rlm1 differentially regulate genes involved in cell wall repair and morphogenesis. Chaperone components may also be required for Slt2-independent functions of MBF

Table 1

Yeast strains

Strain	Genotype	Reference
W303	MAT**a** *ura3-52 trp1 leu2-3,112 his3-11,15 ade2-1 can1-100*	Rothstein 1991
KMY69	W303 *Sse1Δ::kanMX*	Liu et al. 1999
DS10	MAT**a** *ura3-52 lys1 lys2 trp1-1 his3-11,15 leu2-3,112*	Nicolet and Craig 1989
DTY249	DS10 *sse1Δ::kanMX*	Liu et al. 1999
LSY1	DS10 *swi4Δ::LEU2*	This study
LSY2	DS10 *rlm1Δ::kanMX*	This study
CN11	DS10 *sti1Δ::kanMX*	Nicolet and Craig 1989
BY4741	MAT**a** *his3Δ leu2A met15A ura3A*	Brachmann et al. 1998
LSY4	BY4741 *sse1Δ::kanMX*	This study
LSY5	BY4741 *swi4Δ::kanMX*	This study
LSY6	BY4741 *swi6Δ::kanMX*	This study
LSY7	BY4741 *swi6Δ::kanMX swi4Δ::LEU2*	This study
LSY8	BY4741 *swi6Δ::kanMX Sse1Δ::LEU2*	This study
LSY9	BY4741 *sse1Δ::kanMX swi4Δ::LEU2*	This study
LSY10	BY4741 *mbp1Δ::kanMX*	This study
LSY11	BY4741 *mbp1Δ::kanMX Sse1Δ::URA3*	This study
LSY12	BY4741 *mbp1Δ::kanMX swi4Δ::LEU2*	This study
LSY13	BY4741 *swi6Δ::kanMX mbpIΔ::HIS3*	This study
LSY14	BY4741 *rlm1Δ::kanMX*	This study
LSY15	BY4741 *rlm1Δ::kanMXswi4A::LEU2 mbp1Δ::HIS3*	This study
LSY16	BY4741 *rlm1Δ::kanMX sse1Δ::LEU2*	This study
LSY17	BY4741 *mbp1Δ::kanMXswi4Δ::LEU2 sse1Δ::URA3*	This study
LSY18	BY4741 *rlm1Δ::kanMX swi4Δ::LEU2*	This study
BY2207	BY263 *SLT2-13xMyc::kanMX*	B. Andrews
LSY19	BY263 *SLT2-13xMyc::kanMX sse1Δ::LEU2*	This study
JN516	*MAT**a** leu2-3,-112 his3-1 ura3-5 trp1Δ1 lys2 ssa2::LEU2 ssa3::TRP1 ssa4::LYS2*	E. Craig
ssa1-45	*MAT**a** leu2-3,-112 his3-1 ura3-5 trp1Δ1 lys2 ssa1-45 ssa2::LEU2 ssa3::TRP1 ssa4::LYS2*	E. Craig