Running title: Hsp70 control mat formation in yeast

Key words: Ssa1, Fes1, Sse1, Ydj1, Flo11, fungal biofilm

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Abstract

The yeast *Saccharomyces cerevisiae* has been used as a model for fungal biofilm formation due to its ability to adhere to plastic surfaces and to form mats on low-density agar Petri plates. Mats are complex multicellular structures composed of a network of cables that form a central hub from which emanate multiple radial spokes. This reproducible and elaborated pattern is indicative of a highly regulated developmental program that depends on specific transcriptional programming, environmental cues and possibly cell-cell communication systems. While biofilm formation and sliding motility were shown to be strictly dependent on the cell-surface adhesin Flo11p, little is known about the cellular machinery that controls mat formation. Here we show that Hsp70 molecular chaperones play key roles in this process with the assistance of the nucleotide exchange factors Fes1p and Sse1p, and the Hsp40 family member Ydj1p. The disruption of these cofactors completely abolished mat formation. Furthermore, complex interactions among SSA genes were observed: mat formation depended mostly on *SSA1* while minor defects were observed upon loss of *SSA2*; additional mutations in *SSA3* or *SSA4* further enhanced these phenotypes. Importantly, these mutations did not compromise invasive growth nor Flo11p expression, suggesting that Flo11p-independent pathways are necessary to form mats.
70-kDa heat-shock proteins (Hsp70s) are a ubiquitous family of molecular chaperones that play essential housekeeping functions in protein folding, synthesis, transport across biological membranes, and degradation. They are also involved in quality control processes such as protein refolding after a stress injury, and control the activity of regulatory proteins in signal transduction pathways (MAYER and BUKAU 2005). This functional pleiotropy is achieved through the evolutionary amplification and diversification of HSP70 genes, co-factors that recruit and regulate Hsp70s for specific cellular functions, and cooperation of Hsp70s with other chaperone systems such as TRiC/CCT or Hsp90 (MAYER and BUKAU 2005). All of these cellular activities depend on the ability of Hsp70s to interact with hydrophobic peptide stretches of proteins in an ATP-dependent manner. Hsp70s are composed of a highly conserved N-terminal 44-kDa ATPase domain, an 18-kDa peptide-binding domain, and a C-terminal 10-kDa variable ‘lid’ domain. In the ATP-bound state, Hsp70s display fast on and off rates of peptide binding, whereas in the ADP-bound state these constants are slowed (McCARTY et al. 1995; SCHMID et al. 1994). The modulation of the affinity for polypeptide substrates is triggered by a conformational change in the lid that is induced by ATP hydrolysis (JIANG et al. 2005; ZHU et al. 1996). The weak intrinsic ATPase activity of Hsp70s is stimulated by the Hsp40/DnaJ family of co-chaperones such as Ydj1p in yeast, whereas ADP-ATP exchange is catalyzed by several evolutionary unrelated classes of nucleotide exchange factors (KABANI et al. 2003; MAYER and BUKAU 2005). These include GrpE homologues in prokaryotes, and the Bag1 (Bcl2-associated athanogen 1), HspBP1/Fes1 and Hsp110 families in eukaryotes. The reasons and biological implications for such a diversity of eukaryotic nucleotide exchange factors are still not understood (KABANI et al. 2003; MAYER and BUKAU 2005).
The cytosol of the yeast *Saccharomyces cerevisiae* contains four classes of Hsp70s representing a total of 10 proteins (FRYDMAN 2001). The Ssa proteins are encoded by two constitutively expressed genes (*SSA1, SSA2*) and two stress-inducible genes (*SSA3, SSA4*) (WERNER-WASHBURNE et al. 1987). These essential canonical Hsp70s are involved in protein folding, protein translocation across the endoplasmic reticulum and mitochondrial membranes, and in quality control process such as endoplasmic-reticulum associated degradation (ERAD) (NISHIKAWA et al. 2005; YOUNG et al. 2003). As only one Ssa protein is sufficient to support yeast viability if expressed at sufficiently high levels, it is generally assumed that Ssa proteins are functionally redundant (WERNER-WASHBURNE et al. 1987). However, it is possible that individual Hsp70 isoforms evolved to perform specialized activities or to handle different type of substrates. In support of this hypothesis, the overexpression of Ssa1p but not Ssa2p was shown to cure yeast cells from the [URE3] prion (SCHWIMMER and MASISON 2002). In human cells, where up to six Hsp70s can coexist simultaneously in the cytosol, the highly homologous inducible Hsp70 and constitutive Hsc70 were shown to play non-overlapping essential functions in cancer cells growth and survival (ROHDE et al. 2005). The Ssb1/2 and Ssz1 proteins are ribosome-associated Hsp70s that cooperate in the folding of emerging nascent polypeptide chains (YOUNG et al. 2004). The Hsp110-family members Sse1/2 are divergent Hsp70s that co-operate in folding processes with Hsp70 and Hsp90 (GEOECKELER et al. 2002; OH et al. 1997; OH et al. 1999; SHANER et al. 2005; YAM et al. 2005), and function as nucleotide exchange factors for Ssa and Ssb proteins (DRAGOVIC et al. 2006; RAVIOL et al. 2006). The respective contribution of Sse1/2p and Fes1p to the cellular physiology is not clearly established. The overexpression of Fes1p only partially alleviated the lethality of a Δsse1Δsse2 mutant suggesting that these proteins play both distinct and overlapping functions (RAVIOL et al. 2006; SHANER et al. 2006). The yeast *Saccharomyces cerevisiae* also contains another Hsp70 nucleotide exchange factor
which is the Bag1-domain containing protein Snl1p. Snl1p is tethered to the ER and nuclear envelope membranes through a transmembrane domain and its physiological role seems restricted to the nuclear pore complex assembly (Sondermann et al. 2002).

In the present paper, we provide evidence for the implication of Hsp70s and co-chaperones in mat formation, a poorly understood biological phenomenon that is observed when Σ1278b yeast cells are cultivated on the surface of low-agar (0.3%) Petri plates where they form a complex multicellular structure. The mature mat is composed of a central hub made of a network of cables from which emanate multiple radial spokes, and a leading edge (or rim) that is smooth in appearance (Fig. 1) (Reynolds and Fink 2001). The radial symmetry of the mats and the reproducibility of the number of spokes and diameter of these structures are the hallmarks of a regulated developmental program that seems to be controlled by environmental cues (Reynolds and Fink 2001).

A specific transcriptional program is induced upon yeast growth on low-agar plates and controls mat formation (Reynolds 2006). The transcriptional profiles of mat cells resembles that of post-log-phase cells with the noticeable difference that protein synthesis genes continue to be highly expressed, while these are normally down regulated upon entry into the diauxic shift phase (Reynolds 2006).

The formation of mats occurs by sliding motility and depends on the viscosity of the medium, the availability of rich sources of nutrients and was shown to be strictly dependent on the expression of Flo11p, a cell surface adhesion glycoprotein (Fig. 1) (Reynolds and Fink 2001). Flo11p is also required for adhesion to plastic, haploid invasive growth, and diploid filamentous growth upon nitrogen starvation (Lo and Dranginis 1998; Reynolds and Fink 2001; Verstrepen et al. 2004). The expression of FLO11 is complex and depends on crosstalk between the cAMP-Protein Kinase A (PKA) and Mitogen Activated Protein Kinase (MAPK) signal transduction pathways that converge on the unusually large promoter of this
gene (Rupp et al. 1999; Sengupta et al. 2007). Moreover, epigenetic control of \textit{FLO11} expression by the histone deacetylase Hda1p results in a variegated expression of Flo11p at the cell surface of a clonal population derived from a single haploid cell (Halme et al. 2004).

Until now, all mutations known to affect mat formation resulted from the downregulation of \textit{FLO11} and therefore also affected invasive and filamentous growth (Reynolds 2006; Reynolds and Fink 2001). In the present report, we show that mutations in the Hsp70 system specifically compromised mat formation, but not invasive growth through a Flo11p-independent way. Moreover, we show that specific Hsp70 isoforms are required for this function and that the Fes1p and Sse1/2p nucleotide exchange factors play essential non-redundant roles in this process.
MATERIALS AND METHODS

Yeast strains, plasmids, media and growth conditions

All S. cerevisiae strains used in this study are described in Table 1 and were constructed in the Σ1278b derivatives TBR1 (MATα), TBR2 (MATa), or JK371 (Flo11p-HA) strains (generous gifts from Todd B. Reynolds and Jinmi Kim). Gene deletions were made using standard techniques (LONGTINE et al. 1998) and completely removed the indicated open reading frames from the start to the stop codons (construction details are available upon request). Yeast strains were propagated on YPD medium (1% yeast extract, 2% bacto peptone, 2% glucose, 2% bacto agar) or Synthetic Complete (SC) medium (1.7 g/L Yeast Nitrogen Base without amino-acids and ammonium sulfate (Difco), 5 g/L ammonium sulfate, 2% glucose, 2% bacto agar) supplemented with uracil, leucine, and histidine when appropriate.

The pGPD416-FES1, pGPD416-SSE1 and pGPD416-FLAG-SSE2 plasmids, a generous gift from Kevin A. Morano, are described in (SHANER et al. 2006).

For mat assays, low-agar YPD plates containing only 0.3% bacto agar were poured and left for 2-3 days at room temperature. The indicated yeast cells were then inoculated with a toothpick on the center of the plates that were then wrapped in parafilm and incubated at 23° for 14 days (REYNOLDS and FINK 2001). For each strain, at least 8 mats were grown at 23° and the number of spokes and diameter measured after 7 days. We only considered those spokes that fully extended from the central hub to the external rim with the characteristic white color that contrasts well with the rest of the mat.

For invasive growth assays, the indicated strains were heavily streaked on YPD plates and allowed to grow for 5 days at 23°. The plates were photographed, and then washed under a
gentle stream of water before being photographed again. The plates were further washed and rubbed with a gloved finger to remove the remaining attached cells and photographed again.

**Preparation of protein extracts and western blot analysis**

Protein extracts were prepared from mats grown for 7 days as indicated above. Mat cells were harvested from 2 to 4 low-agar plates with a spoon (REYNOLDS 2006), placed into 50 mL conical tubes containing 20 mL of cold water, vortexed to homogenize cells and agar and then washed twice with cold water. Aliquots of 50 OD$_{600\text{nm}}$ units of cells were resuspended in 600 µl of cold lysis buffer (50 mM Tris-Cl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1 mM PMSF). Glass beads were added and cells were broken by repeated vortexing for 30 sec with 1 min on ice between each vortexing. Cellular debris and glass beads were removed by centrifugation at 500 x g and the crude extracts were then centrifuged at 13,000 x g and at 4° for 15 min. The crude membrane pellet (P13) was then resuspended in 20 µl of lysis buffer. Proteins in the supernatant (S13) were precipitated with trichloroacetic acid (TCA) at a final concentration of 5% on ice for 30 min. The protein pellets were recovered by centrifugation at 13,000 x g and at 4°, washed twice with cold acetone then resuspended in 20µl of TCA sample buffer (80 mM Tris-Cl, pH 8.0, 8 mM EDTA, 120 mM dithiothreitol, 3.5% SDS, 0.29% glycerol, 0.08% Tris base, 0.01% bromophenol blue).

Equal amounts of proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and analyzed by Western blting using 12CA5 mouse monoclonal anti-HA antibodies (Roche Applied Science) and rabbit polyclonal anti-BiP antibodies (a generous gift from Jeffrey L. Brodsky, University of Pittsburgh). Immunoblots were developed using enhanced chemiluminescence reagents (Pierce) and a LAS-3000 imager (Fuji).
Indirect immunofluorescence

Indirect immunofluorescent staining of HA-tagged Flo11p was performed essentially as described elsewhere (Guo et al. 2000; Halme et al. 2004). Briefly, cells were isolated from colonies on plates and fixed in phosphate-buffered saline (PBS) containing formaldehyde (3.7%) for 1h. The cells were then washed with PBS and incubated for 1 h in PBS containing 2% bovine serum albumin (BSA). The cells were pelleted and resuspended in PBS containing 2% BSA and 12CA5 mouse monoclonal anti-HA antibody (Roche Applied Science; 1:1000 dilution) for 1 h. The cells were then washed with PBS containing 2% BSA three times and resuspended in PBS containing 2% BSA and goat anti-mouse Alexa Fluor 595-conjugated IgG (Jackson ImmunoResearch; 1:1000 dilution). After 20 min of incubation, the cells were washed three times with PBS containing 2% BSA.
RESULTS AND DISCUSSION

The nucleotide exchange factors Fes1 and Sse1 play critical roles in mat formation

In order to identify physiological functions for the Hsp70 nucleotide exchange factors Fes1p and Sse1/2p in yeast, we constructed null alleles of the respective genes in the Σ1278b strain background. This strain has the ability to undergo a dimorphic transition from yeast to pseudohyphal growth as well as to invade agar, to adhere to plastic surfaces or to form mats on low-agar Petri plates (Fig. 1). We found that ∆fes1 and ∆sse1 were both severely impaired in mat formation (Fig. 2 and Fig. 3 for quantitative measures), yet exhibited different morphologies. The mats formed by the ∆fes1 mutant were small and did not present the characteristic spokes and substructures, even after 14 days at 23° (Fig. 2 and Supplementary Fig. S2). They were however easily distinguishable from ∆flo11 mats that had a smooth and brilliant aspect (Fig. 1). In contrast, the deletion of SIL1 (also known as SLS1), the gene encoding the homologue of Fes1p in the endoplasmic reticulum (KABANI et al. 2000; TYSON and STIRLING 2000), did not affect mat formation (data not shown), suggesting a specific involvement of cytosolic chaperones in this process.

It is noteworthy that the growth rates at 23° of ∆fes1 and most of the other mutants described in this study were not significantly altered compared to the wild-type strain (Supplementary Fig. S1), which indicates that the observed defects in mat formation are not due to a reduced fitness or sickness of these strains. Indeed, even after prolonged periods of time, these mutants were not able to form mats (photographs at 14 days in Fig. 2). We previously showed that a ∆fes1 mutant is thermosensitive in two genetic backgrounds (W303-1b and RSY801) (KABANI et al. 2002), but this is not the case in the Σ1278b background where this mutation does not impede growth at 37° (Supplementary Fig. S1). In contrast, the ∆sse1 mutant in the
Σ1278b background displayed the previously reported slow growth phenotype at all temperatures tested (Supplementary Fig. S1) (MUKAI et al. 1993). This suggests a different utilization of nucleotide exchange factors among strains or species. For example, the deletion of \textit{SLS1/SIL1} in the yeast \textit{Yarrowia lipolytica} resulted in a thermosensitive phenotype whereas no obvious phenotype could be detected in several \textit{Saccharomyces cerevisiae} strains (BOISRAI et al. 1996; KABANI et al. 2000), and we showed that the \textit{Yarrowia lipolytica FES1} gene is essential (our unpublished data). Regardless, the strong defect in mat formation observed for \(\Delta\text{sse1}\) contrasted with the overall healthiness of this strain, and indicates a critical role for this factor in a specific cellular response induced by growth on low-agar plates.

The mats produced by the \(\Delta\text{sse1}\) mutant also had a small diameter compared to wild-type, contained no spokes but showed some level of substructures (Fig. 2 and Supplementary Fig. S2). In contrast, mats formed by the \(\Delta\text{sse2}\) mutant were normal, which is expected because \textit{SSE2} is expressed at low levels in wild-type cells and Sse1p alone is usually sufficient to fulfill Hsp110 activity (MUKAI et al. 1993; RAVIOL et al. 2006; SHANER et al. 2006; YAM et al. 2005). Importantly, the strong defect in mat formation observed for the \(\Delta\text{sse1}\) mutant suggested either that Hsp110 levels are limiting for mat formation in this strain or that Sse2p can not replace Sse1p in this particular cellular pathway. A functional distinction between these two Hsp110 paralogs has been suggested by previous studies that showed that Sse1 is able to form stable complexes with both Ssa and Ssb-type Hsp70s, while Sse2 was only able to bind to the Ssa-type (SHANER et al. 2006; YAM et al. 2005). Regardless, the strong and distinct morphological defects in mat formation observed upon loss of \textit{FES1} or \textit{SSE1} suggest that these two classes of nucleotide exchange factors play important roles in this process where they can not totally replace each other, at least at their normal physiological levels. This is in accordance with previous observations from Bukau and collaborators who showed
that the lethal combination of Δsse1 and Δsse2 mutations can be only partially rescued by the overexpression of FESI; the resulting strain grew much slower at 30° than a similar strain expressing wild-type levels of Sse1p and its viability was significantly reduced in stress conditions (RAVIOL et al. 2006). A recent study reported that while SSE1 and FESI are both required for the propagation of [URE3], only the overexpression of the former cured yeast cells from this prion, again arguing against a complete functional redundancy of these two nucleotide exchange factors (KRYNDUSHKIN and WICKNER 2007)

To further investigate the contributions of nucleotide exchange factors in mat formation, we asked whether the overexpression of Fes1p would compensate for the loss of Sse1p and vice versa. The Δfes1 and Δsse1 strains were transformed with the pGPD416-FES1, pGPD416-SSE1 and pGPD416-FLAG-SSE2 plasmids that allow the constitutive overexpression of Fes1p, Sse1p and Sse2p, respectively (SHANER et al. 2006). These plasmids are centromeric and we reasoned that they would not be significantly lost as cells divide to produce a mat on YPD. As shown in Fig. 4, the Δsse1 mutant formed mats when transformed with pGPD416-SSE1 or pGPD416-FLAG-SSE2, but not with the empty vector. This result shows that Sse2 can replace Sse1 in its function in mat formation, and suggests that the strong defect observed for the Δsse1 mutant results from the decrease in overall Hsp110 levels. Interestingly, the overexpression of FESI efficiently complemented the Δsse1 mutant and allowed mat formation (Fig. 4), suggesting as previously reported (RAVIOL et al. 2006) that the functions of Fes1p and Sse1/2 partly overlap. Thus, Fes1p can replace Sse1/2p to allow mat formation but, strikingly, the opposite is not true. Indeed, while pGPD416-FES1 complemented the Δfes1 mutant as expected, pGPD416-SSE1 and pGPD416-FLAG-SSE2 did not (Fig. 4). These results further demonstrate that separate cellular functions for Fes1p and Sse1/2p exist where these two classes of nucleotide exchange factors can not replace each other. As a control, we overexpressed FES1, SSE1 or SSE2 in a wild-type background and did not
observe any noticeable effect on growth nor on the ability to form mats (Supplementary Figure S3).

**Δydj1 is affected in mat formation**

Hsp70s are also regulated by DnaJ-like proteins that contain a 70 amino-acids J-domain that is required for binding to Hsp70s and to activate their ATPase activity. We therefore investigated the contribution of the main Hsp40-family co-chaperone Ydj1p in mat formation. The Δydj1 mutant totally failed to form mats and spread irregularly on low-agar Petri plates, even after 14 days of incubation (Fig. 2 and Supplementary Fig. S2). The brilliant and spongy aspect of Δydj1 mats was different from those observed for Δfes1 and Δsse1 mats. YDJ1 was previously isolated in a transposon-mutagenesis screen for dia (dig into agar) mutants that enhance invasive growth; an ydj1-100 mutant had a hyper-invasive phenotype due to elevated levels of the Flo11p adhesin (PALECEK et al. 2000). We made a similar observation with our Δydj1 mutant that showed increased adherence to agar (see below). While Flo11p is absolutely required for mat formation, it may impede sliding motility if expressed at high levels by preventing cells from moving along the agar surface. Fink and collaborators showed that epigenetic control of FLO11 expression results in a variegated expression of Flo11p in a cell population (HALME et al. 2004). Whether the expression of FLO11 is differentially and strictly controlled in the different regions of the mats is an attractive hypothesis that needs future investigations. Ydj1p has also been recently implicated in the cell-wall integrity pathway as a Δydj1 was shown to be sensitive to cell-wall disrupting agents and its slow growth compensated by osmo-stabilizing reagents such as sodium chloride or sorbitol (WRIGHT et al. 2007). Unfortunately, mat formation does not occur on NaCl or Sorbitol containing medium (our personal observations) preventing us from testing whether these
compounds could rescue mat formation by the Δydj1 mutant. Moreover, the overexpression of *FES1, SSE1* or *SSE2* in the Δydj1 mutant had no effect on mat formation (data not shown).

From these data, we can not formally conclude on a specific involvement of Ydj1p in mat formation. The yeast *Saccharomyces cerevisiae* contains at least 22 J-domain containing proteins, the functions of many of these remaining unclear (SAHI and CRAIG 2007; WALSH et al. 2004). Future studies will be required to investigate the respective contributions of these co-chaperones in mat formation.

**Complex interactions among Hsp70 isoforms during mat formation**

Because mutations in known Hsp70 cofactors affected mat formation (Fig. 2), we next asked whether mutations in the constitutively expressed *SSA1* and *SSA2* genes or in the conditionally expressed *SSA3* and *SSA4* genes will also result in similar phenotypes. As shown in Fig. 5 (and Supplementary Fig. S4), a Δssa1 strain was significantly affected in mat formation but to a lesser extent than a Δfes1 or Δsse1 mutant. A Δssa1 mutant formed significantly smaller mats than the wild-type strain and with much lesser spokes, even after 14 days of incubation (Fig. 5 and Fig. 3). Mats formed by a Δssa2 mutant had an almost normal diameter but showed a slight decrease in the number of spokes, and the difference with the wild-type was less obvious after 14 days of incubation (Fig. 5 and Fig. 3). Importantly, the level of expression of *SSA2* was shown to be several times that of *SSA1* in standard growth conditions (ELLWOOD and CRAIG 1984; WERNER-WASHBURNE et al. 1987), yet only the absence of the latter resulted in a marked defect in mat formation. This further emphasizes the possibility that the highly homologous Ssa1p and Ssa2p proteins may play distinct physiological roles. A functional distinction among these two Hsp70 isoforms has been suggested by earlier studies that showed that the overexpression of Ssa1p, but not Ssa2p, was
able to cure cells from the [URE3] prion (Schwimmer and Masison 2002). To our knowledge, this is the first description of a phenotype associated with the deletion of SSA1 in an otherwise wild-type background. Indeed, most mutations in SSA1, such as the thermosensitive allele ssa1-45 allele (Becker et al. 1996), result in detectable phenotypes only when at least SSA2 and SSA4 have been also deleted. Thus, mat formation appears as a sensitive mean to explore chaperone function in the cell.

In contrast, the deletion of SSA3 or SSA4 did not affect mat formation (Fig. 5, Fig. 3 and Fig. S4); which is not surprising given that these two Hsp70s are only expressed in stress conditions or in stationary phase (Boorstein and Craig 1990; Werner-Washburne et al. 1987). However, it should be noted that mats present a transcriptional profile that is very similar to stationary-phase cells, with the noticeable exception that genes required for protein synthesis continue to be actively expressed (Reynolds 2006). Therefore, SSA3 is one gene that is overexpressed in mats compared to planktonic log-phase cells (Reynolds 2006), yet it seems dispensable for mat formation.

We then asked whether deletions in SSA3 or SSA4 genes would result in synthetic effects with mutations in SSA1 or SSA2, and this is was indeed the case. As shown in Fig. 3 and Fig. 6, the additional disruption of SSA3 or SSA4 in a ∆ssa1 or ∆ssa2 context resulted in a further reduction in the number of spokes and diameter of the mats. The loss of SSA3 seemed to induce more pronounced effects than SSA4 in these double mutants, consisting with its greater expression in mats (Reynolds 2006). Surprisingly, defects in mat formation were more pronounced when the double mutants were constructed in an isogenic MATα strain, and this was most striking for the ∆ssa2∆ssa4 mutant that was more severely impaired in mat formation in a MATα compared to a MATα background (compare Fig. 6 and Supplementary Fig. S5). Because we analyzed at least two independent clones for each gene deletion and for each background; these differences are unlikely to be due to secondary mutations that arose
after the transformation procedure. It is known that the mating type and ploidy affect the morphology of mats (Reynolds and Fink 2001), and biological differences were observed between \textit{MATa} and \textit{MATa} cells in \textit{Cryptococcus neoformans} (Lee et al. 2005).

Regardless, our observations suggest that the Hsp70 machinery effectively controls mat formation, and highlight the intricate interactions among members of the SSA subfamily that may cooperate in this complex biological process.

\textbf{Mutations in the Hsp70 machinery do not affect invasive growth, nor the expression and cellular localization of Flo11p}

Few studies addressed the molecular mechanisms that control mat formation and until now all known mutations in this process decreased or abolished \textit{FLO11} expression, which in turn affected invasive growth and adherence to synthetic surfaces (Reynolds 2006; Reynolds and Fink 2001). We therefore tested our mutants in a standard agar invasion assay (See Material and Methods section) where a \textit{∆flo11} mutant is readily washed away from a YPD plate with a gentle stream of water, whereas the wild-type strain remains attached to the agar surface (Lo and Dranginis 1998) (Fig. 7). Interestingly, none of our mutants in the Hsp70 system was affected in invasive growth (Fig. 7), nor in adherence to polypropylene or polystyrene plastic surfaces (data not shown), suggesting that these mutations do not result in decreased Flo11p expression at the cell surface. Moreover, we observed an increased invasiveness for the \textit{∆ydj1} mutant that was most apparent when the plates were washed with water and further rubbed with a gloved finger to remove more effectively non-invasive cells (Fig. 7). This behavior is in agreement with increased Flo11p expression in \textit{∆ydj1} cells (Palecek et al. 2000) (see below).
The fact that mutations in the Hsp70 system compromise mat formation without affecting invasive growth suggests that the expression and the cell-surface localization of Flo11p occur normally in those mutants. To verify this hypothesis, we deleted the SSA1, SSA2, FES1, SSE1 and YDJ1 genes in a Σ1278b-derivative strain expressing a hemagglutinin (HA)-tagged Flo11 protein (Park et al. 2006). These mutants behaved as those made in the wild-type background with respect to mat formation and invasive growth (data not shown).

Total lysates were then prepared from these strains and separated into crude membrane (P13) and soluble (S13) fractions as described in the Materials and Methods section. Equal amounts of proteins were separated by SDS-PAGE and analyzed by Western blotting with anti-HA antibodies to detect Flo11p-HA, or anti-BiP (the endoplasmic reticulum Hsp70 family member) antibodies as a control. As shown in Fig. 8A, the Flo11p-HA protein was expressed in all strains, and was essentially detected in the P13 fractions as expected for a plasma membrane/cell-wall-associated GPI-anchored protein. As expected from its hyperinvasive phenotype and from an earlier report (Palecek et al. 2000), the Δydj1 strain contained significantly higher levels of Flo11p-HA protein (Fig. 8A). The Δsse1 strain also contained high levels of Flo11p-HA, probably due to the elevated PKA signaling that was reported for this strain (Trott et al. 2005). Surprisingly, in spite of its elevated Flo11p levels, the Δsse1 mutant was only slightly hyperinvasive compared to the Δydj1 strain (Fig. 7). This could be due to the very slow growth of this mutant that might impede foraging into the agar. Alternatively, Δsse1 may be deficient in other cell-surface determinants that are required for agar invasion. In support of this hypothesis, we observed that Δsse1 cells are hypersensitive to cell-wall disrupting agents (see below).

In light of these data, the observed defects in mat formation upon deletion of SSA1, FES1, SSE1 or YDJ1 can not be attributed to lowered Flo11p levels. It remained formally possible that while Flo11p was normally expressed, its maturation or cellular localization was affected...
by mutations in the Hsp70 system. We therefore analyzed the cellular distribution of the Flo11p-HA protein in cells harvested from colonies grown in regular YPD plates by indirect immunofluorescence (Guo et al. 2000; Halme et al. 2004). As shown in Fig. 8B, the distribution of Flo11p-HA at the cell-surface was comparable between wild-type and mutant strains. We obtained identical results using the same cells that were grown as mats on low-agar YPD plates, and we failed to observe any differences in the expression of Flo11p in the hub, spokes or rim regions of the mats (data not shown).

Thus, mutations in the Hsp70 machinery do not affect the expression, maturation and cellular localization of Flo11p. However, we cannot exclude that subtle differences in Flo11p folding or post-translational modifications such as O-glycosylation exist in these strains that could account for the observed phenotypes.

Because mat formation is tightly linked to cell-surface events and because a Δydj1 mutant was shown to display defects in cell-wall integrity, we checked whether mutations in other components of the Hsp70 machinery would result in cell-wall associated defects. We found that only Δsse1 and Δydj1 were hypersensitive to SDS and Congo Red (Figure S6 and data not shown), indicating that mat defects in the other mutants are not linked to major perturbations in cell-wall integrity.

The spreading of mats is correlated to the number of spokes

The phenotypic characterization of the mutants described in this study implied a quantification of the average number of spokes and diameter reached by each mat after 7 days of incubation at 23° (see Material and Methods section). The number of spokes formed by a wild-type strain is rather constant and is known to depend on both carbon sources (Palecek
et al. 2002) and mating type, as MATa mats produce more spokes than MATα mats (REYNOLDS and FINK 2001).

We ranked each strain according to the number of spokes and the diameter of mats, from the highest to the lowest, and plotted these values on a graph (Fig. 3). Strikingly, we observed that the average diameter reached by individual mats mirrored the average number of spokes. A linear regression analysis of the data revealed a linear relationship between the average diameter and the number of spokes with an R-squared value of ~0.96 (p<0.0001) if the strains that never form spokes but displayed variable diameters (i.e. Δfes1, Δydl1, Δsse1) were excluded from the analysis. If these strains were also taken into account, the R-squared value drops to ~0.8 (p<0.001) but remains nevertheless highly significant.

It is tempting to speculate that spokes are the main structures that control the spreading of the mats and further emphasize the highly organized nature of these multicellular structures. In support with this hypothesis, mats formed on other carbon sources such as galactose or glycerol do not produce spokes and are significantly smaller than mats formed on glucose or fructose (PALECEK et al. 2002). However, because mats are also a physical phenomenon, spokes may be the result of a complex equation with many parameters such as growth rate, cell-cell interactions, adhesion forces, sliding motility and cell-surface proteins production. Further investigations will be required to distinguish between the physical and biological contributions to mat formation. Because molecular chaperones dysfunction may impact growth rate, cell-wall assembly and signaling pathways, they constitute a solid starting point to address these questions.

**Concluding remarks**
In this study, we provided evidence for a role of the Ssa-subfamily of Hsp70 molecular chaperones in mat formation and highlighted a number of important features. First, in agreement with other studies (KRYNDUSHKIN and WICKNER 2007; RAVIOL et al. 2006; SHANER et al. 2006), we showed that the biological functions of Fes1p and Sse1p are mainly distinct since the overexpression of Sse1p did not compensate for the loss of Fes1p in our mat assays. The defects in mat formation in the ∆sse1 and ∆fes1 strains are unlikely to be due to alterations in a common pathway. Because ∆sse1 cells are much sicker than ∆fes1 cells, mat defects may be a secondary effect of more general perturbations in cellular physiology. Another explanation is that the elevated Flo11p levels in the ∆sse1 as well as the ∆ydj1 mutants, by increasing adherence to the agar and foraging, impede the cells to slide along the surface. Because the overexpression of Fes1p allowed mat formation in a ∆sse1 mutant and because the strong mat phenotype contrasted with the apparent healthiness of the ∆fes1 strain, we propose that Fes1p plays a more specific role in mat formation. Future work will be required to ask whether the separate biological functions observed for Sse1p and Fes1p can be attributed to their respective engagement in specific chaperone-containing complexes or their interaction with particular regulatory proteins in distinct cellular pathways.

Second, our data are in agreement with the possible existence of a functional specialization among Ssa-proteins suggested by earlier studies, and challenges the generally admitted idea that Hsp70 isoforms are functionally redundant and only differ by their expression pattern. The fact that any of Ssa1-4 (WERNER-WASHBURNE et al. 1987) or even mammalian Hsp70 orthologues (TUTAR et al. 2006) allow the viability of ∆ssa1∆ssa2∆ssa3∆ssa4 yeast cells if expressed at sufficient levels suggest that their general house-keeping and chaperoning functions are mostly conserved. However, the existence of specialized functions of individual isoforms remains an attractive hypothesis that have major significance in human cells were up to six Hsp70 isoforms potentially coexist in the cytosol (ROHDE et al. 2005; TAVARIA et al.
Furthermore, we showed that the defects in mat formation observed upon deletion of the SSA1 and SSA2 genes were significantly aggravated by a deletion of either one of the inducible SSA3 and SSA4 genes. These data agree with a functional distinction between constitutive Hsc70s and inducible Hsp70s as previously shown (Tutar et al. 2006) but also attractively suggest a possible cooperation between these isoforms in handling different client proteins, their respective contributions being dictated by the cellular conditions and hence their respective cellular levels.

Third, we showed that while attachment to the surface of the low-agar plates is strictly required (Reynolds and Fink 2001), FLO11-independent pathways affect mat formation. Whether mats represent a purely physical phenomenon, a regulated developmental program resulting in specialized regions within the mat, or a combination of these two aspects is still an open issue, but we clearly showed that this biofilm-like behaviour is highly sensitive to changes in the genotype that do not otherwise result in dramatic changes in growth rates or cell-wall integrity. We believe that mats constitute a remarkable biological model to investigate the functions of molecular chaperones and their engagement in specific cellular pathways and protein complexes.
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Author’s contributions: M.K. conceived and designed research. J-M.B. provided new reagents/analytical tools. C.N.M. and M.K. performed experiments and analyzed data. MK wrote the paper.
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Table 1: Yeast strains used in this study

Fig. 1: The S. cerevisiae Σ1278b strain form mats on low-agar plates. A wild-type strain or a Δflo11 mutant (both MATα) was inoculated at the center of YPD plates containing 0.3% agar and grown at 23° for 7 or 14 days. Photographs of the whole plates and of a magnified portion of the plates are shown (note that in Fig. 1, 2, 3, 4 and 6 the photographs at 7 and 14 days do not necessarily show the same plate).

Fig. 2: The Δfes1, Δsse1 and Δydj1 mutants are affected in mat formation. Mats were grown as indicated in Fig. 1 (see Supplementary Fig. S2 for MATa versions of these mutants, and Fig. 3 for quantitative measures)

Fig. 3: Quantification of the diameter and number of spokes for each mat. For each indicated strain, 8 mats were grown in parallel at 23° and the diameter (filled triangles) and number of spokes (filled circles) measured after 7 days (the indicated numbers correspond to the average +/- S.E.).

Fig. 4: The overexpression of SSE1 or FES1 does not equally complement the mat defect of the Δfes1 and Δsse1 mutants. The Δfes1 and Δsse1 strains were transformed with pGPD416 (control), pGPD416-SSE1, pGPD416-FLAG-SSE2 or pGPD416-FES1 (see Materials and Methods) and mats were then grown as indicated in Fig. 1. (see Supplementary Fig. S3 for the wild-type control)
**Fig. 5:** Mutations in SSA1-4 genes differently affect mat formation. Mats were grown as indicated in Fig. 1 (see Supplementary Fig. S4 for MATa versions of these mutants, and Fig. 3 for quantitative measures)

**Fig. 6:** Mutations in SSA3 or SSA4 affect mat formation in a Δssa1 or Δssa2 context. Mats were formed as indicated in Fig. 1 (see Supplementary Fig. S5 for MATa versions of these mutants, and Fig. 3 for quantitative measures)

**Fig. 7:** Mutations in the Hsp70 system do not affect invasive growth. The indicated strains were heavily streaked on YPD plates containing 2% agar and grown for 5 days at 23°. The plates were photographed, washed with water to remove unattached cells and photographed again. To remove more effectively the remaining cells, the plates were rubbed with a gloved finger under a gentle stream of water and photographed again.

**Fig. 8:** Mutations in the Hsp70 do not affect Flo11p expression or cellular localization. (A) Cell lysates were prepared from the indicated strains (grown as mats on low-agar YPD plates at 23°C) expressing HA-tagged Flo11p or from the untagged wild-type strain (control), and subjected to differential centrifugation at 13000 g to generate a crude membrane pellet (P13) and a supernatant (S13) containing cytosol and light vesicles. Equal amounts of proteins from each fractions were resolved by SDS-PAGE and analyzed by Western blotting with mouse anti-HA and rabbit anti-BiP antibodies (B) The localization of Flo11p-HA was visualized by indirect immunofluorescence. The indicated strains were harvested from YPD plates, fixed, treated with mouse anti-HA antibody and stained with Alexa Fluor 595-conjugated goat anti-mouse IgG antibody.
Table 1: Yeast strains used in this study

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